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Mauro Maccarrone *Editor*

Endocannabinoid Signaling

Methods and Protocols

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Edited by

Mauro Maccarrone

Department of Medicine, Campus Bio-Medico University of Rome, Rome, Italy

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Editor
Mauro Maccarrone
Department of Medicine
Campus Bio-Medico University of Rome
Rome, Italy

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Preface

Since the isolation and characterization of the active phytocannabinoids in the cannabis (*Cannabis sativa*) plant, including the most psychoactive ingredient Δ^9-tetrahydrocannabinol (THC), and after the identification and cloning of the target of THC, the type-1 cannabinoid receptor, and of its endogenous counterparts collectively termed "endocannabinoids (eCBs)," many efforts have been produced to study the different physiological functions modulated by what is now known as "endocannabinoid system (ECS)." eCBs are derivatives of arachidonic acid, resembling other lipid transmitters such as prostaglandins or leukotrienes. They are conjugated with ethanolamine to form fatty acid amides, or with glycerol to form monoacylglycerols; so far, *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) represent the best studied and most active members of each class, respectively. In the last 20 years, an ever-growing interest towards the manifold activities of eCBs in pathophysiology has boosted the development of chemical, biochemical, and molecular biological assays to specifically measure the endogenous levels of eCBs, along with the specific activity of distinct elements of the ECS. These include membrane and nuclear receptors (and signaling pathways thereof), biosynthetic and hydrolytic enzymes, as well as membrane transporters and oxidative enzymes. Not only the functional activity of these entities but also their gene and protein expression has attracted attention, because its regulation has emerged as a major determinant of eCB biological activity. Therefore, a manual that puts together all major methodologies to interrogate ECS and eCB signaling appears timely and will help chemists, drug designers, biochemists, molecular biologists, cell biologists, pharmacologists, and (electro)physiologists to navigate the *mare magnum* of endocannabinoid research.

Rome, Italy ***Mauro Maccarrone***

Contents

Contributors

Sharon Anavi-Goffer • *Department of Behavioral Sciences, Ariel University, Ariel, Israel; Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK*

Clotilde B. Angelucci • *Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy*

Michiel G.J. Balvers • *Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands; Clinical Chemistry and Haematology Laboratory, Gelderse Vallei Hospital, Ede, The Netherlands*

Monica Bari • *Department of Experimental Medicine and Surgery, Tor Vergata University of Rome, Rome, Italy*

Natalia Battista • *Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy*

Laura Bindila • *Institute for Physiological Chemistry, University Medical Center, Mainz, Germany*

Tiziana Bisogno • *Endocannabinoid Research Group, Institute of Biomolecular Chemistry, National Research Council, Pozzuoli, Italy; Center of Integrated Research, Campus Bio-Medico University of Rome, Rome, Italy*

Maria Grazia Cascio • *School of Medicine, Medical Sciences & Nutrition, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Scotland, UK*

Valeria M. Catani • *Department of Experimental Medicine and Surgery, Tor Vergata University of Rome, Rome, Italy*

Andrea Chicca • *Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, Bern, Switzerland*

Claudio D'Addario • *Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy; European Center for Brain Research (CERC)/Santa Lucia Foundation, Rome, Italy*

Enrico Dainese • *Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy*

Monica Feole • *Department of Medicine, Campus Bio-Medico University of Rome, Rome, Italy*

Filomena Fezza • *Department of Experimental Medicine and Surgery, Tor Vergata University of Rome, Rome, Italy*

Marina Gabaglio • *Department of Biotechnology and Life Sciences, University of Insubria, Busto Arsizio (VA), Italy*

Valeria Gasperi • *Department of Experimental Medicine and Surgery, Tor Vergata University of Rome, Rome, Italy*

Jürg Gertsch • *Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, Bern, Switzerland*

Noortje van Gils • *Department of Medicinal Chemistry, Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands*

Laura H. Heitman • *Department of Medicinal Chemistry, Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands*

Paul F. Hollenberg • *Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA*

Kwang-Mook Jung • *Department of Anatomy and Neurobiology, University of California, Irvine, Irvine, CA, USA*

Philip J. Kingsley • *A.B. Hancock Jr. Memorial Laboratory for Cancer Research, Department of Biochemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt Center in Molecular Toxicology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN, USA; Department of Bio Chemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt Center in Molecular Toxicology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN, USA*

Shalley N. Kudalkar • *A.B. Hancock Jr. Memorial Laboratory for Cancer Research, Department of Biochemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt Center in Molecular Toxicology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN, USA*

Jarmo T. Laitinen • *School of Medicine, Institute of Biomedicine/Physiology, University of Eastern Finland, Kuopio, Finland*

Beat Lutz • *Institute for Physiological Chemistry, University Medical Center, Mainz, Germany*

Mauro Maccarrone • *Department of Medicine, Campus Bio-Medico University of Rome, Rome, Italy; European Center for Brain Research/IRCCS Santa Lucia Foundation, Rome, Italy*

Ken Mackie • *Department of Psychological and Brain Sciences, Indiana University, Bloomington, IN, USA*

Pietro Marini • *School of Medicine, Medical Sciences & Nutrition, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Scotland, UK*

Lawrence J. Marnett • *A.B. Hancock Jr. Memorial Laboratory for Cancer Research, Department of Biochemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt Center in Molecular Toxicology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN, USA; Department of Chemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt Center in Molecular Toxicology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN, USA; Department of Pharmacology, Vanderbilt Institute of Chemical Biology, Vanderbilt Center in Molecular Toxicology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN, USA*

Nicolina Mastrangelo • *Department of Medicine, Campus Bio-Medico University of Rome, Rome, Italy*

Aniello Schiano Moriello • *Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche (CNR), Pozzuoli, Italy*

Dina Navia-Paldanius • *School of Medicine, Institute of Biomedicine/Physiology, University of Eastern Finland, Kuopio, Finland*

Simon Nicolussi • *Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, Bern, Switzerland*

Sergio Oddi • *Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy; European Center for Brain Research/Santa Lucia Foundation IRCCS, Rome, Italy*

Daniela Parolaro • *Department of Biotechnology and Life Sciences, University of Insubria, Busto Arsizio (VA), Italy; Fondazione Zardi-Gori, Milano, Italy*

Roger G. Pertwee • *School of Medicine, Medical Sciences & Nutrition, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Scotland, UK*

Luciano De Petrocellis • *Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche (CNR), Pozzuoli, Italy*
Daniele Piomelli • *Department of Anatomy and Neurobiology, University of California, Irvine, Irvine, CA, USA; Department of Pharmacology, University of California, Irvine, Irvine, CA, USA; Department of Biological Chemistry, University of California, Irvine, Irvine, CA, USA; Drug Discovery and Development, Istituto Italiano di Tecnologia, Genoa, Italy*
Pamela Prini • *Department of Biotechnology and Life Sciences, University of Insubria, Busto Arsizio (VA), Italy*
Mariangela Pucci • *Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy*
Mark Rau • *Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, Bern, Switzerland*
Ruth A. Ross • *Department of Pharmacology and Toxicology, Faculty of Medicine, University of Toronto, Toronto, ON, Canada; Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK*
Tiziana Rubino • *Department of Biotechnology and Life Sciences, University of Insubria, Busto Arsizio (VA), Italy*
Annalaura Sabatucci • *Faculty of Bioscience and Technology for Food Agriculture and Environment, University of Teramo, Teramo, Italy*
Juha R. Savinainen • *School of Medicine, Institute of Biomedicine/Physiology, University of Eastern Finland, Kuopio, Finland*
Manuel Sergi • *Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy*
Natasha T. Snider • *Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*
Marjolein Soethoudt • *Department of Bio-Organic Synthesis, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands; Department of Medicinal Chemistry, Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands*
Mario van der Stelt • *Department of Molecular Physiology, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands*
Antonio Totaro • *European Center for Brain Research/Santa Lucia Foundation IRCCS, Rome, Italy*
Kazuhito Tsuboi • *Department of Biochemistry, Kagawa University School of Medicine, Kagawa, Japan*
Natsuo Ueda • *Department of Biochemistry, Kagawa University School of Medicine, Kagawa, Japan*
Toru Uyama • *Department of Biochemistry, Kagawa University School of Medicine, Kagawa, Japan*
Jim Wager-Miller • *Department of Psychological and Brain Sciences, Indiana University, Bloomington, IN, USA*
Vyvyca J. Walker • *Department of Medicine – Nephrology, University of Alabama at Birmingham, Birmingham, AL, USA*
Renger F. Witkamp • *Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands*
Guss van Zadelhoff • *Cellular Protein Chemistry, Faculty of Science, Utrecht University, Utrecht, The Netherlands*
Erica Zamberletti • *Department of Biotechnology and Life Sciences, University of Insubria, Busto Arsizio (VA), Italy*

Chapter 1

Need for Methods to Investigate Endocannabinoid Signaling

Mauro Maccarrone

Abstract

Endocannabinoids (eCBs) are endogenous lipids able to activate cannabinoid receptors, the primary molecular targets of the cannabis (*Cannabis sativa*) active principle Δ^9-tetrahydrocannabinol. During the last 20 years, several *N*-acylethanolamines and acylesters have been shown to act as eCBs, and a complex array of receptors, metabolic enzymes, and transporters (that altogether form the so-called eCB system) has been shown to finely tune their manifold biological activities. It appears now urgent to develop methods and protocols that allow to assay in a specific and quantitative manner the distinct components of the eCB system, and that can properly localize them within the cell. A brief overview of eCBs and of the proteins that bind, transport, and metabolize these lipids is presented here, in order to put in a better perspective the relevance of methodologies that help to disclose molecular details of eCB signaling in health and disease. Proper methodological approaches form also the basis for a more rationale and effective drug design and therapeutic strategy to combat human disorders.

Key words Anandamide, 2-Arachidonoylglycerol, Enzyme assays, Immunochemical assays, Intracellular trafficking, Localization, Metabolic routes, Oxidative pathways, Receptor binding assays, Signal transduction

1 A Modern View of the Endocannabinoid System

Two G protein-coupled receptors, termed type-1 (CB_1) and type-2 (CB_2) cannabinoid receptors, are activated by Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of cannabis (*Cannabis sativa*) extracts like hashish and marijuana [1]. Endogenous counterparts of THC [collectively termed "endocannabinoids (eCBs)"], their target receptors, and the enzymes responsible for their synthesis and degradation form an entirely new endogenous signaling system, also known as the "endocannabinoid system (ECS)" [2–4].

The most important eCBs are two arachidonic acid derivatives: *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), shown in Table 1.

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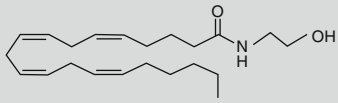
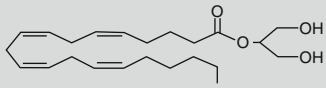
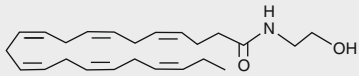
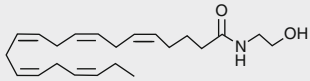
Table 1
Endocannabinoids (eCBs), their molecular targets, and their biosynthetic and catabolic enzymes

Bioactive lipids	Molecular targets	Biosynthetic enzymes	Catabolic/oxidative enzymes
ω – 6 eCBs			
	CB_1 CB_2 TRPV1 PPARα PPARγ GPR55	NAT iNAT NAPE-PLD ABHD4 *Lyso*-PLD GDE1	FAAH NAAA LOXs COX-2 Cyt P_{450}
N-arachidonoylethanolamine (anandamide, AEA)		PTPN22	
	CB_1 CB_2	PLCβ DAGLα	MAGL FAAH
2-Arachidonoylglycerol (2-AG)	TRPV1	DAGLβ	ABHD6
ω – 3 eCBs			
	CB_1 CB_2	Possibly as for other NAEs	Possibly as for other NAEs
N-docosahexaenoylethanolamine (DHEA)	PPARγ		
	CB_1 CB_2	Possibly as for other NAEs	Possibly as for other NAEs
N-eicosapentaenoylethanolamine (EPEA)	PPARγ		

Abbreviations: *ABHD4/6/12* α/β-hydrolase domain 4/6/12, *CB1* type-1 cannabinoid receptors, *CB2* type-2 cannabinoid receptors, *COX-2* cyclooxygenase-2, *Cyt P450* cytochrome P_{450}, *DAGLα/β* diacylglycerol lipase α/β, *FAAH* fatty acid amide hydrolase, *GPR55* orphan G protein-coupled receptor 55, *LOXs* lipoxygenases, *MAGL* monoacylglycerol lipase, *NAAA N*-acylethanolamine-hydrolyzing acid amidase, *NAPE-PLD N*-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D, *NAT N*-acyltransferase, *iNAT* Ca^{2+}-independent *N*-acyltransferase, *PLCβ* phospholipase Cβ, *lyso-PLD lyso*-phospholipase D, *PPARα/γ* peroxisome proliferator-activated receptor α/γ, *PTPN22* protein tyrosine phosphatase, non-receptor type 22, *TRPV1* transient receptor potential vanilloid type 1 channel

They belong to the large families of *N*-acylethanolamines and 2-monoacylglycerols, respectively. Besides these *ω* – 6 (*n* – 6) fatty acid compounds, two metabolically important *ω* – 3 (*n* – 3) fatty acid ethanolamines have been discovered: *N*-eicosapentaenoylethanolamine (EPEA) [5] and *N*-docosahexaenoylethanolamine (DHEA) [6], also shown in Table 1. The latter two substances have been proposed as additional CB_1/CB_2 agonists [7], but their pharmacology and biological relevance remain to be clarified.

The actions of eCBs and congeners are controlled through not yet fully characterized cellular mechanisms that include key agents responsible for their biosynthesis, degradation, and oxidation.

Remarkably, during the last few years multiple pathways have been described for the metabolism of AEA (Fig. 1), and of 2-AG (Fig. 2), as detailed in a recent review [8].

Briefly, the main route for AEA biosynthesis consists of two enzymatic reactions. The first is a fatty acyl chain transfer from membrane phospholipids to a phosphatidylethanolamine, resulting in the formation of *N*-acylphosphatidylethanolamine (NAPE), by a yet-unidentified Ca^{2+}-dependent *N*-acyltransferase (NAT) [9], or by a Ca^{2+}-independent counterpart (iNAT) [10]. The second step is catalyzed by a NAPE-specific type D phospholipase (NAPE-PLD) that is the most relevant enzyme among multiple players in AEA formation from NArPE [9, 10], as shown in Fig. 1.

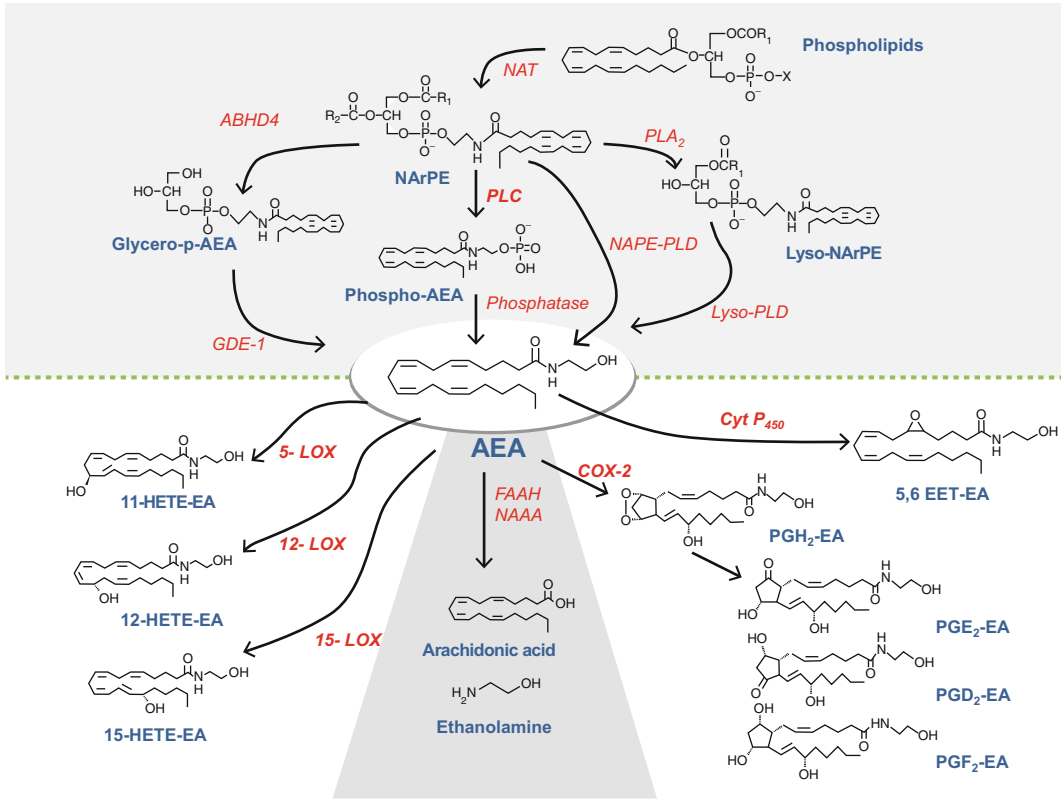


Fig. 1 Alternative biosynthetic, degradative, and oxidative pathways of AEA and congeners. *AA* arachidonic acid, *ABHD4* α/β-hydrolase domain 4, *pAEA* phospho-AEA, *COX-2* cyclooxygenase-2, *Cyt P450* cytochrome P_{450}, *EET-EA* epoxyeicosatrienoyl ethanolamides, *EtNH2* ethanolamine, *FAAH* fatty acid amide hydrolase, *GP-AEA* glycerophospho-AEA, *GDE1* glycerophosphodiester phosphodiesterase 1, *12-HAEA* 12-hydroxyanandamide, *12-LOX* 12-lipoxygenase, *NAAA N*-acylethanolamine-hydrolyzing acid amidase, *NAPE-PLD N*-acyl-phosphatidyl ethanolamine-hydrolyzing phospholipase D, *NAT N*-acyltransferase, *iNAT* Ca^{2+}-independent *N*-acyltransferase, *lyso-NArPE lyso-N*-arachidonoylphosphatidylethanolamine, *NArPE N*-arachidonoylphosphatidylethanolamine, *pNArPE N*-arachidonoylethanolamine plasmalogen, *PLA2* phospholipase A_2, *PLC* phospholipase C, *lyso-PLD lyso*-phospholipase D, *PMF2α* prostamides F2α, *PTPN22* protein tyrosine phosphatase, non-receptor type 22

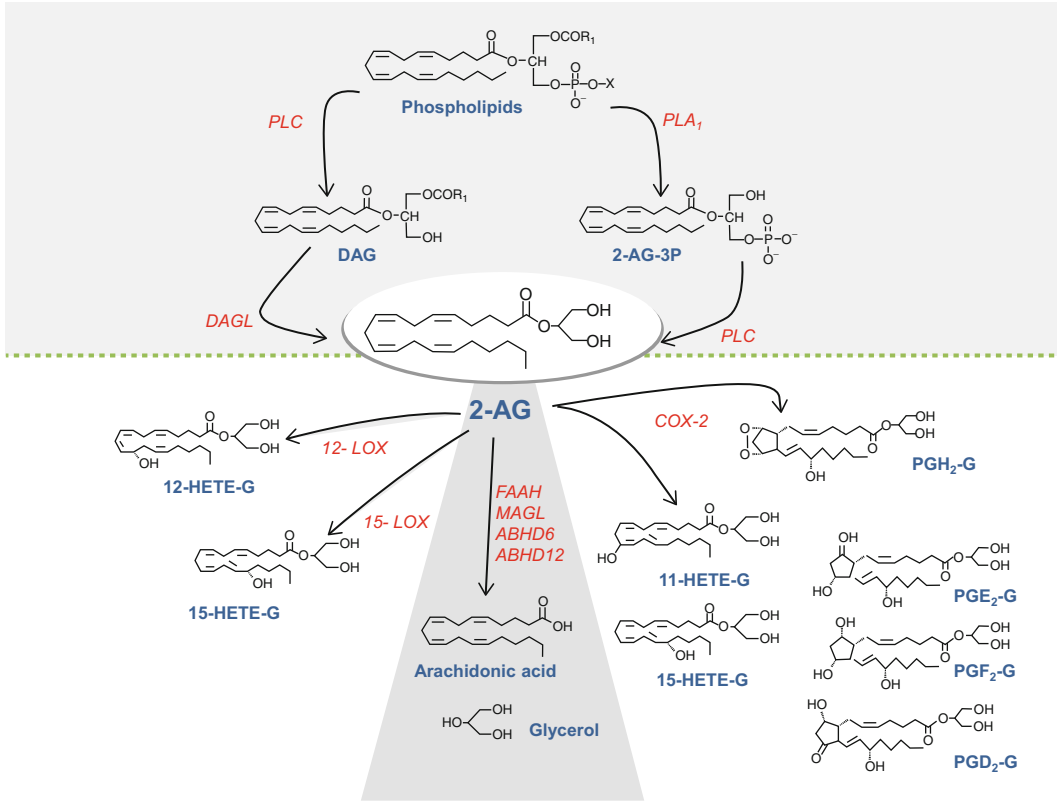


Fig. 2 Alternative biosynthetic, degradative, and oxidative pathways of 2-AG and congeners. *AA* arachidonic acid, *2-AG-3P* 2-arachidonoylglycerol-3-phosphate, *COX-2* cyclooxygenase-2, *DAG* diacylglycerol, *DAGL* diacylglycerol lipase, *12-HETE-G* 12-hydroxy-arachidonoyl-glycerol, *ABHD6/12* α/β-hydrolase domain 6/12, *12-LOX* 12-lipoxygenase, *MAGL* monoacylglycerol lipase, *PLC* phospholipase C, *PLCβ* phospholipase Cβ, *PGE2-G* prostaglandin glycerol E_2-G

As for the biosynthesis of 2-AG and congeners, the best known biosynthetic pathway requires the combined action of phospholipase C (PLC) and diacylglycerol lipase (DAGL that is present in two forms, α and β) [11], but alternative pathways of 2-AG biosynthesis are also known, as shown in Fig. 2.

Degradation of eCBs and congeners can start with their transmembrane uptake, a process that remains highly debated because true "eCBs membrane transporters (EMT)" have not yet been cloned; however, EMT activity and pharmacological inhibition have been repeatedly described [12]. Once inside the cell, eCBs are hydrolyzed to terminate signal transduction. The main catabolic enzyme of AEA is fatty acid amide hydrolase (FAAH) [13], a widely distributed intracellular membrane-bound serine hydrolase [14]. An additional lysosomal cysteine hydrolase termed

N-acylethanolamine-hydrolyzing acid amidase (NAAA) is also known [15], and cleaves AEA and congeners under acidic conditions (Fig. 1). As for 2-AG and congeners, monoacylglycerol lipase (MAGL) is the main responsible for their degradation, along with two additional serine hydrolases, known as α/β-hydrolase domain 6 and 12 (ABHD6 and ABHD12) [16].

Moreover, the oxidative metabolism of eCBs has (patho)physiological relevance, because it leads to the production of new biologically active metabolites [17]. In particular, AEA and 2-AG are metabolized by lipoxygenases (LOXs) [18] and by cyclooxygenase-2 (COX-2) [17–19], and additionally AEA can be oxygenated also by cytochrome P450 [20], as shown in Figs. 1 and 2.

Incidentally, it should be stressed that an emergent issue is how eCBs can reach their distinct sites of action within the cell (e.g., membrane or nuclear receptors, or metabolic enzymes) at the right time and at the right concentration, in order to trigger the appropriate response to a stimulus [21]. In this context, the existence of intracellular storage organelles (adiposomes or lipid droplets) [22], as well as of constitutive intracellular transporters (AEA intracellular transporters, AITs), has been reported for AEA [2]. A functional role for these AITs in eCB signaling has been recently documented [23], providing a proof of concept that indeed they can drive eCBs towards distinct transduction pathways. This is particularly striking in the central nervous system, where at each synapse distinct ECS elements in different neuronal and non-neuronal cells contribute to proper neurotransmission [24, 25]. The same complexity in other organs of our body (e.g., cardiovascular, digestive, musculoskeletal, immune, and reproductive systems) has been the subject of a comprehensive review [26].

Finally, strong pharmacological and biochemical evidence has demonstrated that eCBs are able to interact also with non-CB_1/non-CB_2 receptors, further increasing the complexity of the ECS and of the signaling pathways trigged thereof (Fig. 3). In particular, the best known of these targets is the transient receptor potential vanilloid type 1 (TRPV1) channel, which is activated by both AEA [27] and 2-AG [28]. Other potential receptors activated by eCBs are peroxisome proliferator-activated receptor (PPAR) α and γ [29], and the orphan G protein-coupled receptor GPR55 [30].

In Table 1 old and new members of the ECS are listed together. Unsurprisingly, ECS has been shown to regulate different physiological processes in the central nervous system [2–4] and at the periphery [26], thereby suggesting that its signaling may foster the development of pathway-selective drugs for therapeutic benefit [2–4].

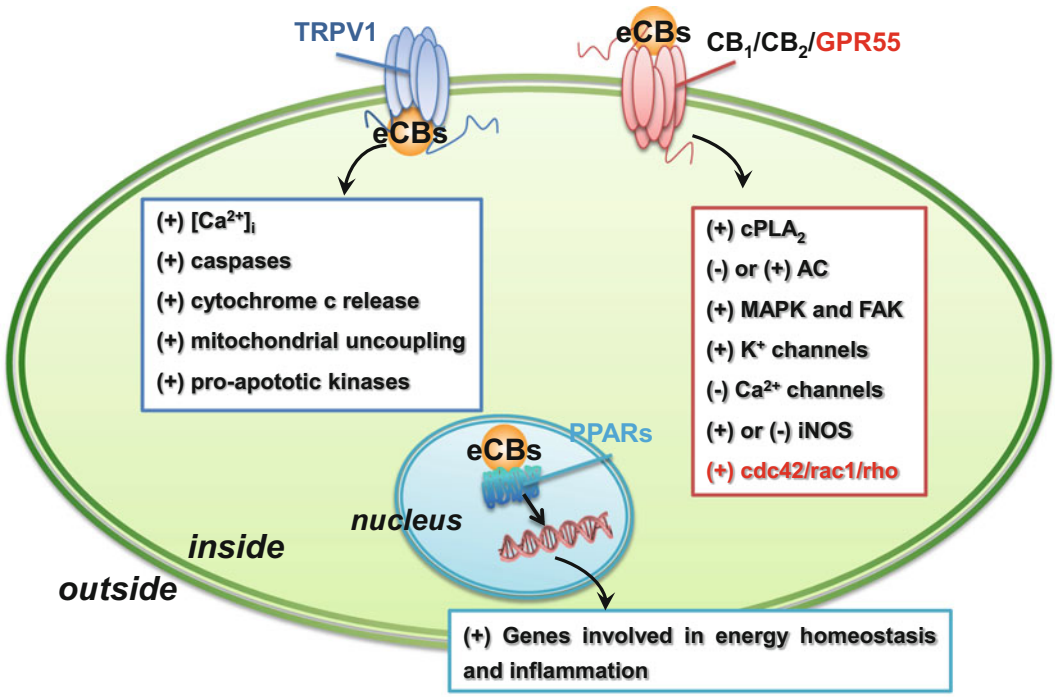


Fig. 3 Signal transduction pathways triggered by endocannabinoids through their main target receptors. *AC* adenylyl cyclase, *CB1* type-1 cannabinoid receptor, *CB2* type-2 cannabinoid receptor, *cPLA2* cytosolic phospholipase A_2, *eCBs* endocannabinoids, *FAK* focal adhesion kinase, *GPR55* orphan G protein-coupled receptor 55, *MAPK* mitogen-activated protein kinase, *iNOS* inducible nitric oxide synthase, *PPARs* peroxisome proliferator-activated receptors, *TRPV1* transient receptor potential vanilloid type 1 channel

2 Conclusions

Taken together, it appears all the more important to develop methods and protocols that allow to properly assay activity and location of the different ECS elements, possibly with specifications that make the same method fully effective in different cells, tissues, and organisms. For most ECS elements reliable methods are indeed available, and will be presented in this theme issue on "Endocannabinoid signaling: Methods and protocols" by those who developed and/or improved them over the last few years. Such a book is a manual that puts together all current methodologies to investigate eCB signaling in a timely manner. Thus, I believe that it will help chemists, drug designers, biochemists, molecular biologists, cell biologists, pharmacologists, and (electro)physiologists to successfully navigate with appropriate tools the *mare magnum* of eCB research.

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References

1. Pertwee RG, Howlett AC, Abood ME et al (2010) International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2. Pharmacol Rev 62:588–631
2. Maccarrone M, Guzmán M, Mackie K et al (2014) Programming of neural cells by (endo) cannabinoids: from physiological rules to emerging therapies. Nat Rev Neurosci 15:786–801
3. Di Marzo V, Stella N, Zimmer A (2015) Endocannabinoid signalling and the deteriorating brain. Nat Rev Neurosci 16:30–42
4. Di Patrizio NV, Piomelli D (2012) The thrifty lipids: endocannabinoids and the neural control of energy conservation. Trends Neurosci 35:403–411
5. Artmann A, Petersen G, Hellgren LI et al (2008) Influence of dietary fatty acids on endocannabinoid and N-acylethanolamine levels in rat brain, liver and small intestine. Biochim Biophys Acta 1781:200–212
6. Lucanic M, Held JM, Vantipalli MC et al (2011) N-acylethanolamine signalling mediates the effect of diet on lifespan in Caenorhabditis elegans. Nature 473:226–229
7. Brown I, Cascio MG, Wahle KW et al (2010) Cannabinoid receptor-dependent and -independent anti-proliferative effects of omega-3 ethanolamides in androgen receptor-positive and -negative prostate cancer cell lines. Carcinogenesis 31:1584–1591
8. Fezza F, Bari M, Florio R et al (2014) Endocannabinoids, related compounds and their metabolic routes. Molecules 19: 17078–17106
9. Ueda N, Tsuboi K, Uyama T (2013) Metabolism of endocannabinoids and related N-acylethanolamines: canonical and alternative pathways. FEBS J 280:1874–1894
10. Jin XH, Uyama T, Wang J et al (2009) cDNA cloning and characterization of human and mouse Ca(2+)-independent phosphatidylethanolamine N-acyltransferases. Biochim Biophys Acta 1791:32–38
11. Bisogno T, Howell F, Williams G et al (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. J Cell Biol 163:463–468
12. Chicca A, Marazzi J, Nicolussi S et al (2012) Evidence for bidirectional endocannabinoid transport across cell membranes. J Biol Chem 287:34660–34682
13. McKinney K, Cravatt BF (2005) Structure and function of fatty acid amide hydrolase. Annu Rev Biochem 74:411–432
14. Fezza F, De Simone C, Amadio D et al (2008) Fatty acid amide hydrolase: a gate-keeper of the endocannabinoid system. Subcell Biochem 49:101–132
15. Tsuboi K, Takezaki N, Ueda N (2007) The N-acylethanolamine-hydrolyzing acid amidase (NAAA). Chem Biodivers 4:1914–1925
16. Blankman JL, Simon GM, Cravatt BF (2007) A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem Biol 14:1347–1356
17. Rouzer CA, Marnett LJ (2011) Endocannabinoid oxygenation by cyclooxygenases, lipoxygenases, and cytochromes P450: cross-talk between the eicosanoid and endocannabinoid signaling pathways. Chem Rev 111:5899–5921
18. Van der Stelt M, van Kuik JA, Bari M et al (2002) Oxygenated metabolites of anandamide and 2-arachidonoylglycerol: conformational analysis and interaction with cannabinoid receptors, membrane transporter, and fatty acid amide hydrolase. J Med Chem 45:3709–3720
19. Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871–1875
20. Snider NT, Walker VJ, Hollenberg PF (2010) Oxidation of the endogenous cannabinoid arachidonoyl ethanolamide by the cytochrome P_{450} monooxygenases: physiological and pharmacological implications. Pharmacol Rev 62:136–154

21. Maccarrone M, Dainese E, Oddi S (2010) Intracellular trafficking of anandamide: new concepts for signaling. Trends Biochem Sci 35:601–608
22. Oddi S, Fezza F, Pasquariello N et al (2008) Evidence for the intracellular accumulation of anandamide in adiposomes. Cell Mol Life Sci 65:840–850
23. Kaczocha M, Vivieca S, Sun J et al (2012) Fatty acid-binding proteins transport N-acylethanolamines to nuclear receptors and are targets of endocannabinoid transport inhibitors. J Biol Chem 287:3415–3424
24. Mechoulam R, Hanuš LO, Pertwee R et al (2014) Early phytocannabinoid chemistry to endocannabinoids and beyond. Nat Rev Neurosci 15:757–764
25. Soltesz I, Alger BE, Kano M et al (2015) Weeding out bad waves: towards selective cannabinoid circuit control in epilepsy. Nat Rev Neurosci 16:264–277
26. Maccarrone M, Bab I, Bíró T et al (2015) Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci 36:277–296
27. Di Marzo V, De Petrocellis L (2010) Endocannabinoids as regulators of transient receptor potential (TRP) channels: A further opportunity to develop new endocannabinoid-based therapeutic drugs. Curr Med Chem 17:1430–1449
28. Zygmunt PM, Ermund A, Movahed P et al (2013) Monoacylglycerols activate TRPV1-a link between phospholipase C and TRPV1. PLoS One 8, e81618
29. Pistis M, Melis M (2010) From surface to nuclear receptors: the endocannabinoid family extends its assets. Curr Med Chem 17:1450–1467
30. Ross RA (2009) The enigmatic pharmacology of GPR55. Trends Pharmacol Sci 30: 156–163

Chapter 2

Extraction and Simultaneous Quantification of Endocannabinoids and Endocannabinoid-Like Lipids in Biological Tissues

Laura Bindila and Beat Lutz

Abstract

Extraction and quantification of endocannabinoids (eCBs) from biological tissues are essential to unravel their changes in physiological and pathophysiological conditions. We describe here an analytical protocol for extraction of endocannabinoids, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), endocannabinoid-like lipids such as palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA), as well as arachidonic acid (AA) from biological tissues using liquid-liquid extraction method and simultaneous quantification by liquid chromatography multiple reaction monitoring (LC/MRM).

Key words Endocannabinoids, Endocannabinoid-like lipids, Liquid-chromatography, Multiple reaction monitoring, Lipid extraction

1 Introduction

The endocannabinoids (eCBs), anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), and endocannabinoid-like lipids such as palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA) are bioactive lipids originating from the degradation of phospholipids and serve themselves as substrate to other bioactive lipids, such as the large family of lipid signaling molecules of the prostaglandin class. The endocannabinoid (eCB) system is involved in the regulation of a multitude of physiological and pathophysiological mechanisms in central and peripheral nervous system, immune system and peripheral organs, and is recognized as potential therapeutic target system. Accordingly, the determination of the spatial and temporal dynamics of the eCB levels of various physiological and pathophysiological states in various biological matrices has become a relevant goal in (pre)clinical research.

Mass spectrometry (MS) is nowadays the core technology in molecular characterization of biological matrices, and has been in

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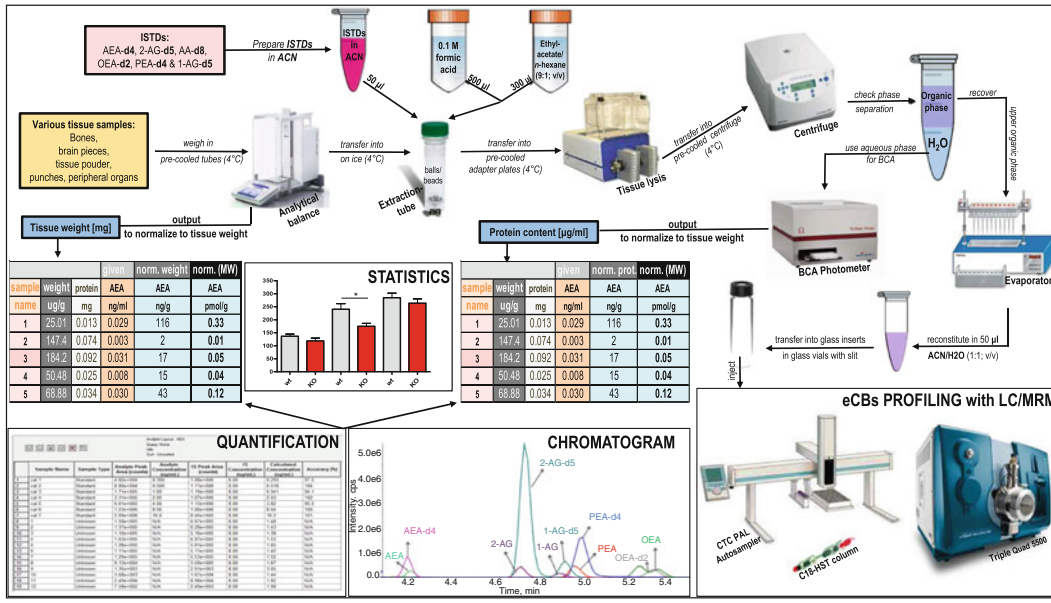
fact the crucial technology in discovery and quantification of endocannabinoids. Advances in the MS technology, particularly in MS-based quantitative assay using liquid chromatography (LC) coupled to multiple reaction monitoring (MRM), have allowed fast and so far the most sensitive quantitative assay for eCBs in various biological matrices, tissues, cells, and biological fluids [1–3]. Main challenges associated with quantification of eCBs stem from their originally low concentration in biological matrices, the diversity of biological matrices where they are present, and their rapidly changing levels during and post sample isolation through ex vivo degradation and synthesis, and chemical modification such as isomerization and oxidation. Such artificial changes in the eCB levels readily occur due to small variations in temperature and time of tissue sampling and handling, materials and equipment for tissue processing, and extraction conditions, making the comparative studies as well as inter- and intra-laboratory reproducibility challenging.

Here we describe an analytical protocol for simultaneous profiling of AEA and 2-AG, 1-AG, PEA, OEA, and AA using liquid-liquid extraction and quantitative assessment by LC/MRM. This protocol is amenable for a variety of biological tissues originating from brain, peripheral organs, bones, fat tissues, and muscles in terms of (a) tissue handling and extraction of eCBs with minimum and controlled ex vivo alteration of their levels, (b) inter- and cross-study reproducibility of the quantitative eCB data, (c) easy adaptability to various type, size, texture, and composition of tissues, and (d) amenability to higher throughput processing [4–7] (Fig. 1).



sample	weight	protein	given AEA	norm. weight AEA	norm. (MW) AEA
name	ug/g	mg	ng/ml	ng/g	pmol/g
1	25.01	0.013	0.029	116	0.33
2	147.4	0.074	0.003	2	0.01
3	184.2	0.092	0.031	17	0.05
4	50.48	0.025	0.008	15	0.04
5	68.88	0.034	0.030	43	0.12

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5	68.88	0.034	0.030	43	0.12

Fig. 1 Schematic representation of the analytical workflow for extraction and quantification of AEA, 2-AG, 1-AG, PEA, OEA, and AA

2 Materials

Prepare all solutions for extraction of eCBs and LC/MRM analysis using LC/MS-grade water and solvents. Use only freshly prepared solutions.

2.1 Tissue Extraction Components

1. Homogenization solution: 0.1 M Formic acid.
2. Extraction solution: Ethylacetate/*n*-hexane (9:1; v/v).
3. Extraction tubes: 1.2 ml Strips (*see* **Note 1**).
4. 1.5 ml Tubes for recovery of the lipid extracts (*see* **Note 2**).
5. Steel balls (*see* **Note 3**).
6. Precision balance maintained at 4 °C.
7. Evaporator.
8. Tissue lyser.
9. Centrifuge.
10. Vortex.

2.2 eCB Internal Standards and Calibration Standards

1. Deuterated standards: AEA-d4, 2-AG-d5, AA-d8, OEA-d2, PEA-d4, 1-AG-d5. Aliquot the original samples in acetonitrile and store at –20 °C (*see* **Note 4**).
2. Standard: AEA, 2-AG, AA, OEA, PEA, 1-AG. Aliquot the original samples in acetonitrile and store at –20 °C (*see* **Note 5**).

2.3 LC/MRM

1. LC solvents: Solvent A 0.1 % formic acid in water; solvent B 0.1 % formic acid in acetonitrile.
2. LC glass vials with siliconized inserts (*see* **Note 6**).
3. LC column: 2.5 μm C18(2)-HST column, 100 mm × 2 mm, combined with a pre-column (C18, 4 mm × 2 mm).
4. Autosampler maintained at 4 °C.
5. LC instrument.
6. 5500 QTrap triple-quadrupole linear ion trap mass spectrometer equipped with a Turbo V Ion Source (AB SCIEX) and polarity switching mode or another triple-quadrupole mass spectrometer.
7. LC quality control sample: Mix deuterated eCB standards and standard eCBs to an equimolar mixture in acetonitrile/water (1:1, v/v). Store at –20 °C before use.
8. Acetonitrile/water solution (1:1, v/v), prepare fresh and maintain at 4 °C.
9. Acetonitrile/isopropanol solution (1:1, v/v), prepare fresh and maintain at 4 °C.

2.4 BCA

1. Fluostar instrument.
2. BCA working reagent and calibrants.
3. BCA well plates.
4. 1.5 ml Tubes.

3 Methods

3.1 Tissue Isolation and Pre-processing

1. Isolate the tissue according to its source within the same time frame for all samples to be investigated (*see* **Note 7**).
2. If necessary to dissect tissue or remove specific (sub)areas of tissues, or perform punches, carry out this step on ice or at cold temperature (*see* **Note 8**).
3. Use precooled 1.5 or 2 ml tubes to transfer the tissue following sampling.
4. Snap freeze immediately the tissue and store at −80 °C (*see* **Note 9**).

3.2 Preliminary Steps for Extraction of eCBs from Tissues

1. Equilibrate extraction tubes to 4 °C for 30 min.
2. Equilibrate balance at 4 °C and calibrate.
3. Precool centrifuge at 4 °C.
4. Prepare fresh homogenization and extraction solution, cool, and keep at 4 °C.
5. Place tubes containing the frozen tissue on dry ice.
6. Cool steel balls at 4 °C.

3.3 Tissue Weighing

1. Tare the cooled extraction tubes on balance (*see* **Note 10**).
2. Transfer the frozen tissue from the original tubes to the extraction tubes and weigh (*see* **Notes 11** and **12**).
3. Place the extraction tubes containing the weighed tissue immediately on dry ice.

3.4 Extraction of eCBs

1. Prepare spiking solution of deuterated internal standards: Set the target concentration of each internal standard in the final extracts (50 μl) for LC/MRM analysis. Prepare a mixture of deuterated eCB standards in acetonitrile to a 20 times higher concentration than the target concentration in the final extracts for LC/MRM. The volume of spiking solution needed is 50 μl × no. of samples + no. of calibration solutions × 50 μl. Keep the spiking solution at 4 °C till extraction of samples (*see* **Note 13**).
2. Add cold steel balls in the extraction tubes.
3. Dilute 1:20 in acetonitrile a volume of spiking solution corresponding to 50 μl × no. of samples. Perform this step on ice.

4. Accurately pipette 50 µl of the diluted cold spiking solution to each sample.
5. Add 500 µl of cold homogenization solution, followed by 300 µl cold extraction solution (*see* **Note 14**).
6. Place the extraction tubes in tissue lyser holder and prepare a counterbalance plate.
7. Homogenize without delay in tissue lyser. Typical settings for homogenization: one cycle of 30 s at 30 Hz (*see* **Note 15**).
8. Transfer immediately the extraction tubes from tissue lyser to precooled (4 °C) centrifuge.
9. Centrifuge the extraction tubes for 10 min at 8000 × *g*, at 4 °C.
10. Transfer the extraction tubes in a precooled metal rack and place them at –20 °C for 10–30 min to freeze the lower aqueous phase.
11. Recover the organic phase into 1.5 ml tubes. These tubes will contain the lipid extracts.
12. Place the tubes containing the organic phase into pre-set evaporator at 37 °C and evaporate to dryness. These tubes will contain the extracted lipids, e.g., eCBs (*see* **Note 16**).
13. Store the remaining aqueous phase from **step 10** at –20 °C (*see* **Note 17**).

3.5 LC/MRM

1. Calibration curve solutions: Prepare fresh, on the day of analysis, a mixture of standard eCBs in acetonitrile in an LC glass vial. Pipette increasing volumes of this standard eCB mixture into LC glass vials in order to create a calibration curve. Add 50 µl of spiking solution of internal standards (*see* Subheading 3.4) to each vial and complement with appropriate volume of acetonitrile up to 500 µl and then add 500 µl of water in each vial to make up for 1 ml each calibration solution (*see* **Note 18**).
2. Accurately pipette cold 50 µl acetonitrile/water into the tubes containing the dry lipid extracts.
3. Vortex for 30 s at room temperature.
4. Centrifuge for 3 min at 10,000 × *g* at 4 °C.
5. Transfer 30 µl of the sample solutions from tubes into LC glass inserts, placed into LC glass vials, and close with open lid caps (*see* **Note 19**).
6. Place the sample-containing LC vials and the vials containing calibration standards into autosampler maintained at 4 °C.
7. Set the following conditions for LC/MRM method (*see* **Note 20**):

   LC gradient: Increase acetonitrile containing 0.1 % formic acid over 2 min from 55 to 90 %, and maintain it at 90 % for 5.5 min.

LC flow rate: 300 µl/min.
LC injection volume: 20 µl; use pre- and post-syringe cleaning.
Ion source: +4800 V for positive ion mode, –4500 V for negative ion mode; curtain gas: 40; temperature (TEM): 550 °C; ion source gas 1 and 2: 50; polarity switching time: 50 ms.
MRM transitions in positive ion mode: AEA, *m/z* 348.3 to *m/z* 62.1; AEA-d4, *m/z* 352.3 to *m/z* 62.1; 2-AG, *m/z* 379.1 to *m/z* 287.2; 2-AG-d5, *m/z* 384.2 to *m/z* 287.2; PEA, *m/z* 300.2 to *m/z* 62.1; PEA-d4, *m/z* 304.2 to *m/z* 62.1; OEA, *m/z 326.2 to m/z 62.1*; *OEA-d2*, *m/z 328.2 to m/z 62.1*.
Compound parameters in positive ion mode: EP: 10 V; dwell time: 20 ms; DP: 70 V; CE: for AEA, AEA-d4, 2-AG, 2-AG-d5 22 V, for OEA, PEA, PEA-d4 21 V and for OEA-d2 23 V; CXP: for AEA, AEA-d4 9 V, for 2-AG, 2-AG-d5 30 V, for OEA, OEA-d2, PEA, PEA-d4 10 V.
MRM transitions negative ion mode AA, *m/z* 303.05 to *m/z* 259.1; AA-d8, *m/z* 311.04 to *m/z* 267.0.
Compound parameters in negative ion mode: EP: –10 V; dwell time: 50 ms; DP: –200 V; CE: –19 V; CXP: –12 V.

8. Equilibrate the LC/MRM system with the instrument default method for equilibration, for 2–5 min.
9. Equilibrate LC/MRM system with the method for eCB analysis for 5–10 min.
10. Verify the performance of the LC/MRM using the LC quality control sample.
11. Write a sample batch for LC/MRM analysis. A minimum of three calibration curves are necessary: one at the beginning of the batch, one in the middle of the batch, and one at the end of the batch. Place a wash run before and after every calibration curve. For the wash run inject 20 µl of acetonitrile/isopropanol (1:1, v/v). Run regularly LC quality control samples (it can also be a calibration solution) between samples without pre- or post-quality control wash run.
    Run minimum five washes: by injecting 20 µl of acetonitrile/isopropanol (1:1, v/v) after batch analysis, followed by control sample of the system (*see* **Note 21**).
12. Following analysis remove the LC glass vials containing the samples from the autosampler, replace the open lid caps with closed lid caps, and store the remaining solution at –20 °C.
13. Quantify the data using the Analyst software.
14. Import the Analyst-calculated concentrations of eCBs into an Excel sheet. Input the tissue weight and/or protein amount corresponding to every sample and normalize the values of the Analyst-calculated concentrations of eCBs to tissue weight and/or protein content.

15. Perform statistical analysis when sample groups are to be compared.

3.6 BCA Assay

1. Remove the extraction tubes containing the aqueous phase from −20 °C and let it thaw for 30 min.
2. Dilute 1:10 a volume of 20 μl aqueous phase and run BCA assay according to the provider's instructions. Repeat the measurement with another dilution rate if the protein values are out of range.
3. Retrieve and store the protein concentration data.

4 Notes

1. 2 ml Precellys tubes can alternatively be used when the tissue samples are large and do not fit into the 1.2 ml strips.
2. When processing more samples, in our hands more than 20, we use microtiter plates or deep well plates to increase the speed of pipetting.
3. Even though one steel ball can be used, we find it more effectively to use small steel balls for homogenization with the tissue lyser. Ceramic beads can alternatively be used.
4. We typically dilute the deuterated standards as obtained from provider in acetonitrile and further aliquot. To avoid isomerization or other chemical modifications we do not thaw and freeze these aliquots for multiple use, but use them one time for tissue extraction.
5. We typically dilute the standards as obtained from provider in acetonitrile and further aliquot. To avoid isomerization or other chemical modifications we do not thaw and freeze these aliquots for multiple use, but use them one time for tissue extraction.
6. When processing more than 20 samples we use coated (e.g., silic-onized) microtiter or deep well plates for LC/MRM analysis to increase the speed of pipetting. Moreover, the storage of the rest samples post-analysis is more space effective.
7. Because postmortem alteration of eCB levels is readily occurring it is essential that tissue sampling is as fast as possible, irrespective of the tissue origin, to prevent such alterations. Moreover, for reliable comparative studies and minimum variability in the eCB levels due to tissue sampling procedure, the time required for sampling has to be equal for all samples to be compared in one or more biological replicate analysis.
8. When tissue dissection or isolation of specific subregions of tissues is necessary, place the original tissue directly on ice to perform such isolation. Also, any transport of the tissue from

one bench or lab to another has to be carried out on ice. For tissues such as brain, we typically freeze the entire brain at −80 °C prior to dissection, and then for brain region isolation we thaw the brain on ice and perform the isolation at 4 °C. This way the time and temperature conditions necessary to perform region isolation can be controlled to be similar and short for all samples to be compared to prevent variability in the rate of ex vivo synthesis/degradation of eCBs.

9. For tissue samples where multiple types of analysis has to be carried out from the same tissue source, but precluding pitfalls due to tissue heterogeneity, we pulverize the tissue and split for the corresponding analysis. When the tissue samples are hard tissues such as bone, or fibrous tissues such as heart and lungs, we cut the tissue in smaller pieces while frozen using a steel scissors or cutter to enhance the homogenization step.
10. Place the balance in the cold room to carry out the weighing or use a balance with a temperature control, maintaining it at 4 °C to avoid tissue thawing. Also, make sure that you do not hold the tubes in your hands more than necessary to avoid thawing of the tissue. The tissue weighing is time consuming, when analyzing large number of samples. We do weigh the tissue only if the biological question specifically requires normalization of the eCB values to tissue weight, or when reference/comparison to previously published data on eCB levels/tissue weight is necessary (*see* **Note 17**).
11. For larger tissues, we reduce the tissue sample by cutting or by pulverization and splitting the sample. We typically do not process tissue samples larger than 30 mg using this extraction method. This is due to increased difficulty for lipid-protein-phase separation under the limited volume of homogenization and extraction solvent used for these tubes and additionally because the homogenization becomes often ineffective, or requires too many homogenization cycles that lead to temperature increase in the sample, hence ex vivo eCB level alteration.
12. For very small tissues, such as biopsies or punches, we do not weigh the tissue at all, because it leads to fast, uncontrollable thawing during handling, which in turn leads to ex vivo degradation/synthesis of the eCBs and consequently to large variability in the eCB levels. In such cases, protein content (determined by a protein assay of the aqueous phase, see below extraction procedure) will be used to normalize the eCB values.
13. The target concentration of the internal standards in the final extracts is set based on preliminary tests on reference tissues. We typically use a target concentration of deuterated standards corresponding to the middle calibration curve of the LC/MRM, which in turn is tailored using reference tissue to encompass the reference values of eCB levels for the tissue type under investigation.

14. Irrespective of the tissue type, we invariably perform tissue lysis/homogenization with extraction solvent included. In our hands, this gave the highest abundance of the endogenous eCBs in LC/MRM analysis, possibly due to a more effective release of eCBs from their location in the tissue.
15. Visually inspect the homogenized samples after one cycle and assess if pieces of tissue are still visible. If so, repeat for one or two cycles of homogenization. Homogenization and extraction solvent volume can additionally be added, if the phase separation is not appropriate that is usually the case for very large samples.
16. If not possible to analyze immediately the samples by LC/MRM, store the dried extracts at −20 °C.
17. The aqueous phase can be used to determine the protein content of the tissue by a protein assay and normalize the eCB values to this content (*see* also **Note 12**) as an alternative to weight for small tissue samples such as biopsies and punches. We typically use normalization to protein content because of increased throughput possible, as compared to weighing.
18. The calibration curve range is tailored to the tissue type using reference tissue. We use the eCB levels determined in a reference.
19. We typically transfer 30 µl of the extracts into LC vials or well plates for analysis. The leftover 20 µl of extracts are immediately frozen and stored at −20 °C for an additional analytical replicate if necessary, or can be used for additional analysis in another ionization mode, if polarity switching is not available on the mass spectrometer.
20. The parameters and conditions for LC/MRM are valid for the lab equipment mentioned here. For other MS, LC instruments, or other LC column provider type all these settings have to be optimized accordingly. When polarity switching mode is not available, you can use the rest of the eCB extract to analyze the sample in the additional ionization mode.
21. Typically, we run a quality control every 20 biological samples and a calibration curve every 40 biological samples.

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References

1. Zoerner AA, Gutzki FM, Batkai S et al (2011) Quantification of endocannabinoids in biological systems by chromatography and mass spectrometry: a comprehensive review from an analytical and biological perspective. Biochim Biophys Acta 1811:706–723
2. Buczynski MW, Parsons LH (2010) Quantification of brain endocannabinoid levels: methods, interpretations and pitfalls. Br J Pharmacol 160: 423–442
3. Lerner R, Lutz B, Bindila L (2013) Tricks and tracks in the identification and qualification of endocannabinoids. eLS. Wiley, Chichester. doi:10.1002/9780470015902.a0023407
4. Duerr GD, Heinemann JC, Gestrich C et al (2014) Impaired border zone formation and adverse remodeling after reperfused myocardial infarction in cannabinoid CB2 receptor deficient mice. Life Sci 14:324–325
5. Lomazzo E, Bindila L, Remmers F et al (2015) Therapeutic potential of inhibitors of endocannabinoid degradation for the treatment of stress-related hyperalgesia in an animal model of chronic pain. Neuropsychopharmacology 40:488–501
6. Jergas B, Schulte K, Bindila L et al (2014) O-2050 facilitates noradrenaline release and increases the CB1 receptor inverse agonistic effect of rimonabant in the guinea pig hippocampus. Naunyn Schmiedebergs Arch Pharmacol 387:621–628
7. Wenzel D, Matthey M, Bindila L et al (2013) Endocannabinoid anandamide mediates hypoxic pulmonary vasoconstriction. Proc Natl Acad Sci U S A 110:18710–18715

Chapter 3

Determination of 2-Arachidonoylglycerol by μSPE-LC-MS/MS

Natalia Battista and Manuel Sergi

Abstract

LC-MS/MS is a powerful analytical technique that provides unequivocal identification and reliable quantification of the analytes, using Selected Reaction Monitoring or Multi Reaction Monitoring acquisition mode.

2-Arachidonoylglycerol (2-AG) is the most abundant endocannabinoid, which plays a major role in a wide variety of physiological and pathological processes. Analysis of 2-AG by means of LC-MS/MS allows the detection of very low concentrations in biological samples. Here, we describe how to determine 2-AG levels in tiny samples of tissues and plasma through LC-MS/MS, by using very quick and easy to perform extraction procedures, with reduced solvent consumption.

Key words 2-AG, μSPE, LC-MS/MS, Plasma, Tissue

1 Introduction

High-performance liquid chromatography (HPLC) is a well-established separation technique that is able to solve numerous analytical problems. Several developments have been done recently to improve HPLC performance, the most important possibly being Ultra High Performance Liquid Chromatography (UHPLC or UPLC) [1, 2]. Yet, due to the cost of UPLC hardware, chromatographic research addressed to improve HPLC performance in terms of faster separation and efficiency. Thus, when UPLC is not available, alternative devices and technologies have been developed for fast chromatography: high-temperature liquid chromatography (HTLC), and use of monolithic supports or of column with superficially porous packing materials based on silica particles with non-porous cores [3]. The latter solution is very interesting, because it allows obtaining high efficiency with common HPLC devices.

Liquid chromatography–mass spectrometry or tandem mass spectrometry (LC-MS or LC-MS/MS) is largely replacing gas chromatography (GC)-MS in most biomedical applications, as

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they do not require derivatization steps and are capable of simultaneous determination in a single analysis with reduced sample pretreatment [4].

Moreover, MS/MS is considered necessary to provide unequivocal identification and quantification of analytes, due to the high selectivity of the MS detector [5]. The quadrupole is the most common mass analyzer, due to its cheapness and high performance allowed by high efficiency transmission. The first implementation of a two-dimensional MS (MS/MS or MS^2) was obtained with a triple quadrupole mass analyzer that is nowadays the most common device. The first and third (Q1 and Q3) quadrupoles are separative, and the second (Q2) is usually a collision cell, where the ions can be fragmented, as depicted in Fig. 1.

MS^2 enables the reduction of background noise and increase in selectivity, two features that also allow different types of experiments: Full Scan (FS), Selected Ion Monitoring (SIM), Product Ion Scan (PIS), Precursor Ion Scan, Selected Reaction Monitoring or Multi Reaction Monitoring (SRM or MRM), the latter being most widely used for the confirmatory analysis of target molecules.

LC and MS coupling is not straightforward, as chromatographic separation occurs with neutral molecules in high operating pressure solution, while MS responds to ions in the gas phase and requires deep vacuum [6].

The most widely used technique is electrospray ionization (ESI), which is suitable for the analysis of medium to highly polar compounds. Atmospheric pressure chemical ionization (APCI) is more efficient for nonpolar (hydrophobic) analytes, which do not readily form ions in solution [7]. Instead, thermally labile compounds may decompose in the ionization source, since APCI requires a heated nebulizer [7].

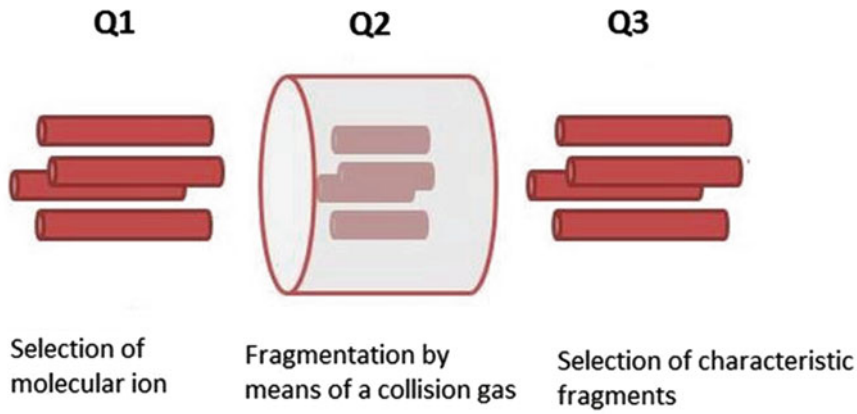

Fig. 1 Schematic description of a triple quadrupole mass spectrometer: first quadrupole (Q1), second quadrupole (Q2), which acts as a collision cell, and third quadrupole (Q3)

2-Arachidonoylglycerol (2-AG) is the most abundant endocannabinoid, identified for the first time in brain tissues [8]. 2-AG plays a major role in a wide variety of physiological and pathological processes [9]. The measurement of circulating levels of 2-AG in several biological matrices, including also fluids such as blood [10–12] and seminal plasma [13], is an essential step to determine both "signaling" and "metabolic-intermediate" levels of this endocannabinoid [14]. Despite great improvements in instrumentation, the quantitation of 2-AG remains an analytical challenge, also because of its instability that makes it easily isomerize into 1-AG [15].

2 Materials

2-AG and [$^2H_8$]-2-AG standard (0.26 mM, 100 μg/ml in acetonitrile).

Acetone, acetonitrile, chloroform, formic acid, and methanol are of RS-Plus grade.

Ultrapure water is produced by a Milli-Q Plus apparatus.

OMIX C_{18} tips.

Kinetex C18-XB chromatographic column (100 × 2.1 mm) packed with 2.6 μm average diameter core-shell particles, and provided with a guard column.

2.1 Specimens Collection

Tissues must be snap-frozen in liquid nitrogen after collection (*see* **Note 1**) and stored at −80 °C (*see* **Note 2**).

2.1.1 Tissue

2.1.2 Plasma

1. Collect blood in a Vacutainer tube with sodium citrate.
2. Centrifuge blood in the collection tube for 15 min at 150 × *g* with brake off switch.
3. Remove the tube from the centrifuge.
4. Transfer plasma (top layer) to a 15 ml centrifuge tube. Be careful not to aspirate cells from the buffy coat (cellular) layer.
5. Centrifuge plasma in the 15 ml centrifuge tube for 15 min at 1000 × *g*.
6. Transfer plasma into a 8 ml glass vial and store at −80 °C (*see* **Note 2**).

3 Methods

3.1 Extraction from Tissue

1. Warm at room temperature and vortex the [$^2H_8$]-2-AG standard (stored at −80 °C).
2. Prepare a solution of internal standard (IS) in ice-cold methanol, in order to add 50 pmoles of [$^2H_8$]-2-AG per sample.

3. Add 1 ml IS solution *per* 8 ml glass vial and keep the vial on ice.
4. Weight the frozen tissue and put it in the vial containing the IS solution.
5. Homogenize the tissue with an Ultra Turrax T25, keeping the vial on ice (*see* **Note 3**).
6. Add 2 ml chloroform per vial with vortexing.
7. Add 1 ml water per vial with vortexing.
8. Centrifuge the mixture at 2000 × *g* at 4 °C for 10 min.
9. Collect the lower organic phase using a Pasteur pipette, and discard the upper aqueous layer and the protein disk.
10. Evaporate the solvent to dryness in the vial under a gentle nitrogen stream.
11. Plug a Pasteur pipette 5–3/4″ length with glass wool.
12. Prepare a suspension of Silica Gel G (60-Å 230–400 Mesh ASTM) in chloroform (1:1, v/v) and load 1 ml of this suspension onto the Pasteur pipette from **step 11**.
13. Dissolve the dried lipids into 1 ml chloroform.
14. Load the sample onto the Silica G gel column.
15. Wash the vial with 1 ml of chloroform and repeat **step 14**.
16. Elute the analyte from the column with 1 ml of chloroform/methanol (9:1, v/v) and recover the eluate. Repeat **step 16**.
17. The collected eluate is dried under a gentle nitrogen stream.
18. Dissolve the dried lipids into 100 μl methanol and transfer the mixture to 1 ml glass vial with 0.1 ml conical glass insert.
19. The vial is transferred into the autosampler for the LC-MS/MS analysis.

3.2 Extraction from Plasma

1. Warm at room temperature and vortex the [$^2H_8$]-2-AG standard stored at −80 °C.
2. Prepare a solution of IS in 200 mM formic acid in ice-cold methanol, in order to add 2 pmoles of [$^2H_8$]-2-AG per sample.
3. Mix 100 μl of plasma with 100 μl of IS solution.
4. Sonicate the sample in an ultrasonic bath for 6 min at room temperature.
5. Centrifuge at 17,500 × *g* for 6 min at 4 °C.
6. Recover the upper phase and transfer into a 1 ml glass vial with 0.1 ml conical glass insert.
7. Condition the μSPE tip by flushing four times a 1:1 ultrapure water/acetonitrile solution and four times a 1:1 ultrapure

water/200 mM formic acid in methanol using an automatic propipette.

8. Load the sample by inserting the OMIX C_{18} tip into the vial containing the sample, and perform five load/release cycles.
9. Wash with 100 μl of a 9:1 (v/v) water/methanol solution (five load/release cycles).
10. Elute the analytes with 50 μl of 10 mM formic acid in methanol. Repeat this step five times, performing five load/release cycles for each step.
11. The collected eluate is transferred into 1 ml glass vial with 0.1 ml conical glass insert.
12. The vial is transferred into the autosampler for the LC-MS/MS analysis.

3.3 LC-MS/MS Analysis

The chromatographic run is carried out by means of a Series 200 system with 2 μ-LC pumps and autosampler from Perkin Elmer (Norwalk, CT, USA). The HPLC system is coupled to an API 2000 triple quadrupole mass spectrometer from ABSciex (Toronto, ON, Canada), equipped with a TurboIonSpray source.

The mobile phases are water (A) and 2.5 mM formic acid in methanol (B), at a flow rate of 0.4 ml min^{-1}. Only 0.1 ml min^{-1} is driven into the ion source by means of a 1:4 split. A gradient elution is applied, so that the organic phase increases from 80 to 84 % in 3 min and then to 100 % in 1 min. After 3 min at 100 % of organic phase, the column is led to the original ratio of 20 % B and 80 % A within 3 min, in order to enable equilibration of the column.

A timed switch valve (10-PORT Valco valve connected to the instrument) drives the effluent to the source only from 3 to 6.5 min. The resulting total run-time is 10 min.

All the analytes are detected in positive ionization mode with a capillary voltage of 5000 V and nebulizer gas (air) at 90 psi, while the turbo gas (nitrogen) is at 40 psi and 400 °C. For each analyte, two MRM transitions are selected. All sources and instrument parameters for the monitored analytes are tuned by injecting standard solutions at a concentration of 100 ng ml^{-1} (containing 10 mM of formic acid) at 10 μl min^{-1} by a syringe pump.

Peak areas for the selected ions are determined using ABSciex package Multiview 1.4 and quantitation is performed by the IS method. The selected transitions, together with the main LC-MS/MS parameters, are reported in Table 1 (*see* **Note 4**).

The quantification of 2-AG is accomplished by the use of deuterium-labeled IS *vs* a calibration curve in methanol acidified with 10 mM formic acid, in a 2.64–528 nM range (*see* **Note 5**).

A chromatogram showing the separation between 2-AG and 1-AG, obtained with the above described experimental conditions, is reported in Fig. 2.

Table 1
LC-MS/MS parameters for 2-AG and its IS

Analyte	RT (min)	Q1 (amu)	DP (V)	FP (V)	EP (V)	Q3 (amu)	CE (V)	CXP (V)
2-AG	4.2	379.1	21	400	7	287.2	21	15
						269.1	26	14
[$^2H_8$]-2-AG	4.2	387.2	21	400	7	295.0	21	15

RT retention time, *Q1* precursor ion mass, *DP* declustering potential, *FP* focusing potential, *EP* entrance potential, *Q3* product ion mass, *CE* collision energy, *CXP* cell exit potential

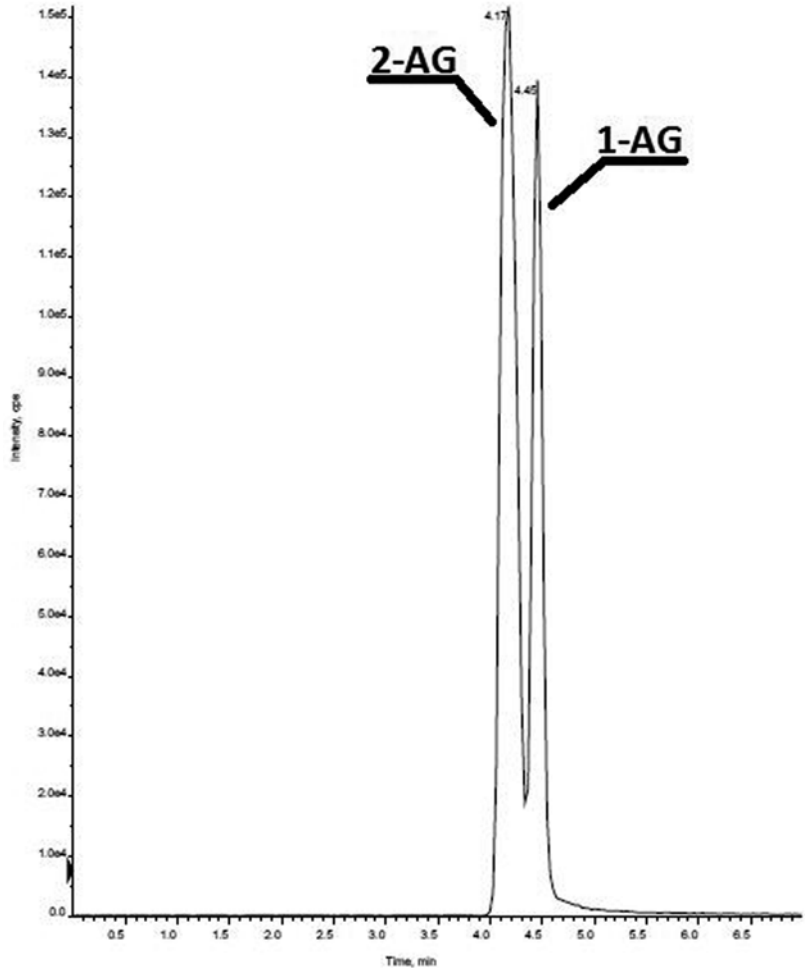

Fig. 2 Chromatogram of 2-AG and 1-AG obtained by LC-MS/MS in MRM acquisition mode

4 Notes

1. Use only Teflon caps, glass vials, and glass pipettes.
2. Biological samples must be snap-frozen at –80 °C immediately after collection.
3. It may be useful to normalize the amount of lipids to the protein content of the samples (cells or tissues). Before starting the extraction procedure, take 20 µl aliquots from the homogenates and determine protein concentration by using a Bradford protein concentration assay.
4. Perform quantitative analysis by selecting the $[M+H]^+$ adduct: 2-AG may form $[M+Na]^+$ adducts which do not lead to any fragmentation. It is important to verify the ratio between the $[M+H]^+$ adduct and the $[M+Na]^+$ adduct, to avoid dramatic loss in sensitivity.
5. Plot calibration curves as the peak area ratio of the analyte over its respective IS against the nominal concentration of the calibrator, by least square linear regression.

References

1. Nguyen DT, Guillarme D, Rudaz S et al (2006) Fast analysis in liquid chromatography using small particle size and high pressure. J Sep Sci 29:1836–1848
2. Wren SA, Tchelitcheff P (2006) Use of ultra-performance liquid chromatography in pharmaceutical development. J Chromatogr A 1119:140–146
3. Guillarme D, Ruta J, Rudaz S et al (2010) New trends in fast and high-resolution liquid chromatography: a critical comparison of existing approaches. Anal Bioanal Chem 397:1069–1082
4. Stout PR, Bynum ND, Mitchell JM et al (2009) A comparison of the validity of gas chromatography- mass spectrometry and liquid chromatography- tandem mass spectrometry analysis of urine samples for morphine, codeine, 6-acetylmorphine, and benzoylecgonine. J Anal Toxicol 33:398–408
5. Maralikova B, Weinmann W (2004) Confirmatory analysis for drugs of abuse in plasma and urine by high-performance liquid chromatography-tandem mass spectrometry with respect to criteria for compound identification. J Chromatogr B Analyt Technol Biomed Life Sci 811:21–30
6. Arpino PJ (1982) On-line liquid chromatography/mass spectrometry? An odd couple! Trends Anal Chem 1:154–158
7. Maurer HH (2007) Current role of liquid chromatography-mass spectrometry in clinical and forensic toxicology. Anal Bioanal Chem 388:1315–1325
8. Sugiura T, Kondo S, Sukagawa A et al (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:89–97
9. Maccarrone M, Guzmán M, Mackie K et al (2014) Programming of neural cells by (endo) cannabinoids: from physiological rules to emerging therapies. Nat Rev Neurosci 15:786–801
10. Thomas A, Hopfgartner G, Giroud C et al (2009) Quantitative and qualitative profiling of endocannabinoids in human plasma using a triple quadrupole linear ion trap mass spectrometer with liquid chromatography. Rapid Commun Mass Spectrom 23:629–638
11. Zoerner AA, Batkai S, Suchy MT et al (2011) Simultaneous UPLC-MS/MS quantification of the endocannabinoids 2-arachidonoyl glycerol (2AG), 1-arachidonoyl glycerol (1AG), and anandamide in human plasma: Minimization of matrix-effects, 2AG/1AG isomerization and degradation by toluene solvent extraction. J Chromatogr B Analyt Technol Biomed Life Sci 883:161–171

12. Sergi M, Battista N, Montesano C et al (2013) Determination of the two major endocannabinoids in human plasma by μ-SPE followed by HPLC-MS/MS. Anal Bioanal Chem 405:785–793
13. Lewis SEM, Rapino C, Di Tommaso M et al (2012) Differences in the endocannabinoid system of sperm from fertile and infertile men. PLoS One 7:e47704
14. Murataeva N, Straiker A, Mackie K (2014) Parsing the players: 2-arachidonoylglycerol synthesis and degradation in the CNS. Br J Pharmacol 171:1379–1391
15. Battista N, Sergi M, Montesano C et al (2014) Analytical approaches for the determination of phytocannabinoids and endocannabinoids in human matrices. Drug Test Anal 6:7–16

Chapter 4

Analysis of Omega-3 Fatty Acid Derived *N*-Acylethanolamines in Biological Matrices

Renger F. Witkamp and Michiel G.J. Balvers

Abstract

The adequate quantification of endocannabinoids can be complex due to their low endogenous levels and structural diversity. Therefore, advanced analytical approaches, such as LC-MS, are used to measure endocannabinoids in plasma, tissues, and other matrices. Recent work has shown that endocannabinoids that are synthesized from n-3 fatty acids, such as docosahexaenoylethanolamide (DHEA) and eicosapentaenoylethanolamide (EPEA), have anti-inflammatory and anti-tumorigenic properties and stimulate synapse formation in neurites. Here, an LC-MS based method for the quantification of n-3 endocannabinoids DHEA and EPEA which is also suited to measure a wider spectrum of endocannabinoids is described. The chapter contains a step-by-step protocol for the analysis of n-3 endocannabinoids in plasma, including sample collection and solid phase extraction, LC-MS analysis, and data processing. Modifications to the protocol that allow quantifying n-3 endocannabinoids in tissues and cell culture media will also be discussed. Finally, conditions that alter endocannabinoid concentrations are briefly discussed.

Key words Endocannabinoids, n-3 Fatty acids, Docosahexaenoylethanolamide, Eicosapentaenoylethanolamide, Solid phase extraction, LC-MS

1 Introduction

According to the current (IUPHAR) classification system, endocannabinoids constitute a relatively small group of fatty acid-derived endogenous ligands of the cannabinoid receptors CB_1 and CB_2 [1]. From a biochemical perspective, these endocannabinoids are part of a large family of structurally related amides, esters, and ethers of fatty acids, which are continuously formed and degraded in a dynamic equilibrium. The so-far best studied group of endocannabinoids is the class of *N*-acylethanolamines (NAEs), which comprises conjugates of fatty acids to ethanolamine, such as arachidonoylethanolamide (AEA, anandamide) [1]. Other examples of endocannabinoids include glycerol esters, such as 2-arachidonoylglycerol (2-AG), and the dopamine conjugate *N*-arachidonoyldopamine (NADA) [1].

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Next to AEA, ethanolamine conjugates have been described for palmitic acid, stearic acid, and the n-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid [2–5]. It has been shown that cells are able to "combine" different fatty acids and biogenic amines, thus producing several possible permutations of fatty acid amides [6, 7]. Studies have also demonstrated that the local relative availability of their fatty acid precursors, which is amongst others determined by diet, plays an important role in determining the pattern of amide conjugates formed [2, 3].

The high diversity and dynamics of endocannabinoids and related compounds in tissues demands for adequate methods for the quantification. Recently published reports are all using advanced analytical chemical techniques, such as gas chromatography coupled to mass spectrometry (GC-MS) or liquid chromatography coupled to mass spectrometry (LC-MS), to meet this challenge [8]. MS-based methods use the molecular mass of a compound to detect it, which allows simultaneously measuring a broad range of compounds in a single sample. The molecular mass of a compound is more specific than, e.g., its ultraviolet (UV) absorbance characteristics, and therefore MS analysis results in higher specificity. Another advantage of MS over optical detectors is that similar molecules of different molecular weight can be measured even when there is no chromatographic separation.

Typically, published methods on the quantification of "classical" endocannabinoids and related compounds are focusing on a limited number of molecules derived from selected precursor fatty acids, such as AEA, 2-AG, palmitoylethanolamine (PEA), and oleoylethanolamine (OEA). However, increasing data underline the (patho-) physiological relevance of compounds derived from other biologically important fatty acids. For instance, n-3 fatty acids are abundantly present in brain and important for brain functioning. We have also shown, using LC-MS, that n-3 fatty acid-derived endocannabinoids such as docosahexaenoylethanolamine (DHEA) and eicosapentaenoylethanolamine (EPEA) are present in mammalian plasma and other tissues, in concentrations similar to that of the "classical endocannabinoids" (*see* Fig. 1 for molecular structures) [4, 5, 9]. Work from our lab and others has also shown that DHEA and EPEA have biological effects on immune cells [10, 11], cancer cells [12, 13], and hippocampal cells [14]. Based on their affinity for CB_1 and CB_2, DHEA and EPEA could be classified as "true" endocannabinoids, although much of their bio-activity appears to be not directly related to cannabinoid receptors alone [11, 12].

In this chapter, we describe a protocol for the quantification of the n-3 fatty acid-derived endocannabinoids DHEA and EPEA in plasma using LC-MS. This method allows determining a wider spectrum of endocannabinoids, including AEA, 2-AG, and NADA, which we will also address briefly where relevant. Depending on

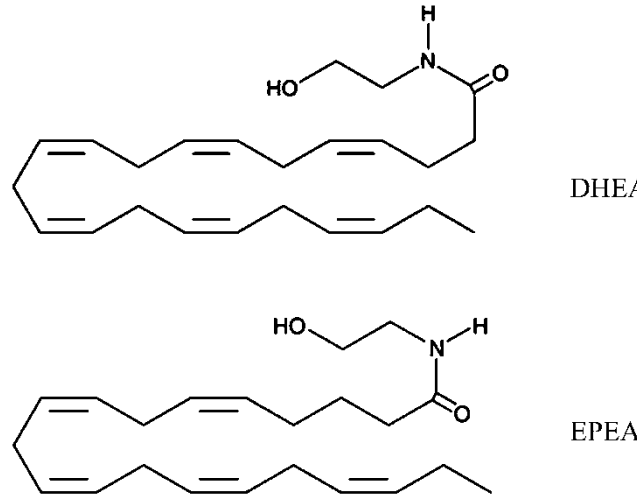

Fig. 1 Molecular structures for DHEA and EPEA

the sensitivity of the LC-MS system, 0.1–1 ml of plasma is sufficient to obtain adequate peaks. Plasma is collected from blood, and a plasma extract is made using acetonitrile (ACN), which is subsequently washed and concentrated using solid phase extraction (SPE). The eluate from the SPE step is evaporated to dryness, reconstituted, and subsequently analyzed using LC-MS. Proper data analysis is also crucial. Modifications to determine endocannabinoid levels in other matrices will also be discussed.

2 Materials

Water and solvents should be of high purity. Different suppliers might use different terminology, such as "LC-MS grade" or "ULC grade." Fresh, ultrapure deionized water is preferred, as it is present in most labs. MQ water of >18 MΩ at 25 °C is a good option.

Polypropylene-based plastics show good recoveries during the sample preparation; glass should be avoided, in particular for the vacuum concentration step [15].

2.1 Analytical Standard Solutions

2.1.1 Calibration Curve Solutions

Prepare a set of calibration solutions in ACN containing the concentrations described in Table 1. Store at −80 °C (*see* **Note 1**). All calibrators have the same concentration of deuterated analogues. During LC-MS analysis, both the intensity of the natural compound (e.g., AEA) and its deuterated analogue (e.g., AEA-d8) will be measured. The AEA:AEA-d8 ration will be proportional to the concentration. Deuterated standards will also be spiked to the plasma samples in exactly the same amounts as present in the calibrators (*see* **Note 2**).

Table 1
Overview of concentrations in calibration curve solutions

	cal8	cal7	cal6	cal5	cal4	cal3	cal2	cal1
Concentration (ng/ml)								
AEA	406.50	135.50	45.17	15.06	5.02	1.67	0.56	0.19
2-AG	5617.00	1872.33	624.11	208.04	69.35	23.12	7.71	2.57
DLE	140.40	46.80	15.60	5.20	1.73	0.58	0.19	0.064
OEA	140.40	46.80	15.60	5.20	1.73	0.58	0.19	0.064
PEA	140.40	46.80	15.60	5.20	1.73	0.58	0.19	0.064
SEA	69.50	23.17	7.72	2.57	0.86	0.29	0.10	0.032
DHEA	140.40	46.80	15.60	5.20	1.73	0.58	0.19	0.064
EPEA	500.00	166.67	55.56	18.52	6.17	2.06	0.69	0.23
AEA-d8	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25
2-AG-d8	750	750	750	750	750	750	750	750
PEA-d4	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
OEA-d4	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25

These are values that are normally used on our lab. Typically, our lower limit of quantification (LLOQ) is in the range of cal1 or cal2

2.1.2 Deuterated Standard Spiking Stock Solution

Prepare the spiking solution containing 312.5 ng/ml AEA-d8, 7500 ng/ml 2-AG-d8, and 312.5 ng/ml OEA-d8 in ACN (*see* **Note 2**). Store at −80 °C.

2.1.3 Reconstitution Solution

Prepare the reconstitution solution containing 12.5 ng/ml PEA-d4 in ACN. Store at −80 °C. It is recommended to make amounts sufficient to run hundreds of samples.

2.2 Solutions and Reagents

1. 100 mM phenylmethanesulfonyl fluoride (PMSF) solution in isopropanol. Store at 4–8 °C. A volume of 10 ml of 100 mM PMSF solution is sufficient to collect hundreds of samples. PMSF is an enzyme inhibitor which also inhibits fatty acid amide hydrolase (FAAH), the principal NAE-degrading enzyme (*see* **Note 3**).
2. Deuterated standard spiking work solution containing 100 μM PMSF. Make a tenfold dilution of the deuterated standard spiking stock solution in acetonitrile, and add 100 mM PMSF to a final concentration of 100 μM. Make this solution fresh for every batch. Ensure that the stock solution has reached room temperature before opening the vial.

3. MQ water with 0.133 % trifluoroacetic acid (TFA). Store at room temperature. Prepare fresh solution for each batch. A bottle containing 1 l of MQ-0.133 % TFA is sufficient for approximately 60 plasma samples (15 ml/sample).
4. 20 % ACN in MQ + 0.1 % TFA. Store at room temperature. Prepare fresh solution for each batch. A bottle containing 100 ml is sufficient for 50 samples (2 ml/sample).
5. 80 % ACN in MQ + 0.1 % TFA. Store at room temperature. Prepare fresh solution for each batch. A bottle containing 100 ml is sufficient for 50 samples (2 ml/sample).
6. LC eluents A: 40 % MQ water : 40 % methanol : 20 % ACN + 0.1 % formic acid (FA).
7. LC eluents B: 70 % methanol : 30 % ACN + 0.1 % FA.

2.3 Consumables

For each plasma sample, the following is required:

1. One plasma collection tube containing ethylenediaminetetraacetic acid (EDTA), minimum volume 5 ml, and a 1.5 ml Eppendorf cup; 1 ml of plasma or less is required (*see* **Note 4**).
2. One 15 ml tube with screw cap (polypropylene plastic).
3. One 50 ml tube with screw cap (polypropylene plastic).
4. One C8 SPE column (Agilent, Bond Elut C8, 200 mg, 3 ml cartridge).
5. One 2 ml Eppendorf cup (polypropylene plastic).
6. One LC injection vial (amber, screw cap) with insert. Most LC systems will accept 12 mm × 32 mm vials (*see* **Note 5**).

2.4 Equipment

1. Fume hood.
2. Pipettes, including a large-volume dispenser.
3. Vortexer.
4. Centrifuge suited for 15 ml volume tubes.
5. SPE high-volume cartridges + adapter.
6. SPE manifold, including vacuum pump and stopcocks.
7. Vacuum concentrator.
8. Chromatography column suited for MS analysis, C8.
9. LC-MS/MS system equipped with an electrospray ionization (ESI) source (*see* **Note 6**).

3 Methods

3.1 Sample Collection

1. Prior to the collection of blood from the subject, pre-cool the centrifuge to 4 °C.

2. Aliquot the PMSF solution; the 100 mM PMSF stock solution needs to be diluted in plasma to a final concentration of 100 μM in isopropanol. Aliquot 1.5 μl of the 100 mM PMSF solution in each 1.5 ml Eppendorf tube that will be used to store the plasma in after collection and centrifugation. Once 1.5 ml plasma is added (**step 5**, below), the final concentration will be 100 μM PMSF.
3. Collect venous blood, e.g., from the antecubital vein, in the EDTA tube (*see* **Note 4**). Measures to limit hemolysis are recommended as this might release degrading enzymes or intercellular endocannabinoids.
4. Immediately after blood collection, spin down the tubes in a centrifuge for 10 min at 2000 × *g* and 4 °C. Tubes that cannot be immediately centrifuged should be stored on ice, but should always be centrifuged within 20 min after collection. Especially when working with a high number of samples, this might lead to some variation in time between blood collection and centrifugation. In our experience, this has never led to any obvious artifactual changes in measured endocannabinoid levels.
5. Immediately after centrifugation, collect the plasma and transfer 1.5 ml to the Eppendorf tube containing the PMSF. Store the sample at −80 °C (*see* **Notes 7** and **8**).

3.2 Sample Extraction

Organic solvents and acids are used during sample extraction. Take all necessary precautions to create a safe working environment. This includes performing all steps with organic solvents and acids in a fume hood, and wearing gloves and safety glasses at all times.

1. Thaw the plasma samples. Ensure that all samples have reached room temperature before proceeding to the ACN extraction step. This may take 30–60 min.
2. While the plasma samples are thawing, prepare the deuterated standard spiking work solution.
3. Transfer 1.0 ml of plasma to a clean 15 ml tube.
4. Add 4.0 ml of the deuterated standard spiking work solution to the 1.0 ml plasma. The ACN will precipitate all proteins and highly lipophilic compounds, while keeping the endocannabinoids in solution. The ACN will completely mix with the plasma. While adding this solution to the plasma, keep the tube on a vortexer running at a gentle pace. Vortexing the sample will improve the reproducibility of the precipitation.
5. Centrifuge the 15 ml tubes at 3000 × *g* for 5 min at room temperature.
6. After centrifugation, collect the supernatant and transfer to a clean 50 ml tube. To each 50 ml tube, add 15 ml of the 0.133 % TFA solution. This will dilute the ACN from **step 4** again to 20 %, which is adequate for the subsequent SPE clean-up.

7. Prepare the SPE manifold; the vacuum pump is connected to the glass manifold, and the SPE columns are connected on the manifold through plastic stopcocks that allow controlling the flow rate of fluids through the column.
8. Activate the C8 SPE column by applying 1 ml of methanol on the column's filter bed. Allow the methanol to run through the filter by gravitational pull; the stopcock has to be in the "open" position. The stopcock should be closed just before the bottom of the meniscus reaches the surface of the filter; the filter should remain wet (*see* **Note 9**).
9. Wash the C8 SPE column by applying 1 ml of MQ water on the filter. Allow the water to run through the filter by using the vacuum pump that creates a mild vacuum (600–700 mBar) in the manifold. The stopcock has to be in the "open" position, and should be closed just before the bottom of the meniscus reaches the surface of the filter. The vacuum pump can be turned off when all columns are washed. Again, the filter should remain wet. There may be some variation in the time needed to clear the water through the filter. It is advised to wash the columns a few at the time, to prevent columns running dry (*see* **Note 10**).
10. Load the 20 ml of sample (from **step 6**) to the SPE column. First, fill the SPE column with 2 ml of sample, then connect the high-volume cartridge and adapter on the SPE column, and add the remaining 18 ml. Open the stopcock and turn on the vacuum pump again. It might take a few minutes before all samples have run through the SPE column's filter; once completed, close the stopcock again. Loading the sample in the 20 % ACN in MQ water + 0.1 % TFA solution is critical for binding of the endocannabinoids to the filter. A higher organic solvent content may prevent the endocannabinoids to bind to the column.
11. Open the stopcocks again and wash the column with 2 ml % ACN in MQ water + 0.1 % TFA solution. This will wash away any unbound residue that remained in the filter; the endocannabinoids will remain bound to the filter. Close the stopcocks once the wash has completed.
12. Prepare the manifold for eluting the SPE columns. Turn off the vacuum pump, and gently release the vacuum from the manifold. Open the manifold, discard the fluid that has accumulated in the manifold; this contains organic solvents and TFA. Adjust the collection vessel rack to accommodate the 2 ml sample collection tubes. Ideally, the outlet tip of the gasket's tube connector should be positioned just over the 2 ml tube. Close the manifold again. Ascertain that all outlet tips are still positioned over the 2 ml collection tubes.
13. Elute the SPE columns by adding 2 ml of 80 % ACN in MQ + 0.1 % TFA on the SPE columns. Open the stopcocks and

activate the vacuum pump again (600–700 mBar); the eluent should drip in the 2 ml collection tube. Close the stopcock immediately when the column is completely eluted to prevent air violently blowing in the sample and spilling it. It is advised to elute the columns a few at the time to prevent this.

14. When all samples are eluted, switch off the vacuum pump and gently release the vacuum. Transfer the 2 ml collection tubes to a vacuum concentrator and evaporate the sample to full dryness. It may take a few hours before the samples are completely dry. Dried extracts can be stored in –80 °C until LC-MS analysis, or immediately analyzed (*see* **Note 11**).

3.3 LC-MS Analysis

Before LC-MS analyses can be performed, a method has to be programmed. Therefore, the different aspects of the method, such as chromatography, ion optics settings, and mass spectrometer scan events, have to be (experimentally) optimized for the LC-MS system. Operating LC-MS systems requires specific training and expertise, and is usually delegated to specialized technicians. Published reports provide a good starting point for optimizing the LC-MS settings, but differences between hardware from different manufacturers may exist. It is therefore difficult to provide a general step-by-step guide on how to perform LC-MS analyses, but some general points of attention will be discussed in this section.

1. The chromatography should be optimized to yield symmetric peaks and minimize ion suppression. We have good experience with gradient elution using eluents that contain water, methanol, and acetonitrile (eluents A and B, *see* Subheading 2). Due to the lipophilic nature of the extract, ion suppression may negatively influence peak heights. Ion suppression can be prevented by modifying the chromatography, hence increasing peak height but usually also analysis time. Special attention should be paid when monoacylglycerol esters such as 2-AG are to be determined; 2-AG undergoes isomerization to 1-AG in aqueous media, which may have to be resolved chromatographically depending on the research question.
2. We have good experiences with various C8 columns for "conventional" high-performance liquid chromatography (HPLC) analysis (e.g., Waters Xterra MS C8) or ultra-performance liquid chromatography (UPLC) analysis (e.g., Waters Acquity C8 BEH UPLC). Generally, UPLC columns can be operated against higher backpressures, allowing higher flow rates, improved separation, and shorter run times. In our hands, a 2.1 × 100 mm UPLC C8 column and a 12-min gradient elution protocol with eluents A and B (*see* Subheading 2.2) resulted in adequate peak shape and recoveries [9]. Never use salt-containing eluents for MS analysis! The chromatography,

together with the sensitivity of the MS, will determine the injection volume of the autosampler. Too high sample volumes may deteriorate peak shape. Typically, depending on the available hardware (and sample loops), the injection volume will range between 2 and 10 µl.

3. All individual compounds should be infused directly into the system in order to tune the MS for the different endocannabinoids. In our hands, the parent of DHEA is visible at *m/z* 372 and EPEA at *m/z* 346, which correspond to their protonated forms. Generally, all ethanolamides, including DHEA and EPEA, yield a dominant *m/z* 62 fragment when subjected to collision-induced dissociation (CID). This fragment corresponds to the ethanolamine moiety. Different classes of endocannabinoids, such as monoacylglycerols or the acyldopamines, yield different fragmentation patterns. Too high CID energies may lead to further fragmentation of the dominant fragment, thus resulting in reduced signal intensities. It is therefore important to select the appropriate optimal CID energy, which has to be determined through experimentation.
4. The performance of an LC-MS method depends on the quality of the sample, the chromatographic separation, and the optimization of MS settings. It is highly recommended to perform a validation to establish the system performance. Items include inter- and intraday accuracy and precision, linearity, limit of quantification/detection (LOQ/LOD), freeze-thaw stability, ion suppression, and recovery.

3.4 Data Processing and Experimental Results

Chromatograms have to be critically reviewed and adjusted if necessary to ensure that high-quality data are reported for further (statistical) processing. Attention has to be paid to a few aspects, such as retention time shifts, quality control (QC) samples, and fit of calibration curves.

1. The accuracy of the calibration curve is essential as it influences the reported concentration for each sample. Usually, each calibration curve is analyzed in duplicate or triplicate for each run, and a single regression equation is generated. The curve fit can be optimized to achieve good accuracy in the concentration range relevant to the "unknown" study samples, e.g., by modifying the curve type or "weighing." The quantification software provides several options to achieve this. In addition, calibration points at concentrations that are not relevant for the study samples may be unselected, to ensure that the calibration curve has optimal fit in the relevant concentration range.
2. Although most software suites allow for automatic peak processing, it is recommended to check all integrations manually. Especially in matrices where low concentrations are expected,

such as in plasma, small background interferences ("noise") may interfere with peak integration and may severely influence the results. We have good experience with using peak areas.

3. Correct for the concentration factor that occurs during sample preparation. The software reports concentrations as they were found in the extract. In case of plasma, 100 µL extracts contains the endocannabinoids from 1 ml of original material, meaning that the reported concentrations are to be divided by 10.
4. It is difficult to give reference values for endocannabinoids in plasma because their levels are modified by a variety of factors (*see* Subheading 3.6). We have found human plasma concentrations for most endocannabinoids in the low ng/ml range [9, 15]. DHEA is present in human plasma whereas EPEA was undetectable [5]. However, EPEA is found in tissues at concentrations in the ng/g range of animals fed a n-3 fatty rich diet [4].

3.5 Modifications to the Sample Preparation Protocol for Matrices Other Than Plasma

We have applied the sample preparation protocol for a variety of matrices, such as adipose tissue, liver, intestinal tissue, and cell culture media. Here we briefly describe modifications to the standard protocol for a selection of matrices other than plasma.

Like most tissues, adipose tissue contains higher concentrations of endocannabinoids compared to plasma. In our experience, adipose tissue did not require SPE sample pre-treatment. A simple extraction step using ACN followed by sonication was sufficient to accurately determine endocannabinoid levels. To 50–100 mg of adipose tissue, 1 ml of ACN containing deuterated standards is added, the sample is sonicated for 5–10 s, and centrifuged for 5 min at 12,000 × *g* on a table-top centrifuge. The supernatant is transferred to a clean 2 ml Eppendorf tube. To the tissue, again 1 ml of ACN is added, sonicated, and centrifuged, and the supernatant is pooled. The 2 ml of ACN extract is evaporated to full dryness, and subjected to LC-MS analysis as described.

Liver and gut tissue contains high concentrations of endocannabinoids. In our experience, 100 mg freeze-dried liver and 50 mg freeze-dried gut tissue contain quantifiable amounts of n-3 fatty acid-derived endocannabinoids. Liver or gut tissue is processed similar to plasma, using SPE. First, the tissue is extracted using 2 × 1 ml of ACN and sonication. The 2 ml of supernatant is diluted with 8 ml of MQ + 0.1 % TFA, and subjected to SPE clean-up similar to plasma.

Cell culture medium may also contain endocannabinoids. In our experience, using 3T3-L1 adipocytes, 2 ml of medium contained quantifiable endocannabinoid levels. To 2 ml of medium, 2 µl of TFA and the deuterated standards are added, and the acidified medium is directly applied onto activated C8 SPE columns and processed as described for plasma. Different strategies may be used to improve endocannabinoid yield from cultured cells, such

as increasing cell numbers, prolonging medium incubation time, or stimulation with ionomycin to stimulate immediate release of endocannabinoids in the medium.

3.6 Factors That Influence Levels of n-3 Endocannabinoids

Generally, endocannabinoid levels depend on a variety of factors, including dietary composition of fatty acids, the presence of inflammation, and postprandial status. DHEA is normally found in human and mouse plasma, but we have not been able to detect EPEA in human plasma [4, 5, 15]. Experience from animal studies demonstrated that EPEA is only detectable in plasma when the animals were fed a diet rich in n-3 fatty acids for 6 weeks [4]. Whether EPEA is present in human plasma after eating n-3 rich diets is not known at present. Both DHEA and EPEA have been consistently found in mouse tissues such as adipose tissue, liver, and gut. Generally, with increased n-3 fatty acid content in the diet, or during inflammation, DHEA and EPEA levels were increased [4, 16]. Also cultured 3 T3-L1 adipocytes released quantifiable amounts of DHEA and EPEA in the medium [5].

3.7 Choice of Target Analytes

The method described in this chapter is suited to quantify the levels of DHEA and EPEA in plasma and other matrices. As mentioned above, using exactly the same sample preparation steps, a wider spectrum of endocannabinoids can be measured, including the classical endocannabinoids AEA and 2-AG. These molecules can be quantified parallel to DHEA and EPEA in the same sample, which requires adding the desired compounds to the calibration curve and MS settings. Most MS triple quadrupole equipment can easily scan 10–20 compounds in one sample. In our experience, quantifying DHEA and EPEA together with the other endocannabinoids provided additional information that helped to understand the dynamic context in which n-3 endocannabinoids are present. For instance, with our more comprehensive analytical approach, we were able to demonstrate that specific fatty acid enriched diets cause a shift in multiple endocannabinoids, rather than affecting only one or two endocannabinoids [4]. It thus seems that establishing a comprehensive "endocannabinoid profile" reveals changes that were not detected using methodology that only focuses on a limited number of endocannabinoids.

4 Notes

1. It is needless to say that stock solutions and calibrators have to be prepared precisely. The concentrations in Table 1 depict the ranges that we routinely measure, but this depends on the MS available in the lab. Therefore, depending on the sensitivity of the available equipment, different concentration ranges may apply in other labs.

2. During LC-MS analysis, the ratio of an endocannabinoid to its deuterated analogue (=isotope ratio) is measured. The ratio is proportional to the concentration in the sample; therefore, it is crucial that the amount of deuterated compound that is spiked to the sample is identical to the amount present in the calibrators. Deuterated standards are normally not present in biological materials and are therefore added to the sample during sample preparation. Measuring isotope ratios has several advantages, because it intrinsically corrects for any sample spill during the sample preparation or any other factors that may vary between samples. It also corrects for so-called ion suppression. Ion suppression is the phenomenon where ionization of the compound of interest is suppressed due to interference of other molecules in the sample, leading to apparently lower peaks. With a carefully selected deuterated analogue that elutes from the LC column at the same time, both molecules are equally affected by ion suppression, thus the isotope ratio remains stable. Different deuterated standards can be used. We have good experiences with using deuterated AEA (AEA-d8) for the quantification of DHEA and EPEA. In the protocol presented here, deuterated 2-AG and NADA are also added to allow for the quantification of a wider range of endocannabinoids.
3. Endocannabinoids are degraded by a variety of enzymes, such as FAAH and monoacylglycerol lipase (MGL). Although FAAH is normally present within the cell, it may also be present in the plasma when cells are lysed, for instance due to mechanical stress during the venapunction. Therefore, as a precaution, it is recommended to store plasma in the presence of FAAH inhibitors, such as PMSF.
4. Different anticoagulants are available to prevent ex vivo blood clotting, such as citrate, heparin, and EDTA. Generally, it is known that the anticoagulant can affect the performance of LC-MS. Anticoagulants may cause interfering peaks in the chromatogram, and it is therefore recommended to investigate the presence of interfering peaks. In our hands, we did not encounter any interferences with EDTA.
5. Different LC systems are available, with either binary or quaternary pumps, autosamplers that accept different types of vials or plates, and operate at different pressures. When using only 2 eluents for the chromatography, a binary LC system is adequate. In the protocol presented here, the dried extract is reconstituted in 100 μl of ACN; for most autosamplers, this means that vials with inserts have to be used. The right type of vial and the appropriate injection needle height have to be selected in the LC systems settings.

6. Mass spectrometry is an advanced analytical technique that allows characterizing and/or quantifying levels of molecules in a certain matrix. Different types of MS machines are available, which all have their particular strengths, weaknesses, and area of application. For accurate and sensitive quantification of multiple compounds, a so-called triple-quadrupole or tandem MS (annotated as MS/MS) is usually the first choice. A tandem MS system consists of three mass filters ("quadrupoles") that are connected in series. The second quadrupole acts as a collision cell. The detector is positioned after the third quadrupole. Intact ions of interest, "parent ions," are selected in the first quadrupole and move on to the collision cell, where they are subjected to collision with inert gas, such as argon. As a result, the parent ions will dissociate into fragments, known as "daughter ions." The daughter ion will continue their flight to the third quadrupole, where only selected daughter ions of one specific mass-over-charge (m/z) ratio will be passed on to the detector. Thus, an ion that hits the detector is a selected fragment from a selected parent ion. Through this "dual selection" a high level of specificity can be achieved.
7. Information on the long-term stability of endocannabinoids in plasma at −80 °C is scarce. However, considering the potential absorption of endocannabinoids to plastics, and the presence of degrading enzymes in the biological matrix, endocannabinoids are generally considered unstable [8]. Therefore, we recommend analyzing the endocannabinoid levels at the earliest convenience.
8. It is needless to say that blood and plasma should be treated as potentially infectious materials. Therefore, wear gloves when handling blood or plasma. It is common to take additional precautions, such as compulsory Hepatitis B vaccination for all personal handling human materials.
9. The methanol unfolds the C8 aliphatic chains in the filter bed, allowing the binding of lipophilic substances to the filter. Hence, this step is known as "activation."
10. This washing step is needed to wash away any overabundant methanol that may prevent compound binding in the sample loading step.
11. The vacuum concentration step is critical for good recoveries. We have compared different techniques to dry the SPE extract, including evaporation under nitrogen flow and freeze-drying. In our hands, we obtained the most robust results and highest recoveries by drying the samples in a vacuum concentrator centrifuge. Please be aware that the vacuum concentrator has to be compatible to work with organic solvents and acids.

References

1. Pertwee RG, Howlett AC, Abood ME et al (2010) International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. Pharmacol Rev 62:588–631
2. Artmann A, Petersen G, Hellgren LI et al (2008) Influence of dietary fatty acids on endocannabinoid and *N*-acylethanolamine levels in rat brain, liver and small intestine. Biochim Biophys Acta 1781:200–212
3. Matias I, Carta G, Murru E et al (2008) Effect of polyunsaturated fatty acids on endocannabinoid and *N*-acyl-ethanolamine levels in mouse adipocytes. Biochim Biophys Acta 1781:52–60
4. Balvers M, Verhoeckx K, Bijlsma S et al (2012) Fish oil and inflammatory status alter the n-3 to n-6 balance of the endocannabinoid and oxylipin metabolomes in mouse plasma and tissues. Metabolomics 8:1130–1147
5. Balvers MGJ, Verhoeckx KCM, Plastina P et al (2010) Docosahexaenoic acid and eicosapentaenoic acid are converted by 3T3-L1 adipocytes to *N*-acyl ethanolamines with anti-inflammatory properties. Biochim Biophys Acta 1801:1107–1114
6. Bradshaw HB, Raboune S, Hollis JL (2013) Opportunistic activation of TRP receptors by endogenous lipids: Exploiting lipidomics to understand TRP receptor cellular communication. Life Sci 92:404–409
7. Di Marzo V, Bisogno T, De Petrocellis L (2007) Endocannabinoids and related compounds: walking back and forth between plant natural products and animal physiology. Chem Biol 14:741–756
8. Zoerner AA, Gutzki F-M, Batkai S et al (2011) Quantification of endocannabinoids in biological systems by chromatography and mass spectrometry: a comprehensive review from an analytical and biological perspective. Biochim Biophys Acta 1811:706–723
9. Balvers MGJ, Wortelboer HM, Witkamp RF et al (2013) Liquid chromatography–tandem mass spectrometry analysis of free and esterified fatty acid N-acyl ethanolamines in plasma and blood cells. Anal Biochem 434:275–283
10. Meijerink J, Plastina P, Vincken J-P et al (2011) The ethanolamide metabolite of DHA, docosahexaenoylethanolamine, shows immunomodulating effects in mouse peritoneal and RAW264.7 macrophages: evidence for a new link between fish oil and inflammation. Br J Nutr 105:1789–1807
11. Meijerink J, Poland M, Balvers MGJ et al (2015) Inhibition of COX-2-mediated eicosanoid production plays a major role in the anti-inflammatory effects of the endocannabinoid *N*-docosahexaenoylethanolamine (DHEA) in macrophages. Br J Pharmacol 172:24–37
12. Brown I, Cascio MG, Wahle KWJ et al (2010) Cannabinoid receptor-dependent and -independent anti-proliferative effects of omega-3 ethanolamides in androgen receptor-positive and -negative prostate cancer cell lines. Carcinogenesis 31:1584–1591
13. Rovito D, Giordano C, Vizza D et al (2013) Omega-3 PUFA ethanolamides DHEA and EPEA induce autophagy through PPARγ activation in MCF-7 breast cancer cells. J Cell Physiol 228:1314–1322
14. Kim HY, Spector AA, Xiong ZM (2011) A synaptogenic amide *N*-docosahexaenoylethanolamide promotes hippocampal development. Development 96:114–120
15. Balvers MGJ, Verhoeckx KCM, Witkamp RF (2009) Development and validation of a quantitative method for the determination of 12 endocannabinoids and related compounds in human plasma using liquid chromatography-tandem mass spectrometry. J Chromatogr B 877:1583–1590
16. Balvers MGJ, Verhoeckx KCM, Meijerink J et al (2012) Time-dependent effect of in vivo inflammation on eicosanoid and endocannabinoid levels in plasma, liver, ileum and adipose tissue in C57BL/6 mice fed a fish-oil diet. Int Immunopharmacol 13:204–214

Chapter 5

Assay of CB_1 Receptor Binding

Valeria M. Catani and Valeria Gasperi

Abstract

Type-1 cannabinoid receptor (CB_1), one of the main targets of endocannabinoids, plays a key role in several pathophysiological conditions that affect both central nervous system and peripheral tissues. Today, its biochemical identification and pharmacological characterization, as well as the screening of thousands of novel ligands that might be useful for developing CB_1-based therapies, are the subject of intense research. Among available techniques that allow the analysis of CB_1 binding activity, radioligand-based assays represent one of the best, fast, and reliable methods.

Here, we describe radioligand binding methods standardized in our laboratory to assess CB_1 binding in both tissues and cultured cells. We also report a high-throughput radioligand binding assay that allows to evaluate efficacy and potency of different compounds, which might represent the basis for the development of new drugs that target CB_1 receptor-dependent human diseases.

Key words Type-1 cannabinoid receptor, CP55.940, Filtration assay, Radioligand-receptor binding, SR141716

1 Introduction

Type-1 cannabinoid receptor (CB_1), the first identified and cloned receptor of Δ^9-tetrahydrocannabinol [1], represents one of the main targets of endocannabinoids [2, 3]. It is highly expressed in brain, but also in peripheral organs (e.g., lung, liver, and kidney) and cells, including T lymphocytes and platelets [4]. Due to its broad localization, CB_1 is involved in the regulation of several physiological processes at both central nervous system and periphery, such as neurogenesis, reward, cognition, learning, memory, immune response, and body energy homeostasis [4–6]. In addition, several pathological conditions have been shown to correlate with up- or downregulation of this receptor. Overactive CB_1 signaling, for example, promotes the development of obesity, insulin resistance, and dyslipidemia [6]. Therefore, the design of novel CB_1 ligands that might be useful

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therapeutics for prevention and/or treatment of CB_1-dependent human disorders is very active [6].

One of the main techniques used for the biochemical identification and pharmacological characterization of CB_1 is based on the use of a radioactive ligand, such as [^{3}H]CP55.940 [2]. The basic outline of radioligand-CB_1 binding assays is very easy and fast to perform: a preparation containing CB_1 is incubated with radioactive (hot) [^{3}H]CP55.940, thus forming the radioactive complex [^{3}H]CP55.940/CB_1. After reaching equilibrium, this complex is usually separated from unbound hot [^{3}H]CP55.940 by membrane filtration (still the most efficient, fast, and convenient method), and quantified by liquid scintillation counting. The use of an unlabeled (cold) CB_1 ligand, such as the antagonist/inverse agonist SR141716 or rimonabant [7], able to selectively displace hot [^{3}H]CP55.940 from CB_1 sites, allows then to assess CB_1 binding activity.

Noteworthy, we observed that, although CB_1 binding assay with filtration might work well with membrane homogenates from both tissues and cells, the most reproducible and reliable results are obtained by working with intact cultured cells without a filtration step.

Against this background, here we describe two relatively simple, but extremely powerful, methods that we have standardized in our laboratory and that we commonly employ to interrogate the presence of CB_1 in unknown samples, as well as to identify potential changes in its activity, under either physiological (e.g., in young versus aged tissues, or during cell differentiation) or pathological conditions (e.g., healthy versus diseased tissues). We also describe a standardized high-throughput CB_1 binding assay that, by employing human CB_1 over-expressing membranes, allows to screen large libraries of compounds. This method is a useful assay to identify novel CB_1 ligands that might represent the basis for developing innovative therapeutics against CB_1-dependent human diseases.

2 Materials

Prepare all buffers and solutions by using ultrapure water and analytical grade reagents. Prepare and store all reagents at –20 °C (unless indicated otherwise).

2.1 Labeled and Unlabeled Compounds

[^{3}H]CP55.940 [5-(1,10-dimethylheptyl)-2-[(1R,5R)-hydroxy-(2R)-(3-hydroxypropyl)-cyclo-hexyl]phenol].

CP55.940.

SR141716, rimonabant [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide] (Cayman Chemicals Ann Arbor, MI, USA).

2.2 Buffers and Equipments

2.2.1 CB_1 Binding Assay for Tissues

Buffer A: 2 mM Tris-EDTA, 320 mM sucrose, 5 mg/ml BSA, 5 mM $MgCl_2$, pH 7.4.

Buffer B: 50 mM Tris–HCl, 2 mM Tris-EDTA, 3 mM $MgCl_2$, pH 7.4.

Buffer C: 50 mM Tris–HCl, 2 mM Tris-EDTA, 3 mM $MgCl_2$, 5 mg/ml bovine serum albumin (BSA), 50 μM phenylmethylsulfonyl fluoride (PMSF) (to add immediately before use), pH 7.4.

Buffer D: 50 mM Tris–HCl, 1 mg/ml BSA, pH 7.4.

0.1 % Triton in Buffer D, pH 7.4.

Glass/Teflon Potter homogenizer.

Ultra-Turrax T 25.

15 ml polypropylene tubes.

Vortex mixer.

Water bath or Thermoblock for 15 ml tubes, or incubator set at 37 °C.

Whatman GF/C Glass Microfiber filters (binder free), diameter 25 mm, with 1.2 μm pore size.

Vacuum filtration manifold, Millipore® model 1225 (Merck-Millipore Co., Darmstadt, Germany).

Vacuum pump.

Liquid scintillation cocktail (Ultima Gold™ XR).

10 ml scintillation vials.

Liquid scintillation β-counter.

2.2.2 CB_1 Binding Assay for Intact Cells

1. Incubation buffer: 50 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 320 mM sucrose, 2 mg/ml BSA, pH 7.4.
2. Washing buffer: 50 mM Tris–HCl, 500 mM NaCl, 1 mg/ml BSA, pH 7.4.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
4. Sterile 24-well plates for cell cultures.
5. Incubator for cell cultures set at 37 °C.
6. Liquid scintillation cocktail (Ultima Gold™ XR).
7. 10 ml scintillation vials.
8. Liquid scintillation β-counter.

2.2.3 High-Throughput CB_1 Binding Assay

1. Binding buffer: 50 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mg/ml BSA, pH 7.4.
2. Pre-soaking buffer for FC-filter plate: 50 mM Tris–HCl, 0.33 % polyethylenimine (PEI), pH 7.4.
3. Washing buffer: 50 mM Tris–HCl, 500 mM $NaCl_2$, 1 mg/ml BSA, pH 7.4.
4. 0.1 % Triton in buffer D, pH 7.4.
5. Incubator set at 37 ° C.
6. Polypropylene 96-well plate with lid.
7. Human CB_1 over-expressing membranes (Merck-Millipore Co., Darmstadt, Germany).
8. MultiScreenHTS 96-well filter plate (Merck-Millipore Co., Darmstadt, Germany).
9. Vacuum pump.
10. FC-96-well plate.
11. Liquid scintillation cocktail (Ultima Gold™ XR).
12. 10 ml scintillation vials.
13. Liquid scintillation β-counter.

3 Methods

3.1 Protocol 1: CB_1 Binding Assay for Tissues

This protocol is recommended for low-throughput assays, or for just a few experiments with tissue samples.

3.1.1 Preparation of Membrane Homogenates

Perform all steps (Fig. 1) on ice and keep samples on ice or in a refrigerated centrifuge, in order to preserve CB_1 functionality.

1. Resuspend tissue (*see* **Note 1**) in pre-cooled buffer A (fresh weight/volume ratio = 1 g/4 ml), and homogenize it on ice (*see* **Note 2**).
2. Centrifuge the membrane homogenate at 1000 *g* and 4 °C for 10 min.
3. Save the supernatant (S1) in a tube on ice.
4. Resuspend the pellet with the same volume of buffer A used in **Step 1**, and centrifuge again at 1000 *g* and 4 °C for 10 min.
5. Save the supernatant (S2) and add it to S1.
6. Repeat **Steps 4** and **5** to obtain S3, and discard the pellet.
7. Centrifuge the collected volume (S1 + S2 + S3) at 39,000 *g* and 4 °C for 30 min.
8. Discard the supernatant.
9. Resuspend the resulting pellet in the smallest possible volume of cold buffer B.

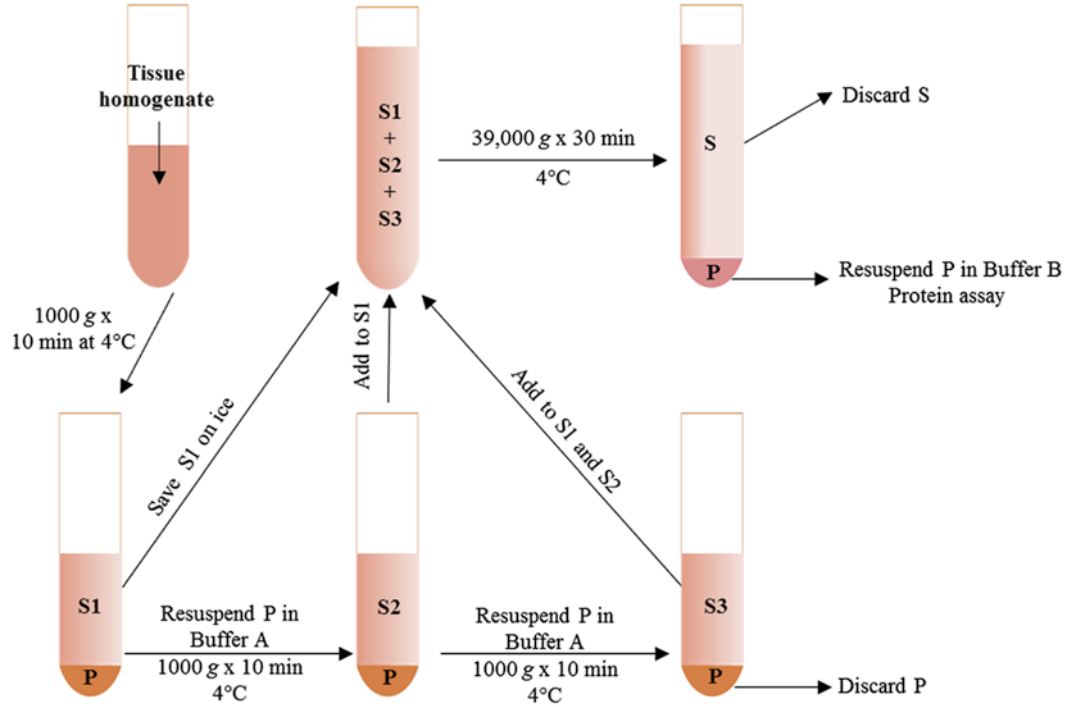


Fig. 1 Schematic representation of membrane homogenate preparation. *See* Subheading 3.1.1 for further details. *P* pellet; *S* supernatant

10. Calculate protein concentration with any commercially available assay, such as Bradford assay.
11. Membrane homogenate is now ready for CB_1 binding assay (*see* **Note 3**).

3.1.2 Assay Using Vacuum Filtration Manifold Millipore® Model 1225

Each condition in the assay must be repeated in triplicate, in order to calculate average and standard error of the mean (S.E.M.).

Prepare also three tubes that contain only pre-warmed buffer C and [^{3}H]CP55.940 (i.e., reaction blank, Bk) (Fig. 2). All incubations are carried out at 37 °C in pre-warmed buffer C, at a final reaction volume of 500 µl.

1. For each sample, prepare three 15 ml polypropylene tubes (in triplicate), marked as TB (total binding), NSB (nonspecific binding), and SR1 (CB_1 antagonist/inverse agonist SR141716). Add in each tube the reagents in the following order:
   (a) Pre-warmed buffer C (up to 500 µl).

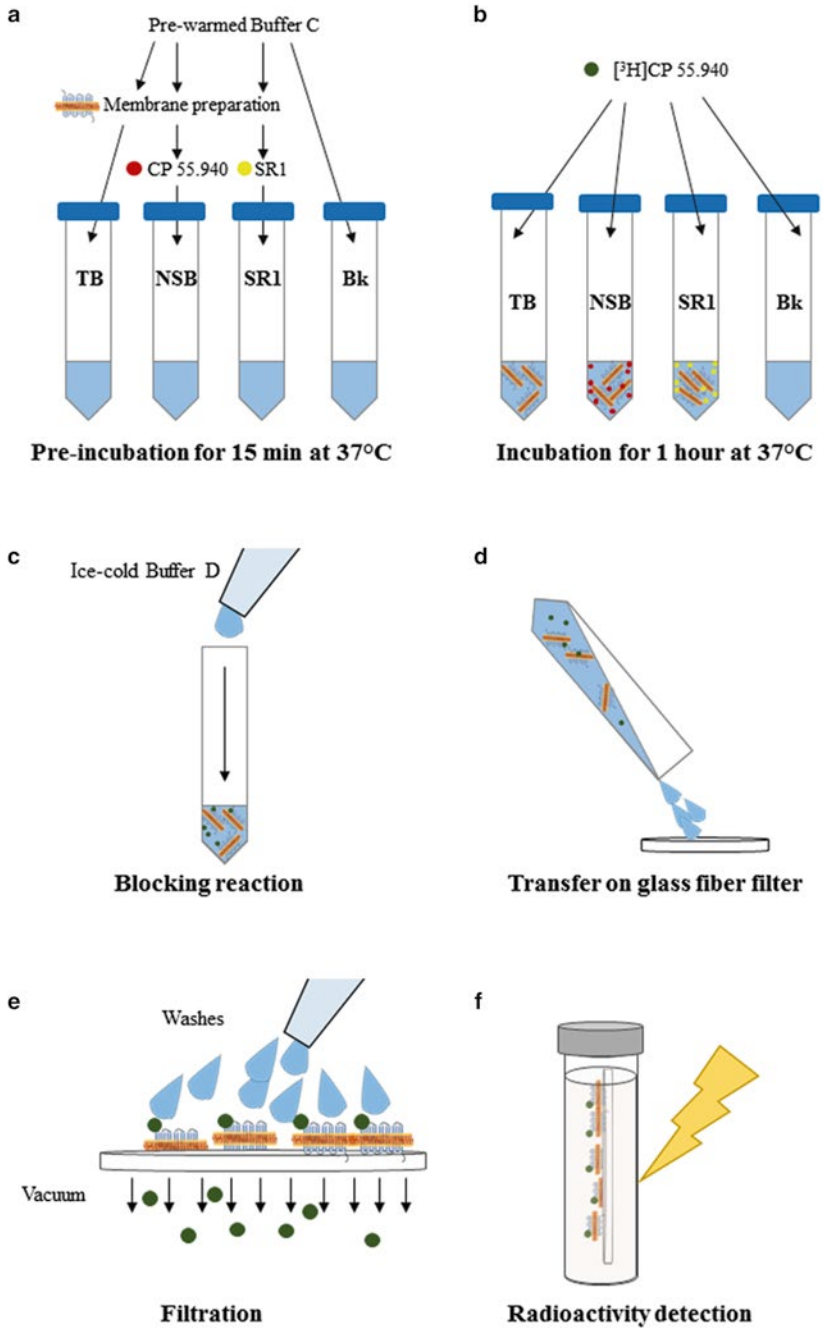


Fig. 2 Schematic representation of the procedure to analyze CB_1 binding activity in membrane homogenates by using filtration assay. (**a**) TB (total binding, that contains buffer C and membrane proteins), NSB (nonspecific binding, that contains buffer C, membrane proteins and cold CP55.940), SR1 (that contains buffer C, membrane proteins and the CB_1 antagonist/inverse agonist SR141716), and Bk (blank of reaction, that contains buffer C only) tubes are pre-incubated at 37 °C for 15 min. (**b**) After adding [^{3}H]CP55.940 to each tube, samples are incubated for 1 h at 37 °C. (**c**) At the end of the incubation, reaction is blocked by adding 2 ml of ice-cold buffer D to the tubes. (**d**) The content of each tube is then transferred to a GF/C Glass fiber filter, placed in the filter plate of vacuum filtration manifold, Millipore® model 1225. (**e**) Vacuum traps the complex [^{3}H]CP55.940/CB_1 receptor onto the glass fiber filter, while unbound radioligand is removed through cycles of washes with ice-cold buffer D and vacuum. (**f**) Each filter is transferred to 10 ml scintillation vial, and residual radioactivity is measured in a liquid scintillation β-counter. *See* Subheading 3.1.2 for further details

(b) Membrane homogenate. For murine brain membranes, we usually use 50–100 μg/test (*see* **Note 4**).

(c) CP55.940 at a final concentration of 1 μM, for NSB (*see* **Note 5**), or SR141716 at a final concentration of 0.1 μM, for SR1 (*see* **Note 6**).

2. Vortex and incubate for 15 min at 37 °C (Fig. 2a).
3. Add [^{3}H]CP55.940. For murine brain membranes, we use it at the final concentration of 0.4 nM (*see* **Note 7**).
4. Vortex and incubate for 1 h at 37 °C (Fig. 2b).
5. In the meantime, place in a Petri dish, with tweezers, as many GF/C glass fiber filters as the number of tubes, and pre-soak them in cold buffer D (*see* **Note 8**).
6. 10 min before terminating the reaction, place (with tweezers) each filter in the numbered filter plate of the vacuum filtration manifold.
7. Turn on vacuum to remove excess of buffer D from glass fiber filters.
8. Block the vacuum.
9. Terminate reaction by adding 2 ml of ice-cold buffer D to each tube (*see* **Note 9**) (Fig. 2c).
10. Vortex and transfer each sample to the corresponding filter plate (Fig. 2d).
11. Apply vacuum (for not more than 2 min) to trap the complex [^{3}H]CP55.940/CB_1 receptor onto the filter, and remove unbound radioligand (*see* **Notes 10** and **11**) (Fig. 2e).
12. Block the vacuum.
13. Wash each tube with 2 ml of ice-cold buffer D.
14. Repeat **steps 11–13**.
15. Wash each filter twice with 2 ml of ice-cold buffer D (Fig. 2e).
16. Apply vacuum.
17. Block the vacuum and allow filters to dry at room temperature (*see* **Note 12**).
18. Transfer with tweezers each dried filter in the corresponding scintillation vial, containing 500 μl of 0.1 % Triton in buffer D.
19. Add 3.5 ml of liquid scintillation cocktail, and read radioactivity by a scintillation β-counter (*see* **Note 13**) (Fig. 2f).

3.2 Protocol 2: CB_1 Binding Assay for Adherent Living Cells

Each condition in the assay must be repeated in triplicate, in order to calculate average and S.E.M.

All incubations are carried out at 37 °C in pre-warmed incubation buffer (final volume of reaction for 24-well plate = 300 μl).

1. Plate cells in order to reach 90–100 % confluence on the day of the assay (*see* **Note 14**). For each sample, prepare three wells (in triplicate), marked as TB (total binding), NSB (nonspecific binding), and SR1 (CB_1 antagonist/inverse agonist). For blanks (Bk), add culture medium to three wells, without cells.
2. On the day of the experiment, carefully wash each well twice with 1 ml of PBS.
3. After removing PBS, add to each well incubation buffer up to 300 μl.
4. Add CP55.940 for NSB (at a final concentration of 1 μM for HeLa cells) (*see* **Note 5**), or SR141716 (at a final concentration of 0.1 μM for HeLa cells), for SR1 (*see* **Note 6**).
5. Incubate for 10 min at 37 °C.
6. Add [^{3}H]CP55.940 (at a final concentration of 2.5 nM for HeLa cells) (*see* **Note 7**).
7. Incubate for 1 h in an incubator set at 37 °C.
8. At the end of the incubation, carefully remove the incubation buffer.
9. Carefully rinse cells 4 times with 1 ml of ice-cold washing buffer.
10. After removing washing buffer, add 300 μl of 0.5 M NaOH, and pipet up and down for several times to lyse cells.
11. Transfer solution to a 10 ml scintillation vial.
12. Add 3.5 ml of liquid scintillation cocktail, and immediately read radioactivity in a scintillation β-counter.

3.3 Protocol 3: CB_1 Binding Assay for High-Throughput Screening

Each condition in the assay must be repeated at least in triplicate, in order to calculate average and S.E.M. Prepare also three wells that will contain only pre-warmed binding buffer and [^{3}H] CP55.940 (i.e., blank of reaction, Bk). All incubations are carried out at 37 °C in pre-warmed binding buffer, at a final reaction volume of 200 μl.

1. Add the reagents to each well of a polypropylene 96-well plate, in the following order:
   (a) Pre-warmed binding buffer (up to 200 μl).
   (b) CB_1 over-expressing membranes (10 μg/well, except for the blanks).
   (c) CP55.940 for NSB, SR141716 for SR1 (*see* **Notes 4** and **5**), or test compounds (*see* **Note 15**).
2. Cover the lid and carefully shake the plate.
3. Incubate for 20 min at 37 °C.
4. Add [^{3}H]CP 55.940 at the final concentration of 2.5 nM, to start the reaction.

5. Cover the lid and carefully shake the plate.
6. Incubate for 1 h at 37 °C.
7. In the meantime, pre-soak FC-filter plate with 200 µl of 50 mM Tris–HCl containing 0.33 % PEI (*see* **Note 8**).
8. 10 min before terminating the reaction, turn on vacuum to remove PEI from filters.
9. Wash filters 3 times in washing buffer.
10. Transfer reaction solution from 96-well plate to the FC-filter plate, and filter by turning on vacuum.
11. Wash 4 times with 300 µl of washing buffer.
12. Allow filters to dry, by placing the plate for 30 min in an incubator at 37 °C.
13. Transfer each filter to the corresponding scintillation vial containing 500 µl of 0.1 % Triton in buffer D.
14. Add 3.5 ml of liquid scintillation cocktail and read radioactivity in a scintillation β-counter (*see* **Note 13**).

3.4 Data Analysis

It is recommended to repeat each condition in the assay at least in triplicate, in order to calculate mean and S.E.M.

3.4.1 Protocols 1 and 2

To calculate CB_1 binding activity (usually expressed as fmol or pmol per mg of membrane protein), do as follows:

1. Subtract disintegrations per minute (DPM) mean value of blank (Bk) from every other mean value.
2. Calculate SB by subtracting DPM relative to NSB from those relative to TB.
3. Knowing that 1 nCi corresponds to 2200 DPM, convert DPM in nCi.
4. To calculate moles of bound [^{3}H]CP55.940 to CB_1, divide the obtained nCi for the specific activity of [^{3}H]CP55.940 (such a value, expressed as Ci/mmol, is reported in the datasheet provided by the manufacturer) (*see* **Note 16**).
5. Divide the moles by the mg of proteins used in the assay (*see* **Note 17**).
6. Calculate SR141716 displacement according to the formula:

$$\%\ \text{of inhibition} = 100 - \frac{[3\text{H}]\text{CP55.940 SB} - \text{SR141716 SB}}{[3\text{H}]\text{CP55.940 SB}}$$

7. Calculate differences among two or more groups by inferential statistics: Student's *t*–test (unpaired or paired data) or

Mann Whitney *U* test/Wilcoxon' signed ranks test; ANOVA (or repeated-ANOVA) with Bonferroni *post hoc* test or Kruskal–Wallis test, followed by Mann–Whitney *U* test.

3.4.2 Protocol 3

To calculate IC_{50} and Ki values of novel CB_1 ligands (agonists/antagonists/inverse agonists), calculate SB of [^{3}H]CP55.940 (see above how to convert DPM of SB into fmol or pmol per mg of membrane proteins), in the presence of unlabeled test compound at each concentration tested. By using GraphPad Prism software for Science (San Diego, CA), plot each value and determine IC_{50} and Ki values by nonlinear regression analysis of one-site competition binding curve. It should be stressed that Ki is dependent on IC_{50} value, as indicated by the Cheng and Prusoff equation:

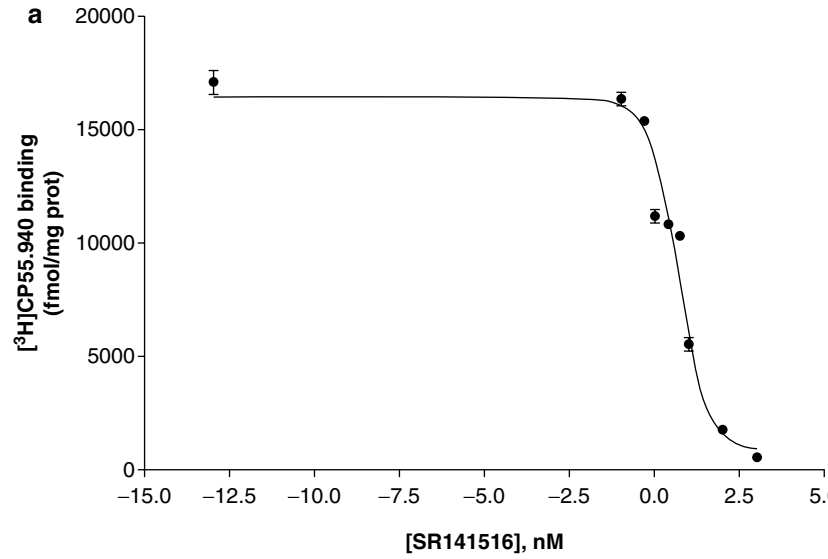
$$\mathrm{Ki} = \mathrm{IC}_{50} / \left[1 + \left([L] / K_D\right)\right]$$

where $[L]$ is the fixed concentration of [^{3}H]CP55.940 used in the assay, and K_D is its equilibrium dissociation constant (*see* Table 1 and Fig. 3). Therefore, prior to performing competition binding studies, you should know (or eventually calculate through saturation binding assay) K_D value of the radioligand used.

Table 1
Experimental data of heterologous competition binding studies performed by incubating membranes over-expressing human CB_1 receptor with 2.5 nM [^{3}H]CP55.940, and increasing concentrations (0–1000 nM) of SR141716

[SR141716][a]	Specific binding of [^{3}H]CP 55.940[b]
0.00	17,088.23 ± 558.42
0.10	16,344.45 ± 281.93
0.50	15,353.87 ± 128.72
1.00	11,165.90 ± 272.95
2.50	10,851.56 ± 92.94
5.00	10,288.43 ± 203.93
10.00	5500.12 ± 291.70
100.00	1725.14 ± 77.32
1000.00	526.40 ± 18.21

Values are the means ± S.E.M. of three independent experiments, each performed at least in quintuplicate
[a]The concentration of SR141716 is expressed as nM
[b]Binding values are expressed as fmol/mg of membrane protein



b

Best-fit values	
Bottom	820.2
Top	16397
$LogIC_{50}$	0.6836
IC_{50}	4.826
Ki	3.666
[^{3}H]CP 55.940 (Constant)	2.500
[^{3}H]CP 55.940 K_D *(Constant)	7.900
Std. Error	
Bottom	474.9
Top	375.8
$LogIC_{50}$	0.06064
95% confidence Intervals	
Bottom	–145.5 to 1786
Top	15633 to 17161
$LogIC_{50}$	0.5603 to 0.8069
IC50	3.633 to 6.411
Ki	2.760 to 4.870
Goodness of Fit	
Degrees of Freedom	34
R^2	0.9569

Fig. 3 Representative heterologous competition curve of SR141716 against [^{3}H]CP55.940 binding to membrane preparations over-expressing CB_1. Binding assays were performed in our laboratory by using MultiScreenHTS 96-well filter plate, as described in Subheading 3.3. (**a**) Human CB_1 over-expressing membranes (10 μg/test) were incubated with 2.5 nM [^{3}H]CP55.940 in the presence of increasing concentrations (0–1000 nM) of unlabeled SR141716. (**b**) Experimental data (reported in Table 1) were used to calculate IC_{50} and Ki values by nonlinear regression analysis of one-site competition binding curve, through GraphPAD Software for Science version 5 (San Diego, CA). *To calculate IC_{50} and Ki values of SR141716, we have previously calculated K_D (obtained value = 7.9 ± 1.9 nM) of hot CP55.940 through saturation binding assays

4 Notes

1. We recommend to work with fresh tissues; if it is not possible, keep tissues frozen at −80 °C until the day of the assay, and thaw them on ice in the presence of pre-cooled buffer A.

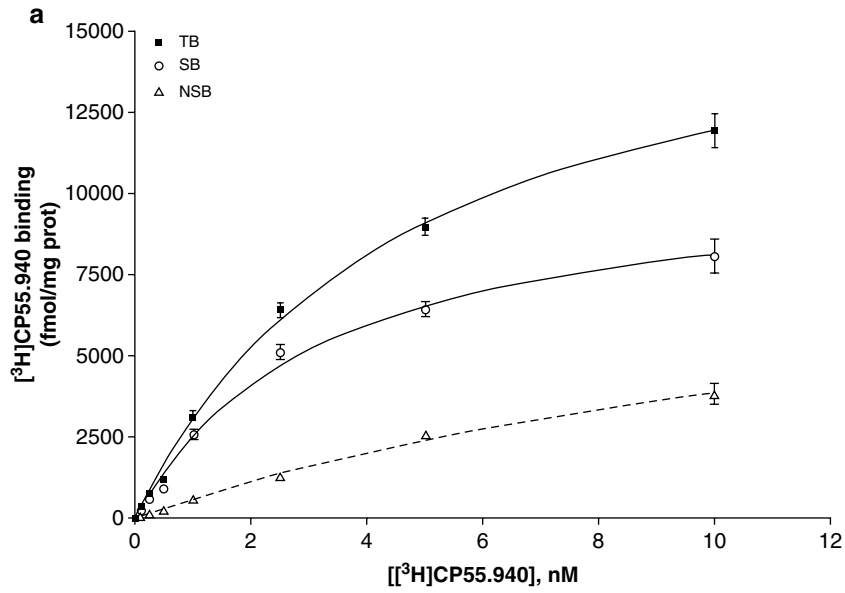
2. For soft tissues (like brain), homogenize samples with a Glass/Teflon Potter homogenizer. To facilitate homogenization of fibrous tissues (e.g., muscle), prior1 to using Glass/Teflon Potter homogenizer, mince samples with scissors and, then, with an Ultra-Turrax T25.
3. Membrane homogenates from fresh tissues can be aliquoted, frozen on dry ice, and stored at −80 °C for no longer than one week. In order to preserve receptor binding activity, do not store membranes from frozen tissues, but proceed immediately with CB_1 receptor binding assay.
4. Protein concentration to be used in the assays depends on CB_1 receptor expression levels; thus we recommend to perform a dose–response curve for unknown biological samples.
5. In addition to binding to receptors of interest (specific binding, SB), radioligands can also interact nonspecifically (nonspecific binding, NSB) with proteins that are not of interest, as well as with the tube walls (during incubation) or with the filters. To overcome this problem, use an excess of CP55.940 at a final concentration that is at least 1000-fold higher than that used for [^{3}H]CP55.940. Indeed, in the presence of an excess of cold CP55.940, CB_1 receptors are virtually all occupied by the unlabeled ligand, and hence the hot ligand can only bind to nonspecific sites. Ideally, NSB should be less than 50 % of the Total Binding (TB).
6. SR141716 is a highly potent antagonist/inverse agonist of CB_1 that displays almost 300-fold selectivity for CB_1 over type-2 cannabinoid receptor (CB_2); Ki values are 1.800 ± 0.075 nM and 514 ± 30 nM, respectively [2]. Therefore, we recommend to use SR141716 at a concentration lower than the Ki value for CB_2.
7. [^{3}H]CP55.940, as other tritiated ligands, has high specific activity; therefore, we recommend to use it at a concentration equal or below its K_D value. For unknown samples, calculate [^{3}H]CP55.940 K_D through saturation binding experiments that measure TB and NSB at various concentrations of hot CP55.940. Calculate K_D and Bmax for specific binding, by using a one-site binding hyperbola nonlinear regression analysis (i.e., with GraphPad Prism), as shown in Table 2 and Fig. 4.
8. To minimize ligand binding to filters, you can also pre-soak glass fiber filters in 0.1–0.5 % PEI (a cationic polymer that neutralizes the negative charge of the glass fiber filter) in buffer D. Prior to filtration of receptor sample, filter PEI solution away, and then wash with ice-cold buffer D for at least three times.

Table 2
Experimental data of saturation binding assay of [^{3}H]CP55.940 to murine brain membrane homogenate

([^{3}H]CP 55.940)[a]	Total binding (TB)[b]	Nonspecific binding (NSB)[b]	Specific binding (SB)[b]
0.10	372.50 ± 17.65	77.49 ± 5.95	295.01 ± 14.62
0.25	751.46 ± 29.94	152.94 ± 11.09	598.52 ± 27.84
0.50	1199.12 ± 19.33	272.49 ± 39.45	926.63 ± 20.10
1.00	3175.15 ± 145.71	596.71 ± 51.70	2578.44 ± 154.68
2.50	6418.74 ± 233.15	1288.23 ± 50.84	5130.51 ± 245.02
5.00	8997.04 ± 267.42	2556.41 ± 85.48	6440.63 ± 376.47
10.00	11,941.20 ± 536.31	3849.46 ± 312.21	8091.74 ± 653.32

Values are the means ± S.E.M. of three independent experiments, each performed in triplicate
[a]The concentration of [^{3}H]CP 55.940 is expressed as nM
[b]Binding values are expressed as fmol/mg of membrane protein

9. A drastically reduced temperature (with ice-cold buffer D) during the washing step will prevent and/or slow down dissociation of bound ligands from CB_1.
10. Control vacuum pump in order to have enough pressure to rapidly filter the samples and prevent ligand dissociation, yet without affecting filter integrity or the amount of membranes retained on the filter. If possible, use a control pressure valve.
11. Time of filtration negatively correlates with ligand K_D; it can range from 20 min for ligands with a $K_D = 0.1$ nM, to 10 s for ligands with a $K_D = 10$ nM.
12. Dry filters require less liquid scintillant to achieve maximum signal than wetted filters. If filters are not completely dried prior to the addition of scintillation liquid cocktail, the residual water present in the filters can interact with the scintillant, thus reducing counting efficiency.
13. Since radioligand embedded within the filter requires some time to become accessible to the liquid scintillant, read radioactivity in the vial at least after 6 h of incubation.
14. We usually plate HeLa cells the day before the experiment, at a concentration of 100,000 cells/well, in a 24-well plate. We recommend to set up a growth curve with different cell types, in order to optimize cell concentration.
15. We advise to use at least 6–8 concentrations for each test compound: the lowest concentration should be at least 1/10 of K_D for CP55.940, while the highest concentration should be approximately 10-fold over such K_D (see Table 2 and Fig. 4).



b

Best-fit values	
Bmax	10773
Kd	3.224
Std. Error	
Bmax	560.8
Kd	0.4104
95% confidence Intervals	
Bmax	9637 to 11909
Kd	2.393 to 4.055
Goodness of Fit	38
Degrees of Freedom	0.9682

Fig. 4 Representative saturation curve of [^{3}H]CP55.940 binding to whole brain membrane homogenates, performed in our laboratory. Binding assays were performed by using a vacuum filtration manifold, Millipore® model 1225, as described in Subheading 3.1.2. (**a**) Increasing concentrations (0–10 nM) of the synthetic agonist of CB receptors [^{3}H]CP55.940 were incubated with whole brain membrane homogenates (50 μg/test), in the absence [Total Binding (*Filled square* TB)] or in the presence of 1 μM unlabeled CP55.940 [Nonspecific Binding (*triangle* NSB)]. NSB mean values were subtracted to relative TB mean values, in order to obtain Specific Binding (circle SB). (**b**) Experimental data (reported in Table 2) were used to calculate Bmax (expressed as fmol/mg membrane protein) and K_D (expressed as nM), by using nonlinear regression analysis for one-site binding hyperbola, through GraphPAD Software for Science version 5 (San Diego, CA)

16. If data are expressed as CPM (counts per min), you need to know the efficiency of your β-counter for [^{3}H], in order to apply the following formula:

    Efficiency = Net CPM of standard / known DPM of standard.

17. In case of CB_1 receptor binding assay performed with intact living cells, data may also be reported as fmol or pmol per 10^6 cell. In this case, it is important to know the exact amount of confluent cells in the well.

References

1. Matsuda LA, Lolait SJ, Brownstein MJ et al (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564
2. Pertwee RG, Howlett AC, Abood ME et al (2010) International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2. Pharmacol Rev 62:588–631
3. Tantimonaco M, Ceci R, Sabatini S et al (2014) Physical activity and the endocannabinoid sytem: an overview. Cell Mol Life Sci 71:2681–2698
4. Gasperi V, Evangelista D, Savini I et al (2015) Downstream effects of endocannabinoid on blood cells: implications for health and disease. Cell Mol Life Sci 72:3235–3252
5. Mechoulam R, Parker LA (2013) The endocannabinoid system and the brain. Annu Rev Psychol 64:21–47
6. Gatta-Cherifi B, Cota D (2015) Newinsights on the role of the endocannabinoid systemin the regulation of energybalance. Int J Obes (Lond). doi:10.1038/ijo.2015.179
7. Rinaldi-Carmona M, Barth F, Heaulme M et al (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett 350:240–244

Chapter 6

The Displacement Binding Assay Using Human Cannabinoid CB_2 Receptor-Transfected Cells

Maria Grazia Cascio, Pietro Marini, and Roger G. Pertwee

Abstract

Displacement binding assays are nonfunctional assays mostly used with the aim of determining whether a certain compound (plant-derived or synthetic) is able to bind to a specific receptor with high affinity. Here, we describe the displacement binding assay that is carried out with a radioligand and CHO (Chinese Hamster Ovarian) cells stably transfected with the human cannabinoid CB_2 receptor.

Key words Binding, Cannabinoid CB_2 receptors, Agonist, Antagonist, Ligand, Radioligand, Affinity

1 Introduction

The aim of radioligand-binding experiments is to study the binding of drugs to specific receptors. Drugs that bind to a receptor and mimic the action of an endogenous ligand by inducing a biological response are defined as *agonists*. On the other hand, *competitive antagonists* are also able to bind to a receptor, but unlike agonists, they do not induce a biological response themselves, but instead, block the action of an agonist or endogenous transmitter. It is difficult to differentiate agonists from antagonists by using radioligand displacement binding assays; thus such compounds are referred to in these assays as *ligands* [1]. To perform displacement binding assays, the values of two parameters need to be known: (1) the *equilibrium dissociation constant* (K_D), which is the concentration of ligand that will occupy 50 % of the receptors, and (2) the B_{max}, which is the total density of the receptors under investigation in that preparation. Specifically, the K_D (unit M) gives an idea about the strength of the binding of a ligand to its receptor; the lower this value, the higher the affinity of the ligand for that particular receptor. The B_{max} is expressed as amount of ligand bound/mg of protein. Importantly, the determination of the maximum density of receptors in a particular tissue can be done using

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a radioligand binding assay. The values of both K_D and B_{max} can be determined using "Saturation Binding Assays," detailed descriptions of which can be found elsewhere [2, 3]. Here we describe the displacement binding assay that is carried out using Chinese Hamster Ovarian (CHO) cells stably transfected with the human cannabinoid CB_2 receptor. This assay requires the use of a radioligand, and hence can only be performed by suitably qualified researchers (registered radiation workers).

Ideal candidates for radiolabeling are compounds with: (a) high affinity for the receptor under investigation (K_D in the low nanomolar range), (b) low nonspecific binding, (c) receptor selectivity, and (d) high specific activity that will increase the sensitivity of the assay. In experiments directed at determining the affinity of certain ligands for cannabinoid CB_2 receptors, we use tritiated CP55,940 (also known as [^{3}H]CP55,940). Thus, CP55,940 is a well-established, high affinity ligand for these receptors that does however lack selectivity (it also binds potently to the cannabinoid CB_1 receptor). Because of this nonselectivity, it is recommended that binding assays with [^{3}H]CP55,940 are performed with, for example, CB_2-transfected cells, or their membranes, rather than with tissues that naturally express both CB_1 and CB_2 receptors. The method used in our laboratory to perform displacement binding assays is very simple and involves three main steps: (1) cell culturing and scraping; (2) performance of the assay; and (3) analysis of the results.

2 Materials

2.1 Cell Culturing

1. Cells: CHO cells transfected with cDNA encoding human CB_2 receptors are commercially available (e.g., ValiScreen Cannabinoid CB_2 (human) cell line, Perkin Elmer, USA).
2. Culture medium: use sterile Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM, supplemented with 1 mM L-glutamine, 10 % fetal bovine serum (FBS), 1 % penicillin–streptomycin, and 0.6 % G418 [3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl[oxy-2-hydroxycyclehexy]oxy-2-(1-hydroxyethyl0oxane-3,4-diol;400 mg/ml].
3. Nonenzymatic cell dissociation solution: dissolve 1 mM EDTA in distilled water, and then sterilize this solution by using an autoclave (*see* **Note 1**).
4. Use phosphate buffer saline (PBS); this is commercially available.
5. For cell culturing use plastic flasks (surface cell culture: 80 cm^2).
6. To expand the cells, use plastic flasks (surface cell culture: 175 cm^2).

2.2 Radioligand Displacement Binding Assay

This assay is carried out in nonsterile conditions; thus, all the following solutions are nonsterile.

1. Test compounds: dissolve the compounds under investigation in the appropriate solvent (e.g., dimethyl sulfoxide; DMSO) at a concentration of 10 or 100 mM, splitting the solution into 50 μl aliquots and storing each aliquot at −20 °C (*see* **Note 2**).
2. Prepare the binding/washing buffer (Tris/BSA buffer) in distilled water as follows: 50 mM Tris HCl, 50 mM Tris Base, 0.1 % w/v bovine serum albumin (BSA), pH 7.4.
3. To target cannabinoid CB_2 receptors, use [^{3}H]CP55,940 as the radioligand (e.g., provided by PerkinElmer).
4. To determine the amount of nonspecific binding, prepare a solution of CP55,940 in DMSO at a concentration of 1 mM.
5. To perform the assay, use plastic 96 deep-well plates.
6. The assay is stopped by vacuum filtration, using a 24-well sampling manifold (e.g., Brandel Cell Harvester) and Brandel GF/B filters (*see* **Note 3**).
7. To quantify the radioactivity, the filters are oven-dried (85 °C, 60 min) and then placed in 5 ml of scintillation fluid (e.g., Ultima Gold XR, PerkinElmer) and counted by liquid scintillation spectrometry (Beta counter).

3 Methods

3.1 Cell Culturing

Perform the following steps under sterile conditions.

1. Monolayers of human CB_2-transfected CHO cells are maintained at 37 °C and in 5 % CO_2 in medium flasks (surface cell culture: 80 cm^2), and are passaged twice weekly using a nonenzymatic cell dissociation solution (1 mM EDTA).
2. When cells reach 80–85 % confluence, remove medium from the flask, wash the cells with 10 ml of PBS, add 5 ml of nonenzymatic cell dissociation solution, place the flask in an incubator (at 37 °C) for 2–3 min, gently detach the cells, and add 5 ml of culture medium.
3. Expand cells by adding 2 ml of medium (containing the cells) to 25 ml of culturing medium by using four big flasks (surface cell culture: 175 cm^2). To keep the cell line, add 1 ml of the medium containing the cells to 25 ml of culturing medium in a medium flask (surface cell culture: 80 cm^2).
4. Place both medium and big flasks in an incubator (37 °C and in 5 % CO_2) until confluence is reached (*see* **Note 4**).
5. When confluence is reached (80–85 %), remove cells from the flasks by scraping and then freeze them as pellets at −20 °C until they are required.

6. On the day of the experiment, defrost cells, dilute in Tris-buffer that does not contain BSA (50 mM Tris HCl and 50 mM Tris Base), and homogenize with a 1-ml handheld homogenizer.
7. Perform a protein assay (e.g., by using a Bio-Rad Dc kit, Hercules, CA, USA) (*see* **Note 5**).

3.2 Displacement Binding Assay

1. The assay is carried out using 96 deep-well plates in a total volume of 500 μl, using Tris/BSA buffer.
2. Using Tris/BSA buffer and stock solutions of each test compound (e.g., 10 mM in DMSO), prepare decreasing concentrations of the test compound so that a dose–response curve can be drawn (*see* **Note 6**). Also prepare a *control* solution by using Tris/BSA buffer, with the same % of vehicle (e.g., DMSO) present in each diluted solution (e.g., 0.1 %).
3. Using Tris/BSA buffer and a stock solution of CP55,940 (1 mM), prepare a solution of CP55,940 (10 μM) that will be used to determine the nonspecific binding (concentration in the assay is 1 μM).
4. Prepare [^{3}H]CP55940 in Tris/BSA buffer at the appropriate concentration (*see* **Note 7**).
5. Using Tris/BSA buffer, prepare a solution of proteins (from the pellet of hCB_2-transfected cells) so that a known amount of proteins can be added (in our experiments we use 25 μg of proteins) (*see* **Note 8**).
6. Pipette 350 μl of Tris/BSA buffer, 50 μl of [H^3]CP55940 at an appropriate concentration (e.g., 0.7 nM), 50 μl of varying concentrations of the test drug, and 50 μl of cell membranes at an appropriate dose (e.g., 25 μg) into the wells of a 96 deep-well plate, progressing along each row from left to right.
7. Incubate the 96 well plates at 37 °C in a darkened water bath for 60 min.
8. Terminate the assay by adding ice-cold Tris/BSA buffer and then performing rapid vacuum filtration with a 24-well sampling manifold (e.g., Brandel Cell Harvester), and Brandel GF/B filter papers that have been soaked in Tris/BSA buffer at 4 °C for 24 h.
9. Wash each well 3/4 times with Tris/BSA buffer.
10. Dry the filter papers in an oven for 60 min at 85 °C.
11. Place each of these filter papers in a different scintillation vial and add 4 ml of scintillation fluid.
12. After soaking each filter paper in the scintillation fluid for 60 min, measure radioactivity with a beta counter. Values will be in cpm (counts per minute).

3.3 Analysis of the Results

1. The percentage (%) of "specific binding" is calculated as follows:

$$\frac{\text{mean cpm for control} - \text{mean cpm for CP55,940}\,(1\ \text{M})}{\text{mean cpm for control}} \times 100$$

2. The percentage of any displacement produced by each concentration of the test compound is calculated as follows:

$$\frac{\text{mean cpm for control} - \text{mean cpm for ligand at a particular concentration}}{\text{mean cpm for control} - \text{mean cpm for CP55,940}\,(1\ \text{M})} \times 100$$

4 Notes

1. The cell dissociation solution is also commercially available.
2. Avoid multiple thawing of the same aliquot, since this may result in the degradation of chemically unstable compounds. We recommend that the same aliquot is not used more than twice.
3. Before use, filters are soaked in Tris/BSA buffer at 4 °C for at least 24 h.
4. CHO cells grow up quickly. Usually, confluence is reached after 3 days.
5. The protein assay can be carried out as follows:
   (a) Prepare standards and unknown(s) (*X*) as shown in the table (Table 1) below (this scheme is suitable for a Biorad Kit).
   (b) Add the KIT reagents (as described by the manufacturer).
   (c) After 15 min read absorbance at 750 nm.
   (d) Construct the BSA standard curve by using the absorbance values of each standard solution (see table above), in order to perform linear regression analysis (e.g., using GraphPad Prism software).
   (e) Determine the concentration (mg/ml) of proteins in your sample (*X*), by using the above BSA standard curve to interpolate the absorbance value of your sample, then multiply the value obtained by 2 (this is the cell dilution factor; e.g., as described above, we added 50 μl of Tris-buffer to 50 μl of cell solution, thus we have diluted the cells by a factor of two).
   (f) Make a solution containing cells at a known concentration for use in the assay.

Table 1
BSA standard curve for the quantification of the content of unknown proteins

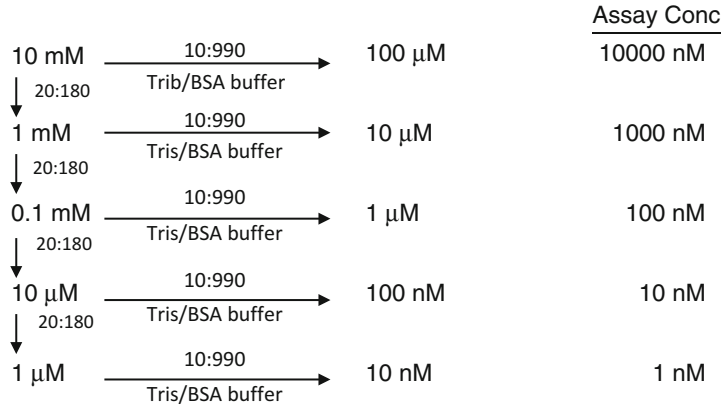
	A	B	C	D	E	F
Conc (mg/ml)	1.5	0.75	0.45	0.3	0.15	*X*
Standard BSA (μl)	100	50	30	20	10	50
Tris-Buffer (no BSA) (μl)	–	50	70	80	90	50

Please note that: (1) A–E are the concentrations of BSA used to build the BSA standard curve, which in turn is used to determine the concentration of proteins in your sample, and (2) by adding 50 μl of Tris-Buffer to 50 μl of cell solution, your sample is diluted twice and this should be taken into account in the calculation of the cell sample concentration

6. To construct a log concentration-response curve for the compound under investigation, prepare a stock solution by dissolving the test compound in DMSO (e.g., 10 mM), and then making serial dilutions to obtain solutions that have one of the following concentrations (*X*): 10, 1 μM, 100, 10, and 1 nM, ensuring that the concentration of vehicle in each solution remains constant (e.g., 0.1 %). Please note that: (1) each of these concentrations should be 10*X*, since 50 μl of each solution is added in the assay in a final volume of 500 μl (thus the solution will be diluted 10 times), and (2) in a sigmoidal log concentration-response curve, these concentrations are expressed as log M (Fig. 1).

   Example of Drug Dilutions (from a 10 mM stock solution in DMSO)

   Please note that the dilutions indicated on the left side of the scheme below should be made by using your stock solution solvent (in this case, DMSO).



7. The concentration of [^{3}H]CP55940 we use in our experiments is 0.7 nM. The binding parameters (B_{max} and K_D) for [^{3}H]CP55940 are determined by fitting data from saturation-

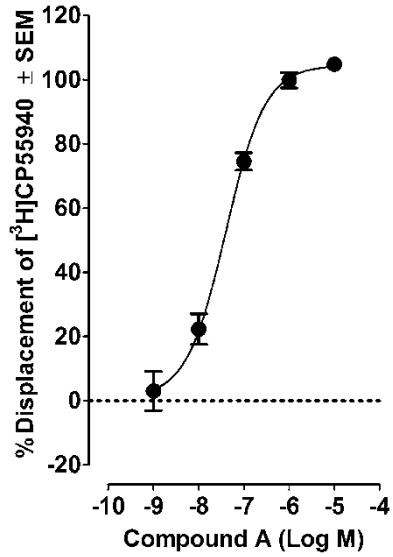


Fig. 1 Displacement of [^{3}H]CP55940 by compound A from specific binding sites on hCB_2 CHO cell membranes. Each symbol represents the mean percentage displacement ± SEM

binding experiments to a one-site saturation plot by using GraphPad Prism. For commercially available cells stably transfected with human CB_2, like the ones we use in our laboratory, these parameters are provided by the manufacturer.

8. Before any displacement binding assay is carried out, the protein content should be optimized by testing increasing concentrations of proteins versus a constant concentration of [^{3}H] CP55,940.

References

1. Bigott-Hennkens HM, Dannoon S, Lewis MR et al (2008) In vitro receptor binding assays: general methods and considerations. Q J Nucl Med Mol Imaging 52:245–253
2. Zettner A (1973) Principles of competitive binding assays (saturation analysis). Equilibrium techniques. Clin Chem 19: 699–705
3. Zettner A, Duly PE (1974) Principles of competitive binding assays (saturation analyses). II. Sequential saturation. Clin Chem 20:5–14

Chapter 7

Assay of TRPV1 Receptor Signaling

Aniello Schiano Moriello and Luciano De Petrocellis

Abstract

The transient receptor potential vanilloid-1 ion channel (TRPV1) is a non-selective ligand-gated cation channel. It is an integrator of a wide variety of exogenous and endogenous physical and chemical stimuli, including capsaicin, noxious heat (>42 °C), and protons (pH < 5.2). TRPV1 is expressed predominantly in primary sensory neurons involved in pain sensation, but also in other neuronal cell types, in the plasma membrane of different non-neuronal cells such as immune cells, keratinocytes, smooth muscle cells, and in the urothelium. Some of these cell types are involved in inflammation. When activated, TRPV1 leads to the gating of cations, including Ca^{2+}, thus generating changes in intracellular Ca^{2+} concentration. Calcium ions play fundamental roles in many cellular processes, virtually in all cells. The use of Ca^{2+} fluorescent indicators is a tool for monitoring intracellular Ca^{2+} concentration.

In this chapter, we describe a method for recording and monitoring Ca^{2+} signals through the single wavelength fluorescent indicator Fluo-4 acetoxymethyl (AM), and the ratiometric fluorescent indicator Fura-2 AM in HEK-293 cells transfected with TRPV1 and other TRP channels. TRPV1 pharmacological modulation may potentially represent a strategy for the control of pain and inflammatory conditions in a variety of diseases and injury states.

Key words Calcium, Fluorescence, TRPV1 channels, TRP channels, Vanilloids, Anandamide

1 Introduction

Calcium ions (Ca^{2+}) play a central role in a multitude of physiological processes. The use of fluorescent Ca^{2+} indicators such as Fluo-4, a brighter and more photostable derivative of Fluo-3 [1], and of ratiometric dye Fura-2, represents a valid approach to study the role of Ca^{2+} in a cellular process. The transient receptor potential (TRP) cation channel superfamily consists of 28 cation channels that have distinct physiological functions, including thermal sensation, chemosensation, and magnesium and iron transport. The TRP channel superfamily can be subdivided into six subfamilies: TRP cation channel subfamily C (canonical; TRPC), TRP cation channel subfamily V (vanilloid; TRPV), TRP cation channel

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subfamily M (melastatin; TRPM), TRP cation channel subfamily A (ankyrin; TRPA), TRP cation channel polycystin subfamily (TRPP), and TRP cation channel mucolipin subfamily (TRPML). The molecular architecture of TRP channels seems to be represented by four subunits, which comprise six transmembrane domains (S1–S6), intracellular N- and C-termini, and a pore-forming loop between S5 and S6 [2]. Members of the TRPV (V1–V4), TRPM (M2, M3, M5, and M8), and TRPA1 that are gated by temperature changes (<15 to >53 °C) are known as thermoTRP channels. Most members of the TRP channel superfamily are cation channels, and some of them show high permeability to Ca^{2+} and Zn^{2+}. Following TRP channels stimulation, an influx of Ca^{2+} via the plasma membrane generates changes in the intracellular Ca^{2+} concentration, $[Ca^{2+}]i$. However, there is evidence that TRP channels are also located in intracellular organelles and serve as intracellular Ca^{2+} release channels [3–6].

TRPV1 was identified by its responsiveness to capsaicin, a naturally derived product from chili peppers that elicits a burning sensation. TRPV1 is also activated by other compounds like resiniferatoxin (RTX), spider, and tarantula toxins. Some endogenous compounds, i.e., the "endovanilloids," may activate TRPV1: in particular, the endocannabinoids, anandamide and 2-arachidonoylglycerol, and the *N*-acyldopamines, *N*-arachidonoyldopamine, and *N*-oleoyldopamine. Moreover, TRPV1 responds to noxious temperatures (>42 °C) and low pH (<5.2), acting as a polymodal nociceptor. TRPV1 displays wide cellular expression in both peripheral and central nervous systems, with the highest expression in sensory neurons. It is well known that a chronic administration of capsaicin produces receptor desensitization, so that previously excited neurons become less sensitive to capsaicin and to other noxious stimuli.

In this chapter, we describe a method for recording Ca^{2+} signals in HEK-293 cells transfected with TRPV1 and other TRP channels, by using the fluorescent indicators Fluo-4 acetoxymethyl (AM) and Fura-2 AM.

2 Materials

1. 100 mm and 6-well plate dishes.
2. HEK-293 cells.
3. Plasmids containing TRP cDNA.
4. Lipofectamine 2000.
5. Cell medium: EMEM, 2 mM Glutamine, 1 % Non-Essential Amino Acids (NEAA), 10 % fetal bovine serum (FBS).
6. Tyrode's solution: 145 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4 adjusted with NaOH (*see* **Note 1**). Store at 4 °C.

7. Fluo-4 AM (10×50 μg) or Fura-2 AM (20×50 μg) (*see* **Note 2**). Store at –20 °C.
8. 20 % Pluronic® F-127 in DMSO solution. Store at room temperature.
9. Ionomycin.
10. Perkin Elmer LS 50B Fluorescence Spectrometer.
11. PTP-1 Fluorescence Peltier System (Perkin Elmer).
12. GraphPad Prism® software.

3 Methods

3.1 Cell Culture and Transfection

Split subconfluent cultures (70–80 %) of HEK-293 (transfected or not) 1:10 to 1:15 by using 0.25 % trypsin/EDTA every 3–4 days, and maintain at 37 °C with 5 % CO_2.

HEK-293 cells are plated in 6-well plate dishes such that they are 70–90 % confluent on the day of transfection. Cells are transfected with the plasmid of interest (TRP construct) using Lipofectamine 2000, according to the following protocol:

(a) The mix containing the plasmid of interest is prepared by diluting 1.6 μg DNA in 50 μl of growth medium in the absence of serum, and is gently mixed.

(b) The mix containing Lipofectamine 2000 is prepared by adding 4 μl of Lipofectamine (1 mg/ml) in 50 μl of medium without serum.

(c) Once prepared, these two mixes are incubated for 5 min at room temperature.

(d) Afterwards, the mix containing Lipofectamine is added to the DNA mix. These two solutions are gently mixed and incubated for 20 min at room temperature to allow formation of the DNA–lipid complex.

(e) 100 μl of DNA–lipid complex is then added to each well containing cells and 900 μl of complete medium (plus serum, *see* **Note 3**), to obtain a final DNA concentration of 1.6 μg/ml.

(f) Cells are incubated at 37 °C and 5 % CO_2 for 24–48 h before adding the required antibiotic to obtain stably transfected clones. In our protocol, stably transfected clones are selected by using 600 μg/ml of Geneticin G-418.

(g) Quantitative real time (qPCR) or Western blot analysis should be carried out routinely, to measure TRP gene over-expression in transfected cells.

3.2 Fluo-4 AM Loading

Prepare Fluo-4 AM stock solution by dissolving 50 μg Fluo-4 AM in 15.2 μl of 20 % Pluronic® F-127 DMSO solution (*see* **Note 4**) + 15.2 μl of DMSO. Store stock solution at −20 °C.

Add 4 μl of Fluo-4 AM stock solution to 1.5 ml of basal EMEM medium (the final Fluo-4 AM concentration is 4 μM).

Take a 100 mm Petri dish with HEK cells at 70–80 % confluence and remove medium (*see* **Note 5**).

Add 4 μM Fluo-4 AM solution.

Keep cells in the dark at room temperature for 1 h (*see* **Note 6**).

Collect the cells by centrifuging for 1 min at 500 × *g*.

Remove supernatant, resuspend cells with 2 ml of Tyrode's solution, and centrifuge for 1 min at 500 × *g*.

Remove supernatant, resuspend cells with 10 ml of Tyrode's solution (*see* **Note 7**). Cells will be suitable for Ca^{2+} spectrofluorimeter assays for up to 2 h (*see* **Note 8**).

To reduce leakage of the de-esterified Fluo-4 probenecid (2 mM), an anion transport inhibitor may be added to the Tyrode's solution containing cells. However, probenecid is a specific activator of TRPV2 [7], and thus it is not appropriate to study this TRP channel.

3.3 Fura-2 AM Loading

Prepare Fura-2 AM stock solution: Dissolve 50 μg Fura-2 AM in 20 μl of 20 % Pluronic® F-127 DMSO solution + 20 μl of DMSO. Store stock solution at −20 °C.

Add 4 μl of Fura-2 AM stock solution to 2 ml of basal EMEM medium (the final Fura-2 AM concentration is 2.5 μM).

Take a 100 mm Petri dish with HEK-293 cells at 70–80 % of confluence and remove medium.

Add 2.5 μM Fura-2 AM solution.

Keep cells in the dark at room temperature for 1 h.

Collect the cells by centrifuging for 1 min at 500 × *g*.

Remove supernatant, resuspend cells with 2 ml of Ca^{2+} free buffer (145 mM NaCl, 2.5 mM KCl, 2.7 mM $MgCl_2$, 10 mM D-glucose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4) (*see* **Note 9**), and centrifuge for 1 min at 500 × *g*.

Remove supernatant, resuspend the cells with 10 ml of Ca^{2+} free buffer. The cells will be suitable for Ca^{2+} spectrofluorimeter assays for up to 2 h.

3.4 Assay of TRP-Mediated Elevation of Intracellular Ca^{2+} in Transfected HEK-293 Cells

The cells (loaded with Fluo-4 AM) are transferred to 1 cm^2 quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS50B; λ_{EX} = 488 nm, λ_{EM} = 516 nm), equipped with PTP-1 Fluorescence Peltier System (PerkinElmer Life and Analytical Sciences) under continuous stirring (*see* **Note 10**). Experiments are carried out by measuring cell fluorescence at 25 °C before and after the addition of various concentrations of test compounds, the effects of which are normalized against the response to 4 μM ionomycin in each experiment. When significant, the increases in fluorescence in

wild-type (i.e., not transfected with any construct) HEK-293 cells are used as baseline, and are subtracted from the values obtained from transfected cells. These increases in fluorescence values may be due to calcium influx that is not mediated by TRP, or to autofluorescence of the test compounds that sometimes occurs at high concentrations. In Figs. 1 and 2 time course examples of the Fluo-4 signals in human TRPV1 over-expressing HEK-293 cells are shown.

The potency of the compounds is determined as EC_{50} value, which is the concentration required to produce half-maximal increase in $[Ca^{2+}]i$. Efficacy is defined as the maximum response elicited by the compounds tested and is determined by comparison with the effect observed with 4 μM ionomycin.

Antagonist/desensitizing behavior is evaluated against the TRP agonist by using the concentration that exerts a submaximal effect, by adding each compound to the quartz cuvette 2–5 min before stimulation of cells with the agonist. IC_{50} is expressed as the concentration that exerts a half-maximal inhibition of the agonist effect, taken as 100 %. Dose-response curves are fitted by a sigmoidal regression with variable slope, using the Prism program (GraphPad Software Inc.). In Figs. 3, 4, and 5 dose-response

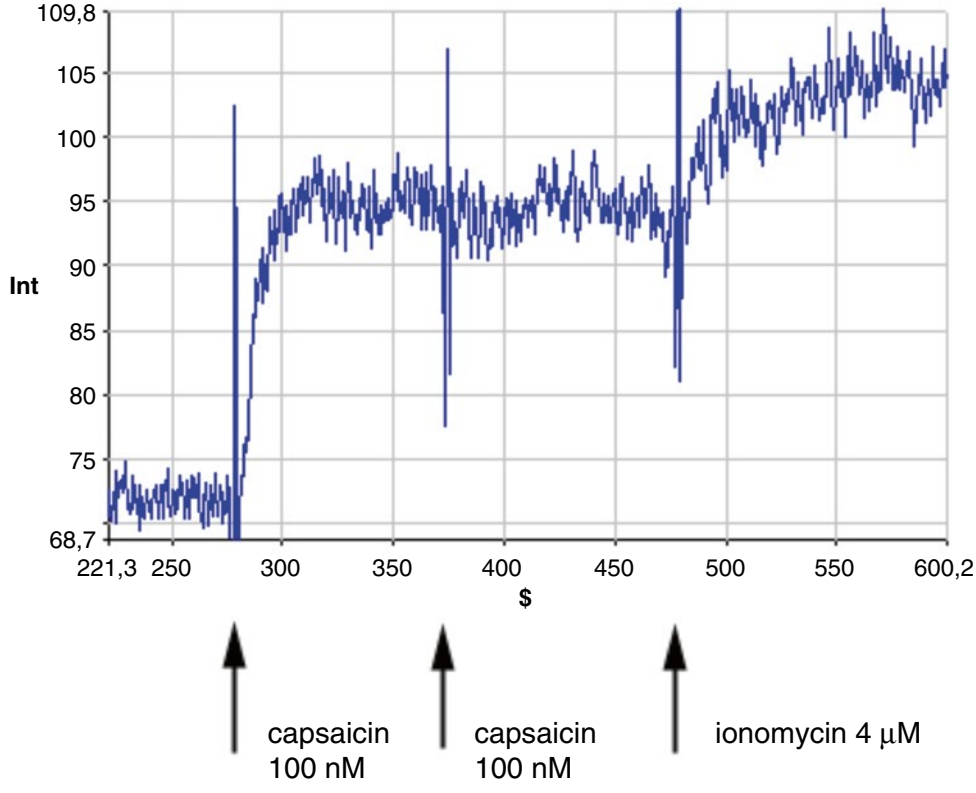

Fig. 1 Time course of the Fluo-4 signals recorded from HEK-293 cells over-expressing the human recombinant TRPV1 as response to two consecutive administration of 100 nM capsaicin and 4 μM ionomycin

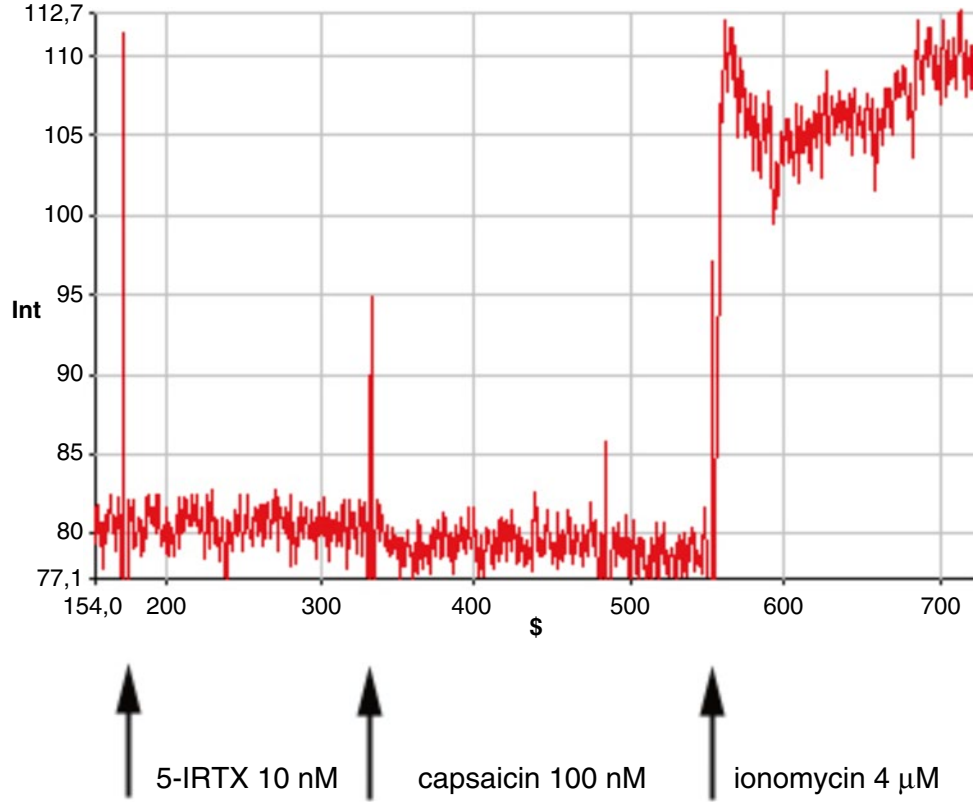

Fig. 2 Time course of the Fluo-4 signals recorded from HEK-293 cells over-expressing the human recombinant TRPV1 as response to 10 nM 5′-iodo-resiniferatoxin (5-IRTX), 100 nM capsaicin, and 4 μM ionomycin

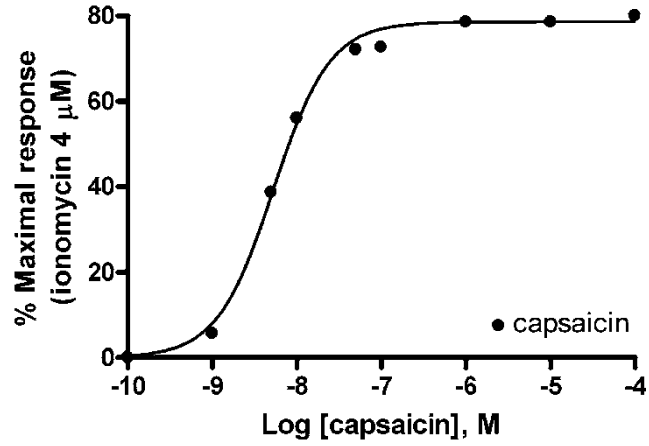

Fig. 3 Effect of capsaicin on intracellular Ca^{2+} elevation in HEK-293 cells over-expressing human TRPV1. Data are expressed as % of the effect observed with 4 μM ionomycin

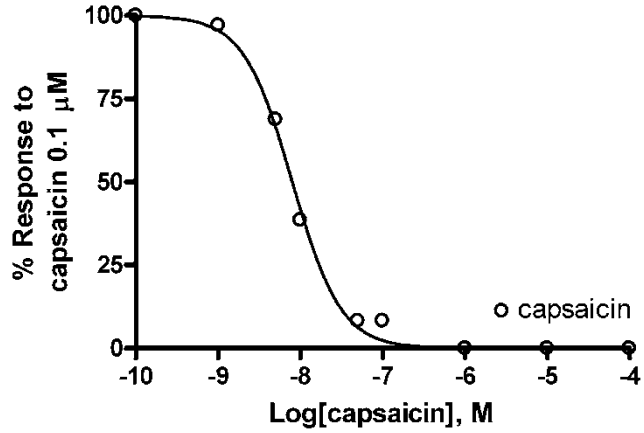

Fig. 4 Desensitization by 5 min pre-incubation with capsaicin of 100 nM capsaicin-induced Ca^{2+} elevation in HEK-293 cells over-expressing the human TRPV1. The effect on $[Ca^{2+}]i$ exerted by 100 nM capsaicin alone was considered as 100 %

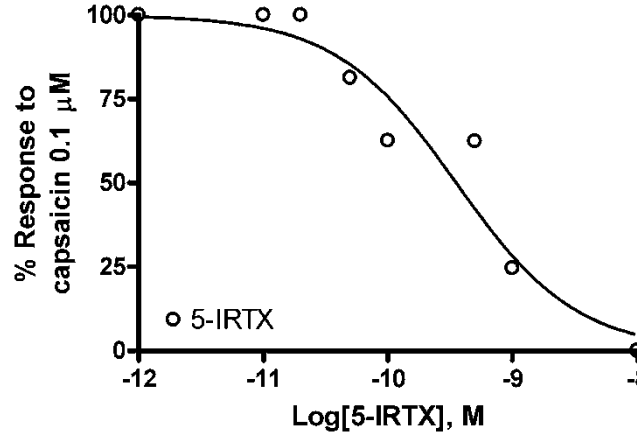

Fig. 5 Effect of a 5 min pre-incubation with the TRPV1 antagonist 5′-iodo-resiniferatoxin (5-IRTX) on the response to 100 nM capsaicin. The effect on $[Ca^{2+}]i$ exerted by 100 nM capsaicin alone was considered as 100 %

curves are shown to evaluate potency or antagonist/desensitizing behavior of test compounds.

TRPV1-expressing HEK-293 cells respond to ethanol in a concentration-dependent fashion [8]; therefore it is important to avoid ethanol and use dimethyl sulfoxide (DMSO) for solubilizing the test compounds (*see* **Note 11**).

To study mobilization of intracellular calcium mediated by TRP, it is better to use the fluorescent probe Fura-2 AM. Indeed, the possibility to obtain ratio measurements is an important property of this probe. The use of the 340/380 nm excitation ratio allows accurate measurement of the intracellular Ca^{2+} concentration. To monitor

changes in calcium concentration which are independent of the concentration of Fura-2 in the cell, the ratio of the fluorescence intensities at 340 nm (corresponding to the bound form of calcium) and 380 nm (corresponding to the unbound form of calcium) is calculated while the agonists are added to the cell suspension. The data are collected by rapidly measuring the intensity at excitation wavelengths of 340 and 380 nm, while the emission remains at 510 nm. A fast filter accessory allows the collection of data every 40 ms. The latter consists of a filter wheel installed in the excitation monochromator. The filter wheel rotates rapidly and enables each filter to be in the beam coincident with the flash of the xenon lamp. Calcium concentrations can be calculated by calibrating the experimental results and using the Grynkiewicz equation to convert the intensity data to calcium concentrations [9].

The efficacy of the agonists is determined by comparison with the maximum effect on $[Ca^{2+}]i$ of 1 mM carbachol, or of 1 μM thapsigargin in cells that do not respond to carbachol [10].

Changes in $[Ca^{2+}]i$ are expressed as F_{340}/F_{380} ratios.

3.5 Measuring Ca^{2+} in TRP Transfected HEK-293 Cells

By using Fluo-4 to estimate $[Ca^{2+}]i$ from the observed fluorescence signal, one can divide changes in fluorescence by the average resting fluorescence according to the formula:

$$\Delta Ca^{2+} = \Delta F / F = \left(F - F_{rest}\right) / F_{rest}$$

where F is the measured fluorescence intensity of Fluo-4 at any given time, and F_{rest} is the average fluorescence intensity of the dye in the cell before stimulation (e.g., before addition of an agonist).

For single wavelength indicators, to convert Ca^{2+} fluorescence to Ca^{2+} concentration the following calibration formula is generally used:

$$\left[Ca^{2+}\right] = K_d\left[\left(F - F_{min}\right) / \left(F_{max} - F\right)\right]$$

where K_d is the dissociation constant of the Ca^{2+} indicator, F is the fluorescence value measured at any time during the recording, F_{min} is the fluorescence in the absence of Ca^{2+}, and F_{max} is the fluorescence intensity of the Ca^{2+} saturated dye. F_{min} and F_{max} are empirically determined in approximately zero and saturating Ca^{2+} [11]. It should be noted that K_d of Fluo-4 AM is 345 nM.

3.6 Study of Other TRPs Using the Calcium Assay

The same test can be used also for other TRPs, as follows.

For TRPV2 we showed that rat TRPV2-HEK-293 cells exhibit a sharp increase in intracellular $[Ca^{2+}]i$ upon application of 2-aminoethoxydiphenyl borate or LPC, two well-established TRPV2 activators [12, 13]. We determined the concentration for half-maximal activation to be 3,400 ± 0.025 μM for LPC [14].

In the case of TRPV3, rat TRPV3-expressing HEK-293 cells were first sensitized with the agonist 2-aminoethoxydiphenyl borate

(100 μM). Antagonist/desensitizing behavior was evaluated against 100 μM thymol, always by adding the test compounds to the quartz cuvette 2–5 min before stimulation of cells with agonists [15]. We did not use 2-aminoethoxydiphenyl borate only, because this compound, although more potent than thymol or carvacrol (EC_{50} = 20 μM and maximal effect 42.1 % of ionomycin, under the conditions of our assay), is not selective [16].

In the case of rat TRPV4-expressing HEK-293 cells, the agonist used was 1 μM 4α-phorbol 12,13-didecanoate (4α-PDD), able to elevate intracellular Ca^{2+} with a potency (EC_{50}) of 0.46 ± 0.07 μM [16].

In the case of rat TRPM8-expressing HEK-293 cells, antagonist/desensitizing behavior was evaluated against 0.25 μM icilin and/or 20 μM menthol by adding the test compounds to the quartz cuvette 5 min before stimulation of cells with agonists. For icilin, efficacy is 75.1 ± 1.1; potency EC_{50} is 0.11 ± 0.01 μM; desensitization IC_{50} is 0.0580 ± 0.0019 μM, while for menthol potency EC_{50} is 40.9 ± 15.1 μM [17–20].

It is noteworthy that in the case of TRPM8 the spectrofluorimeter (Perkin-Elmer LS50B) should be equipped with PTP-1 Fluorescence Peltier System; indeed, TRPM8 is gated by low temperature (<25 °C) and during the cold season it could be already activated and consequently desensitized (*see* **Note 12**).

In the case of HEK-293 cells stably over-expressing recombinant rat TRPA1, the effects of TRPA1 agonists are expressed as a percentage of the effect obtained with 100 μM allyl isothiocyanate. For this substance efficacy is 65.9 ± 0.5; potency EC_{50} is 1.41 ± 0.04 μM; desensitization IC_{50} is 1.71 ± 0.06 μM [21].

4 Notes

1. Prepare Tyrode's solution using ultrapure water.
2. Modification of carboxylic acids of Fluo-4 with acetoxymethyl (AM) ester groups results in an uncharged molecule that can permeate cell membranes. Inside the cell, the AM lipophilic groups are cleaved by nonspecific esterases, forming a charged form. Fluo-4 exhibits large fluorescence intensity increases (>100-fold) upon Ca^{2+} binding.
3. Eukaryotic cells are efficiently transfected with Lipofectamine 2000, according to the manufacturer's instructions, in medium devoid of serum, which must be replaced after 4–5 h post-transfection with fresh medium containing serum. However, the Lipofectamine 2000's efficiency remains high under both cell transfection conditions.
4. Pluronic® F-127 is a nonionic, surfactant polyol that facilitates the solubilization of water-insoluble dyes, and is used to help dispersion of AM ion indicators.

5. Cellular health before transfection is important. Use HEK-293 cells at early passages to have a better transfection efficiency and expression levels. The cells should be maintained in exponential growth avoiding 100 % confluence, in order to maintain the ability to resume an exponential growth after passaging.
6. Load Fluo-4 AM at room temperature, rather than at 37 °C, to avoid subcellular compartmentalization of the indicator, a problem with the AM ester loading technique that may interfere with the measurement of cytosolic Ca^{2+}.
7. It is important to use a medium without serum for loading cells, and to leave out bovine serum albumin (BSA) in the assay buffer because the presence of BSA can influence the assay. Indeed, it was found that increase of $[Ca^{2+}]i$ induced at TRPV1 by the bioactive lipid anandamide is potently reduced by BSA, which is normally used to avoid lipophilic substances from sticking to plastic and glassware [22]. This finding may be explained by suggesting that BSA prevents the uptake of AEA by HEK-TRPV1 cells, thus interfering with the carrier-mediated internalization of this compound, which is necessary to observe activation of TRPV1 at an intracellular site [23]. Additional studies have shown that BSA is necessary for anandamide release from cells, because otherwise this compound is immediately taken up from the incubation medium [24]. This observation may explain why a potency of anandamide at TRPV1 from five to tenfold lower than that reported by us has been observed by others [25–27], who added BSA to their TRPV1 assay protocols.
8. The cells are usable for hours after Fluo-4 AM loading; however, because of the slow leakage of de-esterified indicator, it is better to measure cell fluorescence for 2–3 h after loading, thus avoiding a marked variation of Ca^{2+} signals.
9. EGTA strongly chelates calcium ions, leaving the probe in its unbound state.
10. It is very important that the cells are under continuous stirring, to keep them in suspension during the time course of the experiment.
11. It is noteworthy that TRPV1-expressing HEK-293 cells respond to ethanol in a concentration-dependent mode. Ethanol potentiates the response of TRPV1 to capsaicin, protons and heat [8]. For this reason, it is important to avoid ethanol to solubilize the compounds to be tested, using dimethyl sulfoxide (DMSO) instead or, when unsuitable, methanol at a 0.1 % concentration.
12. Thermo TRPs are activated by changes in the environmental temperature, from noxious cold (<15 °C) to injurious heat (>42 °C). In particular, for TRPM8 the thermal activation threshold is ≤25 °C; therefore it is important to keep experimental temperature constant.

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References

1. Paredes RM, Etzler JC, Watts LT et al (2008) Chemical calcium indicators. Methods 46: 143–151
2. Gaudet R (2008) TRP channels entering the structural era. J Physiol 586:3565–3575
3. Moran MM, McAlexander MA, Bíró T et al (2011) Transient receptor potential channels as therapeutic targets. Nat Rev Drug Discov 10:601–620
4. Vennekens R, Owsianik G, Nilius B (2008) Vanilloid transient receptor potential cation channels: an overview. Curr Pharm Des 14:18–31
5. De Petrocellis L, Schiano Moriello A (2013) Modulation of the TRPV1 channel: current clinical trials and recent patents with focus on neurological conditions. Recent Pat CNS Drug Discov 8:180–204
6. Gees M, Colsoul B, Nilius B (2010) The role of transient receptor potential cation channels in Ca^{2+} signaling. Cold Spring Harb Perspect Biol 10:a003962
7. Bang S, Kim KY, Yoo S et al (2007) Transient receptor potential V2 expressed in sensory neurons is activated by probenecid. Neurosci Lett 425:120–125
8. Trevisani M, Smart D, Gunthorpe MJ et al (2002) Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. Nat Neurosci 5:546–551
9. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
10. Marini P, Schiano Moriello A, Cristino L et al (2009) Cannabinoid CB1 receptor elevation of intracellular calcium in neuroblastoma SH-SY5Y cells: interactions with muscarinic and delta-opioid receptors. Biochim Biophys Acta 1793:1289–1303
11. Takahashi A, Camacho P, Lechleiter JD et al (1999) Measurement of intracellular calcium. Physiol Rev 79:1089–1125
12. Neeper MP, Liu Y, Hutchinson TL et al (2007) Activation properties of heterologously expressed mammalian TRPV2: evidence for species dependence. J Biol Chem 282: 15894–15902
13. Monet M, Gkika D, Lehen'kyi V et al (2009) Lysophospholipids stimulate prostate cancer cell migration via TRPV2 channel activation. Biochim Biophys Acta 1793:528–539
14. De Petrocellis L, Ligresti A, Schiano Moriello A et al (2011) Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. Br J Pharmacol 163:1479–1494
15. Ortar G, Morera L, Schiano Moriello A et al (2012) Modulation of thermo-transient receptor potential (thermo-TRP) channels by thymol-based compounds. Bioorg Med Chem Lett 22:3535–3539
16. De Petrocellis L, Orlando P, Schiano Moriello A et al (2012) Cannabinoid actions at TRPV channels: effects on TRPV3 and TRPV4 and their potential relevance to gastrointestinal inflammation. Acta Physiol 204:255–266
17. De Petrocellis L, Ortar G, Schiano Moriello A et al (2015) Structure–activity relationships of the prototypical TRPM8 agonist icilin. Bioorg Med Chem Lett 25(11):2285–2290. doi:10.1016/j.bmcl.2015.04.032
18. De Petrocellis L, Starowicz K, Schiano Moriello A et al (2007) Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): effect of cAMP, cannabinoid CB(1) receptors and endovanilloids. Exp Cell Res 313:1911–1920
19. De Petrocellis L, Vellani V, Schiano-Moriello A et al (2008) Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. J Pharmacol Exp Ther 325:1007–1015
20. Ortar G, De Petrocellis L, Morera L et al (2010) (-)-Menthylamine derivatives as potent and selective antagonists of transient receptor potential melastatin type-8 (TRPM8) channels. Bioorg Med Chem Lett 20: 2729–2732
21. Del Prete D, Caprioglio D, Appendino G et al (2015) Discovery of non-electrophilic capsaicinoid-type TRPA1 ligands. Bioorg Med Chem Lett 25:1009–1011
22. De Petrocellis L, Davis JB, Di Marzo V (2001) Palmitoylethanolamide enhances anandamide

stimulation of human vanilloid VR1 receptors. FEBS Lett 506:253–256

23. Ligresti A, De Petrocellis L, Hernán Pérez de la Ossa d et al (2010) Exploiting nanotechnologies and TRPV1 channels to investigate the putative anandamide membrane transporter. PLoS One 5, e10239
24. Di Marzo V, Fontana A, Cadas H et al (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372:686–691
25. Smart D, Gunthorpe MJ, Jerman JC et al (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). Br J Pharmacol 129:227–230
26. Ross RA, Gibson TM, Brockie HC et al (2001) Structure-activity relationship for the endogenous cannabinoid, anandamide, and certain of its analogues at vanilloid receptors in transfected cells and vas deferens. Br J Pharmacol 132:631–640
27. Olah Z, Karai L, Ladarola MJ (2001) Anandamide activates vanilloid receptor 1 (VR1) at acidic pH in dorsal root ganglia neurons and cells ectopically expressing VR1. J Biol Chem 276:31163–31170

Chapter 8

A Functional Assay for GPR55: Envision Protocol

Sharon Anavi-Goffer and Ruth A. Ross

Abstract

AlphaScreen® SureFire® assay is a novel technology that combines luminescent oxygen channeling technology, nano-beads, and monocloncal antibodies to detect the level of a selected protein in a volume lower than 5 μl. This method is more sensitive compared with the traditional enzyme-linked immunosorbent assays (ELISA), and can detect an increasing number of new targets. Here, we described a method for AlphaScreen® SureFire® assay that targets ERK1/2 phosphorylation, a primary downstream signaling pathway that conveys activation of GPR55 by L-α-lysophosphatidylinositol (LPI) and certain cannabinoids.

Key words AlphaScreen, Enspire, AlphaLISA, GPR55, Phytocannabinoids, High-throughput screening, Cannabinoid receptor

1 Introduction

AlphaScreen® SureFire® assay is an alternative technology to the traditional ELISA (Fig. 1).

It is faster and more sensitive than ELISA, requires a microquantity of the tested sample, and can be performed in 384 well plates (Fig. 2). This upscale enables the translation to high-throughput screening platforms that employ robotic devices at drug discovery laboratories.

AlphaScreen® SureFire® assay has been applied to study the level of phosphorylated proteins in various fields of research, including GPCR signaling, epigenetics, kinase research, and protein-protein interactions. Using this method, we have recently studied the pharmacological actions of synthetic cannabinoids and phytocannabinoids at the human GPR55 receptor [1]. The latter is activated by the endogenous lipid LPI [1–4], and appears to highly regulate cancer cell function. In selected cancer cells, GPR55 is highly expressed and its activation by LPI increases cell migration, invasion, and proliferation [5–7]. Based on these studies, many resources have been allocated to identify GPR55 inhibitors [1, 8]. Other important

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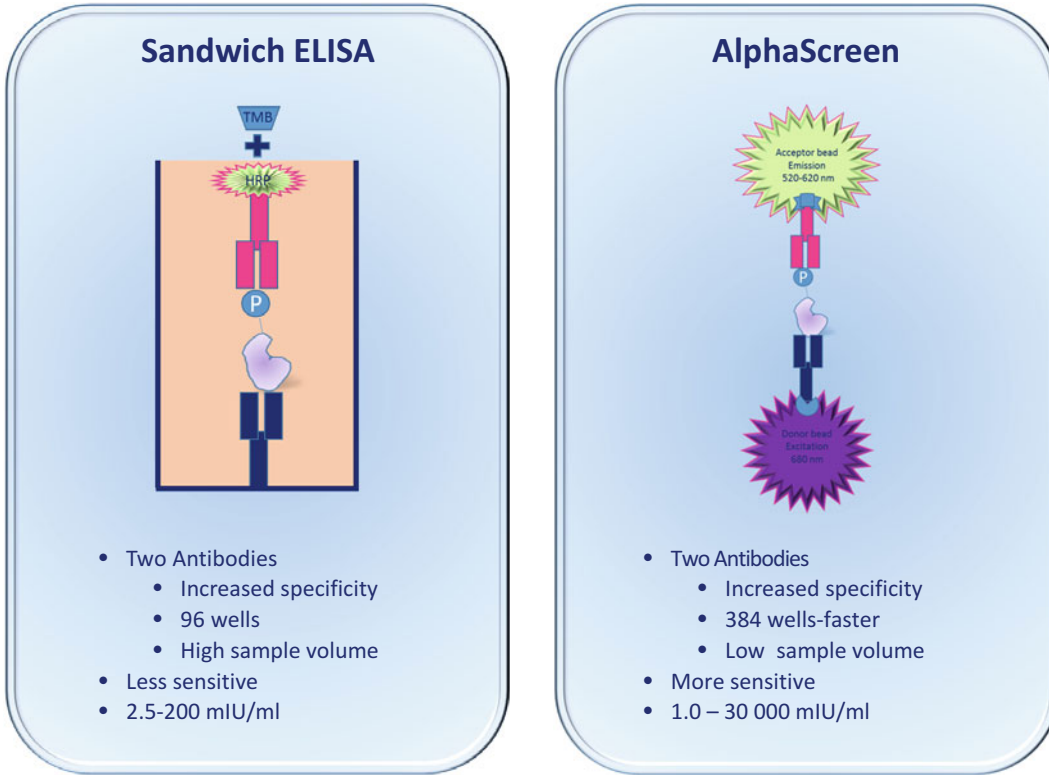

Fig. 1 Time is money. A comparison between traditional sandwich ELISA and AlphaScreen® SureFire® assays. Note the number of samples, time, and sensitivity that AlphaScreen® SureFire® assay offers

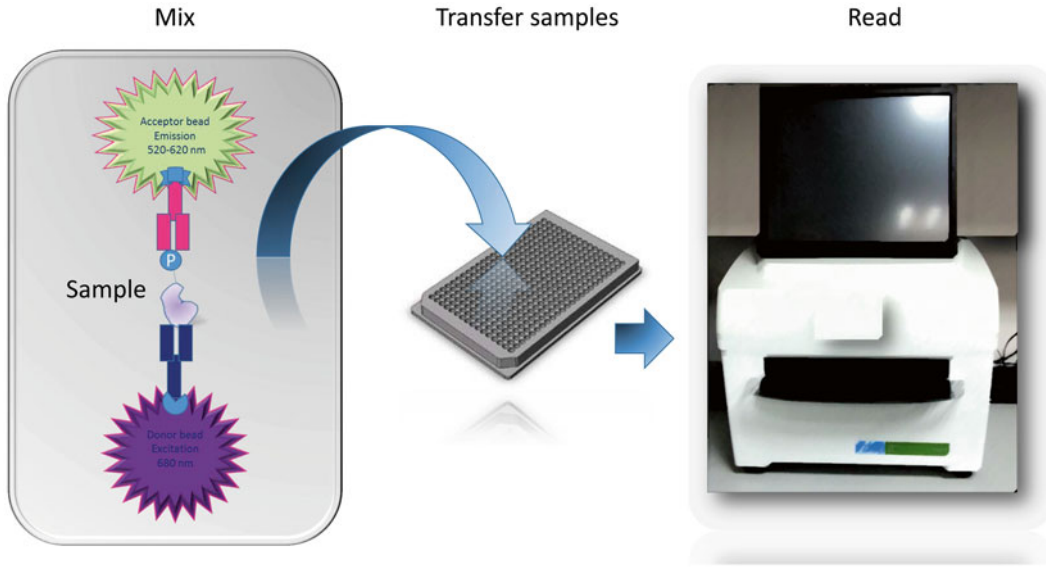

Fig. 2 Illustration of AlphaScreen® SureFire® technology. Steps of the assay are highlighted

clinical targets for identifying GPR55 inhibitors emerged from studies which showed that activation of GPR55 contributes to inflammation and enhances neuropathic pain [9]. We have identified GPR55 enhancers, inhibitors, and modulators of LPI-induced activation of human GPR55 [1]. Our results also suggest that GPR55 has an allosteric binding pocket and point to a cross-talk between the cellular messengers of NFAT-ERK1/2 signaling cascades [1]. Another example is related to the lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) [10]. The latter has been shown to directly interact with C-reactive protein (CRP) in addition to binding to oxLDL [10]. These findings suggest that CRP, a risk factor for cardiovascular events, promotes endothelial dysfunction and amplifies vascular inflammation by engaging LOX-1.

2 Materials

1. *Drugs.* All compounds were dissolved in DMSO, and 10 mM stocks were kept at −20 °C. LPI was stored at −80 °C for up to 3 months.
2. *Cells.* Untransfected HEK293 cells are grown in Dulbecco's modified Eagle's medium (Gibco) containing 2 mM L-glutamine medium (Gibco), and 10 % fetal bovine serum. Cells are slit at a ratio of 1:2 or 1:4, according to the required cell density. For HEK293 cells that stably express the tagged-human GPR55 receptor (hGPR55-HEK293), the GPR55 receptor is tagged with a triple hemagglutinin epitope (HA) at the N terminus (3xHA-GPR55), preceded by the signal sequence from the human growth hormone (HGH; residues 1–33), and is subcloned into pcDNA 3.1 vector. Transfected hGPR55-HEK293 cells are grown in a medium prepared with 500 ml of Dulbecco's modified Eagle's medium/F12 (Gibco), 50 ml of 10 % new born calf serum (Gibco), 5 ml of G-418 (50 mg/ml), 5 ml of L-glutamine (Sigma), and 3 ml of penicillin/streptomycin solution (containing 10,000 units of penicillin (base) and 10,000 μg/ml of streptomycin from Gibco).
3. *Serum-free Medium.* For transfected cells, add to DMEM/F12 medium 5 ml of G418 and 5 ml of L-glutamine. For untransfected cells, add to DMEM/F12 medium 5 ml of L-glutamine without G418.
4. *Assay Medium.* Prepare serum-free/phenol-free "assay medium" with Dulbecco's modified Eagle's medium/F-12 containing 2 nM L-Glutamine (Gibco).
5. *AlphaScreen® Kit.* AlphaScreen® SureFire™ Phospho-ERK½ is from PerkinElmer, and its components are listed in Table 1.

Table 1
Materials supplied with the kit

Lysis buffer ×5	5 × 2 ml	Store at 4 °C
Activation buffer	1 × 2 ml	Store at room temperature or 4 °C Warm up to 37 °C on assay day
Reaction buffer	2 × 1.7 ml	Store at 4 °C
Control un-stimulated cell lysate	50 µl	Aliquot and store at −20 °C
Control stimulated cell lysate	50 µl	Aliquot and store at −20 °C
Protein A IgG kit		Store at 4 °C in the dark

6. *Positive/Negative Control Lysates*. Lysates are provided with the AlphaScreen® Kit. Reconstitute them with double-distilled water (DDW) according to the manufacturer's instructions, and freeze aliquots at −20 °C.
7. *Plates*. Perform the assay in 384 well white ProxiPlates according to the manufacturer's instructions.

3 Methods

1. *Cell Maintenance*. Add cells to 10 ml of medium, centrifuge for 5 min at 200 × *g*, remove supernatant, add 10 ml of fresh growth medium, seed in a T75 flask, place in the incubator in a humidified atmosphere at 37 °C and 5 % CO_2; refresh medium after 24 h.
2. *Plating hGPR55-HEK Cells for Assay*. The following protocol yields about five 96 well plates. Harvest cells from two T75 flasks by washing once with dissociation buffer (from Sigma), add 10 ml of dissociation buffer, collect cells into a 50 ml tube, add 10 ml of growth medium, centrifuge at 200 × *g* for 3 min, resuspend in 20 ml of growth medium. Count cells, seed 40,000 cells/well in a 96 well plate. Cells should be 100 % confluent on the next day.
3. *Prepare hGPR55-HEK for Assay*. Remove the growth medium from the cells, and replace with 100 µl of serum-free medium. Serum-starve the cells for 48 h. Both media should be warmed up to 37 °C (*see* **Note 1**).
4. *ERK1/2 MAP-kinase Phosphorylation Assay*

Follow these steps:

1. Design your assay plate (method described below is for a 96 well plate).
2. Warm up the assay medium to 25 °C.

3. Prepare 1× lysis buffer by diluting 1:5 with DDW the lysis buffer ×5, supplied with the kit. For example, mix 1000 ml of lysis buffer with 4000 ml of DDW. Keep on ice (*see* **Note 2**).
4. Prepare drugs at the required test dilutions. Keep the concentrations of the solvent at a constant level throughout the experiment. Typically, final concentrations of 0.1 or 0.2 % DMSO are used.
5. Label plate and remove medium.
6. Wash once with assay medium.
7. Remove medium from cells.
8. Add the tested drugs at the desire volume.
9. Incubate at 37 °C. Typically, to measure LPI-induced ERK1/2 phosphorylation an incubation of 20 min is recommended. However, the time should be selected based on preliminary experiments.
10. Remove medium from cells.
11. Keep the plate on ice.
12. Add 50 µl/well of 1× lysis buffer.
13. Store at −80 °C for at least 1 h (*see* **Note 3**).

3.1 ERK1/2 Phosphorylation Assay

To test the level of ERK1/2 phosphorylation, follow these steps:

1. Warm up Activation buffer from the kit to room temperature.
2. Thaw the assay plate (from the above **step 13**) at room temperature.
3. Transfer 4 µl of the lysed cells from each well into a well of the 384 ProxiPlate.
4. Transfer 4 µl of control lysate (supplied with the kit) into a well of the 384 ProxiPlate.
5. Prepare AlphaScreen® beads-containing ERK1/2 assay mix, as detailed in Table 2.
6. Add 7 µl per well of ERK1/2 assay mix.
7. Seal the ProxiPlate and protect from light.
8. Incubate the ProxiPlate for 3.5 h at 23–25 °C in the dark.

Table 2
ERK1/2 assay mix

Assay mix for 100 wells
600 µl of Reaction Buffer
100 µl of Activation Buffer
10 µl of Donor Beads
10 µl of Acceptor Beads

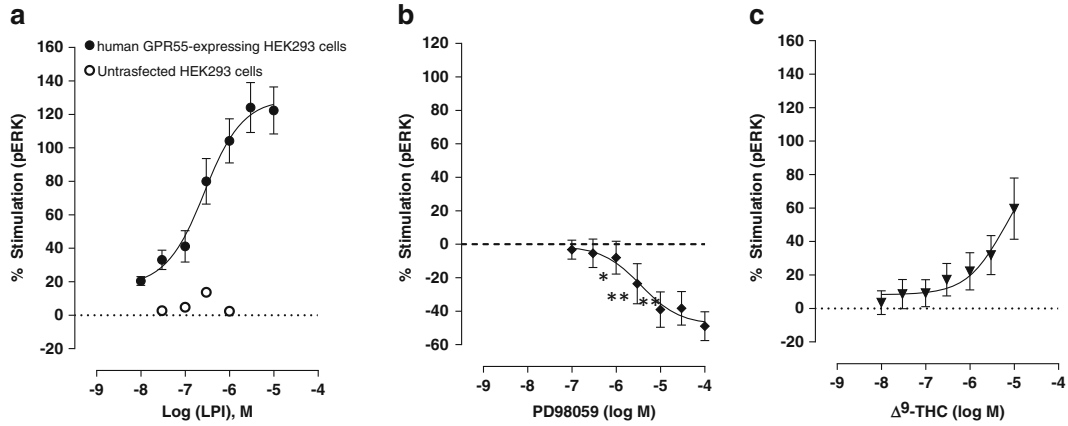

Fig. 3 (**a**) Mean log concentration-response curves of LPI effect on ERK1/2 phosphorylation in hGPR55-HEK293 cells after 20 min ($n = 4$ each in triplicate). No significant differences are observed in basal levels of phosphorylated ERK1/2 in untransfected HEK293 cells and hGPR55-HEK293 cells incubated for 20 min in 0.1 % DMSO ($n = 2$ each in duplicate). (**b**) LPI-induced stimulation of ERK1/2 phosphorylation is attenuated by 10 μM PD98059, a MEK1 inhibitor, that significantly reduces basal pERK levels. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by means of one-sample *t*-test. (**c**) Mean log concentration-response curves of ERK1/2 phosphorylation after 20 min stimulation at 37 °C with Δ^9-tetrahydrocannabinol (Δ^9-THC) ($n = 3$). Each *symbol* represents the mean percentage change in bound phosphorylated ERK1/2 ± SEM over the basal level

9. Incubate plate at room temperature and read with the Envision system (PerkinElmer), by using AlphaScreen® settings.

3.2 Data Analysis

Raw data can be presented as "Envision units," defining basal level (in the presence of vehicle) as zero. Results are presented as means and variability as SEM or 95 % confidence limits (CL) of the percent stimulation of phosphorylated ERK1/2 above the basal level. Data can be analyzed by using nonlinear analysis of log agonist *versus*-response curves with Prism 5.0 program (GraphPad, San Diego, CA) (Fig. 3).

When curves cannot be fitted by nonlinear analysis of log agonist versus-response curves, statistical significance of the stimulation can be determined with an unpaired Student's *t*-test at each specific concentration. Results are considered significant only when F-test comparing the variance is not significantly different.

4 Notes

1. All media should be warmed up to 37 °C, unless specified otherwise.
2. Portions of the diluted lysis buffer can be stored frozen (−20 °C) for subsequent experiments.
3. Lysates can be frozen (−80 °C) at this stage, to be assayed later.

Acknowledgments

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Conflicts of interest: RAR received funding from GW Pharmaceuticals.

References

1. Anavi-Goffer S, Baillie G, Irving AJ et al (2012) Modulation of L-alpha-lysophosphatidylinositol/GPR55 mitogen-activated protein kinase (MAPK) signaling by cannabinoids. J Biol Chem 287:91–104
2. Kotsikorou E, Sharir H, Shore DM et al (2013) Identification of the GPR55 antagonist binding site using a novel set of high-potency GPR55 selective ligands. Biochemistry 52:9456–9469
3. Heynen-Genel S, Dahl R, Shi S, et al (2010) Screening for selective ligands for GPR55 agonists in Probe Reports from the NIH Molecular Libraries Program (Internet), National Center for Biotechnology Information, Bethesda, MD, NBK66152 [bookaccession]
4. Henstridge CM, Balenga NA, Ford LA et al (2009) The GPR55 ligand L-alpha-lysophosphatidylinositol promotes RhoA-dependent Ca2+ signaling and NFAT activation. FASEB J 23:183–193
5. Andradas C, Caffarel MM, Perez-Gomez E et al (2011) The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK. Oncogene 30:245–252
6. Ford LA, Roelofs AJ, Anavi-Goffer S et al (2010) A role for L-alpha-lysophosphatidylinositol and GPR55 in the modulation of migration, orientation and polarization of human breast cancer cells. Br J Pharmacol 160:762–771
7. Pineiro R, Maffucci T, Falasca MT (2011) The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. Oncogene 30:142–152
8. Heynen-Genel S, Dahl R, Shi S, et al (2010) Screening for selective ligands for GPR55 antagonists in Probe Reports from the NIH Molecular Libraries Program (Internet), National Center for Biotechnology Information, Bethesda, MD, NBK66153 [bookaccession]
9. Staton PC, Hatcher JP, Walker DJ et al (2008) The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain. Pain 139:225–236
10. Shih HH, Zhang S, Cao W et al (2009) CRP is a novel ligand for the oxidized LDL receptor LOX-1. Am J Physiol Heart Circ Physiol 296:H1643–H1650

Chapter 9

The Cyclic AMP Assay Using Human Cannabinoid CB_2 Receptor-Transfected Cells

Pietro Marini, Maria Grazia Cascio, and Roger G. Pertwee

Abstract

The cyclic AMP assay is a functional assay that is commonly used to determine the pharmacological behavior (agonists, antagonists, inverse agonists) of G-protein-coupled receptor (GPCR) ligands. Here, we describe the cyclic AMP assay that is carried out with commercially available non-radioligand ready-to-use kits and Chinese hamster ovarian (CHO) cells stably transfected with the human cannabinoid CB_2 receptor.

Key words Cyclic AMP, Cannabinoid receptor type 2, Forskolin (FSK), Agonist, Antagonist, Inverse agonist, 3-Isobutyl-1-methylxanthine (IBMX)

1 Introduction

Cannabinoid CB_2 receptors belong to the superfamily of G-protein-coupled receptors and they are mainly coupled to the $G_{i/o}$ subunit [1]. When activated by CB_2-selective endogenous or synthetic agonists, these receptors are capable of reducing the activity of the enzyme adenylate cyclase that in turn induces a reduction of the production of the second messenger, cyclic AMP (3′,5′-cyclic adenosine monophosphate) [1]. The study of cyclic AMP, which is involved in crucial cellular functions, has over time attracted the interest of scientists, leading to the development of investigational methods that allow the direct assessment of intracellular cyclic AMP levels. In the past, these methods were based mainly on the use of radioligands, and yet they have the disadvantage of lacking high sensitivity. Moreover, the use of radioactive materials is strictly limited to suitably qualified researchers.

Nowadays, these techniques have been replaced, at least in part, by the development of new methodologies that are based on the use of chemiluminescence and offer a number of advantages. Thus, these non-radioligand ready-to-use kits are highly sensitive,

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fast, and very easy to use and clear instructions are provided by the manufacturer. These advantages have recently prompted these kits to be frequently used in research, particularly in laboratories in which in vitro studies aimed at evaluating the pharmacological behavior of plant-derived as well as synthetic CB_2 receptor ligands are being performed [2–6]. Indeed, by measuring cyclic AMP levels, it is possible to establish whether a ligand is capable of activating a receptor (an agonist) or of inhibiting and/or blocking it (an inverse agonist and/or an antagonist) [7]. The measurement of cyclic AMP levels using these kits involves three main steps: (1) cell culturing, counting, and seeding; (2) performing a cyclic AMP assay; and (3) analyzing the results.

In these assays, forskolin (FSK), a ubiquitous activator of adenylate cyclase, is commonly used to raise intracellular levels of cyclic AMP. The competitive nonselective phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), is also used to raise intracellular cyclic AMP levels in the cyclic AMP assay [8].

2 Materials

2.1 Cell Culture

1. Cells: CHO cells transfected with cDNA encoding human CB_2 receptors are commercially available (e.g., ValiScreen Cannabinoid CB_2 (human) cell line, Perkin Elmer, USA).
2. Culture medium: Use sterile Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM, supplemented with 1 mM L-glutamine, 10 % fetal bovine serum (FBS), 1 % penicillin–streptomycin, and 0.6 % G418 (3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl [oxy-2-hydroxycyclehexy]oxy-2-(1-hydroxyethyl)oxane-3,4-diol); 400 mg/ml.
3. Nonenzymatic cell dissociation solution: Dissolve 1 mM EDTA in distilled water, and then sterilize this solution by using an autoclave (*see* **Note 1**).
4. Use phosphate buffer saline (PBS); this is commercially available.
5. For cell culturing use plastic flasks (surface cell culture: 75 cm^2).

2.2 Cyclic AMP Assay

Here we describe the HitHunter® cyclic AMP assay kit according to the vendor's protocol (DiscoveRx Corporation, Ltd, Aston, Birmingham, UK). This assay is carried out in non-sterile conditions; thus all the following solutions are non-sterile.

1. Test compounds: Dissolve the compounds under investigation in the appropriate solvent (e.g., dimethyl sulfoxide; DMSO) at a concentration of 10 or 100 mM, splitting the solution into 50 µl aliquots and storing each aliquot at −20 °C (*see* **Note 2**).

2. Forskolin solution: Dissolve forskolin in DMSO at a concentration of 10 mM and store each aliquot (50 µl) at –20 °C.
3. IBMX solution: Dissolve IBMX in DMSO at a concentration of 100 mM and store each aliquot (50 µl) at –20 °C.
4. Other reagents are included in the kit and they are ready to use (*see* **Note 3**).
5. To seed the cells and carry out the assay, use 96-well clear flat-bottom sterile plates, both with opaque walls (black or white) to prevent well-to-well cross talk and with clear bottoms to permit direct microscopic viewing. The plates should be suitable for top and bottom reading instruments and for reading down to 340 nm (*see* **Note 4**).
6. To seed the cells with a consistent number in each well, use of 8-channel pipettes is suggested.

3 Methods

3.1 Cell Culture

Perform the following steps under sterile conditions.

1. Maintain monolayers of hCB_2-transfected CHO cells at 37 °C and in 5 % CO_2 in medium flasks (surface cell culture: 75 cm^2), and passage them twice weekly using a nonenzymatic cell dissociation solution (1 mM EDTA).
2. When cells reach 80–85 % confluence, remove medium from the flask, wash the cells with 10 ml of PBS, add 5 ml of nonenzymatic cell dissociation solution, place the flask in an incubator (at 37 °C) for 2–3 min, gently detach the cells, add 5 ml of culture medium, and transfer the solution (containing the cells) to a 20 ml of sterile plastic culture tube (clear polystyrene).
3. Count the cells by mixing 10 µl of the solution containing the cells with 10 µl of trypan blue, and then count 10 µl of this mixture by using a hemocytometer chamber and a microscope. The following formula can be used to estimate the number of cells per ml:

$$(\text{cells counted} \times 2) \times 10{,}000.$$

4. Seed 2×10^4 cells/well in 100 µl of complete culture medium in white 96-well plates with clear bottoms, and incubate at 37 °C in 5 % CO_2 for approximately 24 h before continuing with the experiment (*see* **Note 5**).

3.2 Cyclic AMP Assay

Carry out all of the following procedures at room temperature (i.e., from 23 to 27 °C).

1. Prepare a working buffer (A) that contains a 1:1 mixture of DMEM and Ham's F12 medium (without red phenol), the phosphodiesterase inhibitor IBMX (100 µM), and 10 µM for-

skolin (*see* **Note 6**). In addition, prepare a buffer (B) that is buffer A without forskolin.

2. Using buffer A and stock solutions of each test compound (e.g., 10 mM or 100 mM in DMSO), prepare decreasing concentrations of the test compound so that a dose-response curve can be drawn (*see* **Note 7**). Also prepare a *control* solution by using buffer B, with the same % of vehicle (e.g., DMSO) present in each diluted solution.
3. To start the assay, discard medium from each well and wash the cells with DMEM/F-12 medium without FBS and phenol red.
4. Add buffer B to those wells that will be used to determine the basal production of cyclic AMP, then add buffer A to the other wells that will be used to determine the amount of cyclic AMP produced by forskolin, and finally add to each buffer A-containing well a test compound to allow the construction of dose-response curves. The latter will be used to determine whether the test compound is able to inhibit or stimulate forskolin-induced production of cyclic AMP.
5. In parallel, construct a standard curve using known concentrations of cyclic AMP provided by the supplier.
6. Incubate for 30 min at 37 °C and in 5 % CO_2.
7. Add to each well the appropriate mixture of kit components (as described by the manufacturer).
8. Incubate the plates overnight (approximately 12 h) at room temperature in the dark, shaking them gently in a horizontal shaker.
9. Measure the luminescent signal using an appropriate plate reader (e.g., Synergy HT Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA).

3.3 Analysis of the Results

1. Construct a cyclic AMP standard curve by plotting the luminescence values, expressed in relative luminescence units (RLUs), versus each known concentration of cyclic AMP. A standard curve of the kind shown in Fig. 1 should be obtained.
2. Convert this curve into a sigmoidal log dose-response curve (Fig. 2), so that the concentration of cyclic AMP produced by the cells in the presence of forskolin alone or forskolin *plus* the specific CB_2 receptor ligands under investigation can be calculated.
3. To determine the amount of cyclic AMP produced, in ng, a linear regression curve can be plotted (Fig. 3).
4. Results can be expressed either as % inhibition of cyclic AMP production or as % of forskolin-induced cyclic AMP production, using the formulae shown below. Examples of results obtained in this manner are shown in Figs. 4 and 5.

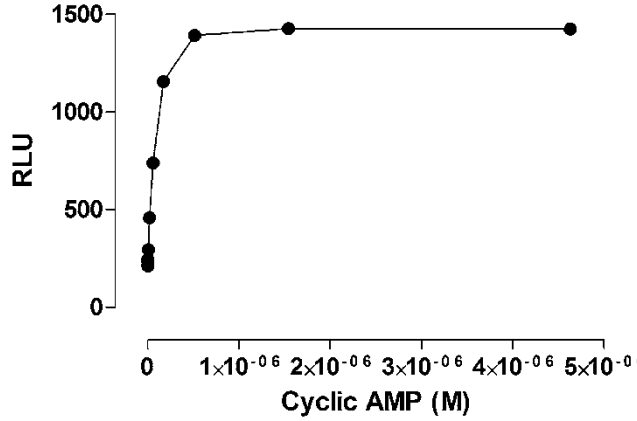

Fig. 1 Cyclic AMP standard curve obtained by plotting the luminescence signals (expressed as RLU values) generated by each standard concentration of cyclic AMP (the standards are provided by the manufacturer)

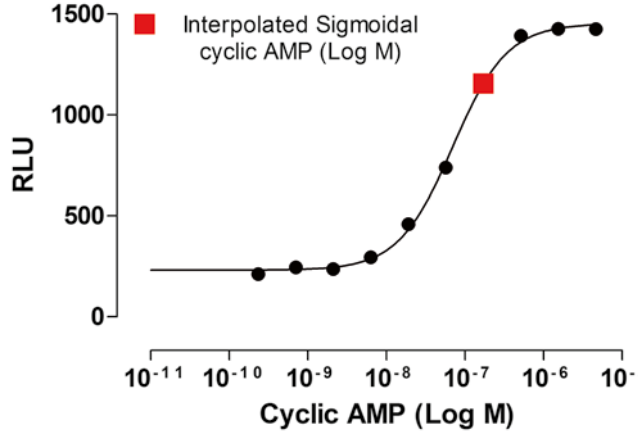

Fig. 2 Sigmoidal cyclic AMP standard curve constructed by converting the cyclic AMP concentrations into log values

% inhibition of cyclic AMP production:

$$\frac{\left(\text{Estimated FSK induced cyclic AMP production}\right)-\left(\begin{array}{l}\text{Estimated ligand}\\ \text{induced cyclic AMP production}\end{array}\right)}{\left(\text{Estimated FSK}-\text{induced cyclic AMP production}\right)}\times 100$$

% of forskolin-induced cAMP production:

$$\frac{\left(\text{Estimated ligand}-\text{induced cyclic AMP production}\right)}{\left(\text{Estimated FSK}-\text{induced cyclic AMP production}\right)}\times 100$$

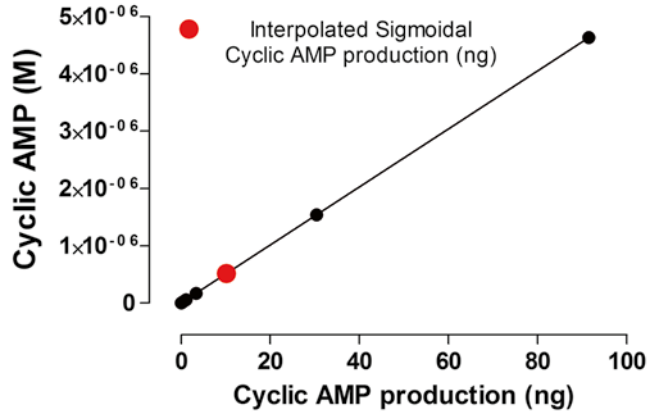

Fig. 3 Linear regression cyclic AMP standard curve obtained by plotting the standard cyclic AMP concentrations (molar) versus the relative amount of cyclic AMP contained in each standard. For instance, the highest concentration of the standard cyclic AMP provided by the manufacturer is 4.63×10^{-6} M in a total volume of 60 μl. The concentration (*M*) is moles/liter, thus multiplying the concentration by the volume (in litre) you get the number of moles. Since the number of moles can be obtained from grams/molecular weight, multiplying the molecular weight by the number of moles, you get the number of grams (that is the amount of cyclic AMP contained in the highest concentration of your standard). Repeat this procedure for each standard provided by the supplier, and report values in the graph. Note that in this figure, the amount of cyclic AMP is expressed in nanograms (ng)

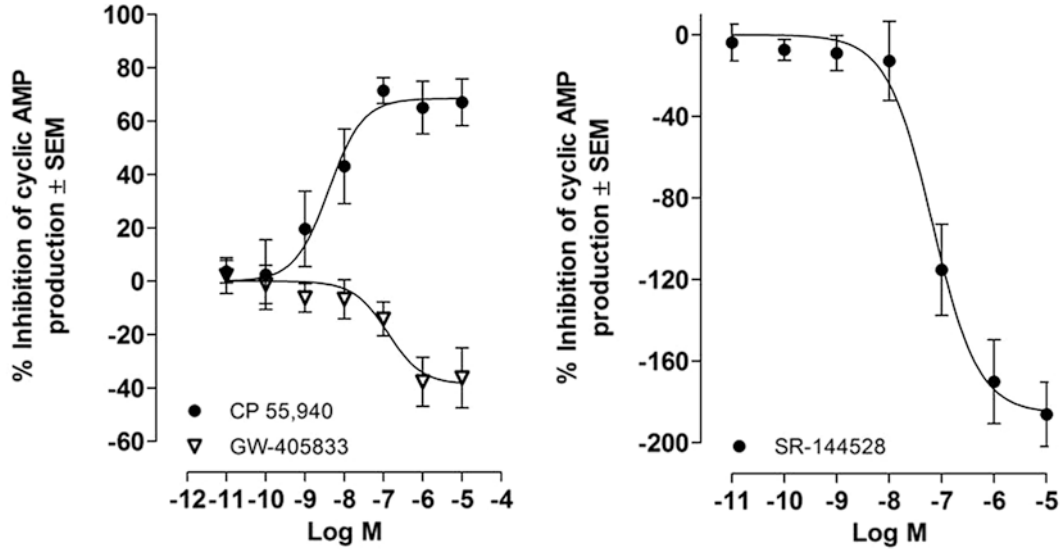

Fig. 4 Typical log concentration-response curves obtained by using non-radioligand ready-to-use kits for cyclic AMP detection. *Left* panel: Typical sigmoidal log concentration-response curves of a cannabinoid CB_2 receptor full agonist (CP-55,940) and a cannabinoid CB_2 receptor antagonist/inverse agonist (GW-405833). *Right* panel: Typical sigmoidal log concentration-response curve of the cannabinoid CB_2 receptor antagonist/inverse agonist, SR 144528. Data are expressed as % inhibition of cyclic AMP production. The concentration of forskolin used in these experiments was 10 μM (EC_{80})

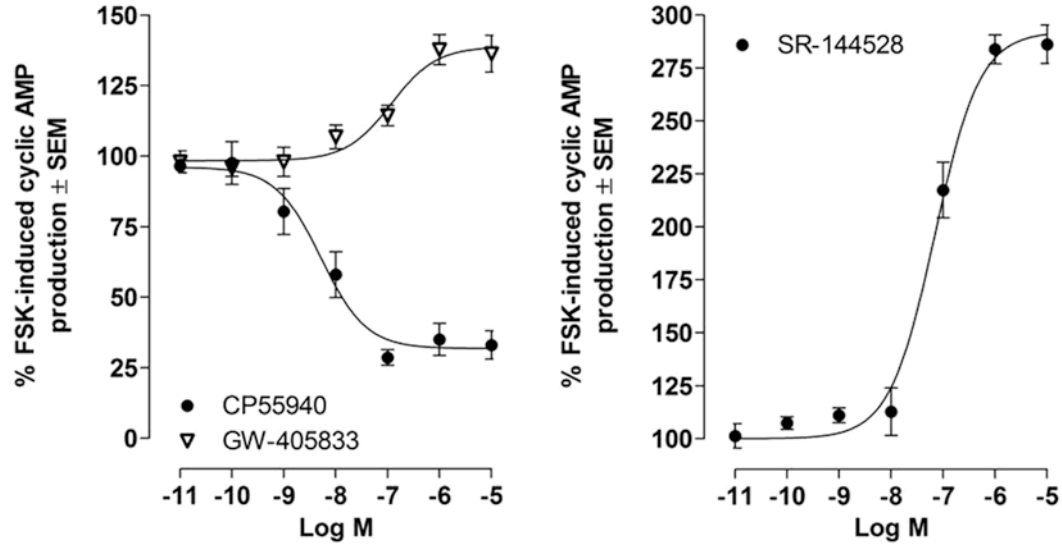

Fig. 5 Typical log concentration-response curves obtained by using non-radioligand ready-to-use kits for cyclic AMP detection. *Left* panel: Typical sigmoidal log concentration-response curves of a cannabinoid CB_2 receptor full agonist (CP-55,940) and a cannabinoid CB_2 receptor antagonist/inverse agonist (GW-405833). *Right* panel: Typical sigmoidal log concentration-response curve of the cannabinoid CB_2 receptor antagonist/inverse agonist, SR 144528. Data are expressed as % of forskolin-induced cyclic AMP production. The concentration of FSK (forskolin) used in these experiments was 10 μM (EC_{80})

4 Notes

1. The cell dissociation solution is also commercially available. We recommend that trypsin is not used to detach the cells, because this enzyme may affect cell functionality.
2. Avoid multiple thawing of the same aliquot, since this may result in the degradation of chemically unstable compounds. We recommend that the same aliquot is not used more than twice.
3. Store reagents at −20 °C and thaw them at room temperature before use. All reagents should be thawed and then frozen no more than three times. We suggest that aliquots are made of all the components of the kit, and that multiple thawing and refreezing are avoided.
4. Black pigment walls have generally low background fluorescence, while dense white pigment walls enhance luminescence signals and reduce both background luminescence and background fluorescence. The manufacturer suggests that white-wall plates are used.

5. The number of cells used in the cyclic AMP assay may vary from lab to lab. We use 2×10^4 cells/well. However, we suggest to optimize the number of cells before further assays are carried out.
6. To determine the optimal concentration of FSK for stimulating adenylate cyclase, and thus cyclic AMP production, cells should be treated with increasing concentrations of FSK. Data needed to construct a log concentration-response curve for FSK-induced cyclic AMP production can be obtained using the following formula:

$$\frac{\left(\text{Estimated cyclic AMP production by specific FSK concentration}\right)}{\left(\text{Estimated basal cyclic AMP production, control sample without FSK added}\right)} \times 100$$

See Fig. 6 for an example of such a log concentration-response curve.

7. To construct a dose-response curve of the compound under investigation, prepare a stock solution by dissolving the test compound in DMSO (e.g., 100 mM), and make serial dilutions as follows: 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, and 0.1 nM. Also ensure that the concentration of vehicle in each solution remains constant.

   Please note that in a sigmoidal log concentration-response curve, these concentrations are expressed as log *M* (Fig. 6).

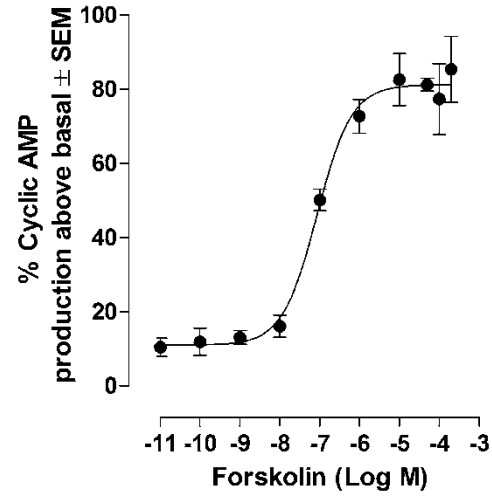

Fig. 6 % of cyclic AMP produced by hCB_2-CHO cells (10,000 cells/well) in response to increasing concentrations of forskolin. The mean EC_{50} value is 90 nM, while the mean EC_{80} is 10 μM

References

1. Pertwee RG, Howlett AC, Abood ME et al (2010) International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2. Pharmacol Rev 62:588–631
2. Gonsiorek W, Lunn C, Fan X et al (2000) Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. Mol Pharmacol 57:1045–1050
3. Yang R, Fredman G, Krishnamoorthy S et al (2011) Decoding functional metabolomics with docosahexaenoyl ethanolamide (DHEA) identifies novel bioactive signals. J Biol Chem 286:31532–31541
4. McGuinness D, Malikzay A, Visconti R et al (2009) Characterizing cannabinoid CB_2 receptor ligands using DiscoveRx PathHunter beta-arrestin assay. J Biomol Screen 14:49–58
5. Mancini I, Brusa R, Quadrato G et al (2009) Constitutive activity of cannabinoid-2 (CB_2) receptors plays an essential role in the protean agonism of (+)AM1241 and L768242. Br J Pharmacol 158:382–391
6. Bolognini D, Cascio MG, Parolaro D et al (2012) AM630 behaves as a protean ligand at the human cannabinoid CB_2 receptor. Br J Pharmacol 165:2561–2574
7. Marini P, Cascio MG, King A et al (2013) Characterization of cannabinoid receptor ligands in tissues natively expressing cannabinoid CB_2 receptors. Br J Pharmacol 169: 887–899
8. Morgan AJ, Murray KJ, Chaliss RA (1993) Comparison of the effect of isobutylmethylxanthine and phosphodiesterase-selective inhibitors on cAMP levels in SH-SY5Y neuroblastoma cells. Biochem Pharmacol 45:2373–2380

Chapter 10

Assay of GTPγS Binding in Autoradiography

Marina Gabaglio, Pamela Prini, Erica Zamberletti, Tiziana Rubino, and Daniela Parolaro

Abstract

Autoradiography of radiolabeled GTPγS ([^{35}S]GTPγS) binding is a relevant method to study the function of G protein-coupled receptors (GPCRs), in tissue sections. Here, we describe the protocol for such a binding autoradiography, suitable to investigate the functionality of CB_1 receptor in tissue slices from rodent brain.

Key words [^{35}S]GTPγS, Binding, Autoradiography, CB_1 receptor, G-proteins, Brain section

1 Introduction

[^{35}S]GTPγS binding autoradiography is a technique used to provide functional information on G protein-coupled receptors (GPCRs). It has been developed to measure the level of G protein activation following agonist occupation of a GPCR. This can be obtained by labeling receptor-coupled G proteins with a non-hydrolyzable radiolabeled analogue of GTP (GTPγS), in the presence of excess GDP [1].

GPCRs represent the largest family of membrane proteins and mediate many cellular processes [2]. Indeed, members of this family include receptors for a great variety of ligands (e.g., hormones, neurotransmitters, lipids, and even for direct sensory stimuli) [3]. The main endocannabinoid receptors, CB_1 and CB_2, belong to the GPCR family.

Structurally, GPCRs consist of an extracellular N terminus, seven transmembrane domains, and an intracellular C terminus. They couple with G proteins that are membrane-anchored heterotrimeric protein molecules responsible for signal transduction and amplification. G proteins are composed by α (45 kDa), β (35 kDa), and γ (8 kDa) subunits. The α and γ subunits are attached to the membrane by lipid anchors, and the former subunit binds either GDP or GTP, depending on whether the protein is inactive (GDP) or active (GTP). In the

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inactive state, the entire G protein-GDP complex binds a GPCR. Upon ligand binding, a conformational change of the GPCR activates the G protein, and GDP bound to the α subunit is physically replaced by GTP. At this point, the G protein subunits dissociate into the GTP-bound α subunit and a β-γ dimer. Both parts remain anchored to the membrane, but not to the GPCR, and can interact with several effector systems. Then, the GTPase activity of Gα subunit hydrolyzes GTP into GDP, allowing the α subunit to reassociate with the β-γ complex, and resetting the G protein to the inactive state [4].

The concept that receptor–G-protein complexes are still able to function in tissue sections came from Zarbin and colleagues in 1983 [5]. In 1986, the first autoradiographic visualization of guanine—nucleotide-binding proteins was obtained using [^{3}H]Gpp(NH)p in a rat brain section [6]. Then, in 1995 for the first time Sim and colleagues performed the [^{35}S]GTPγS binding autoradiography in rat brain cryosections after CB_1 receptor stimulation [7].

2 Materials

Prepare all stock solutions during the days preceding the assay, and store under appropriate temperature conditions.

2.1 Slide Preparation

1. Microscope slides (26×76 mm).
2. Gelatine: Dissolve 2.5 g of gelatine in 0.5 l of heated, deionized water (temperature should not exceed 60 °C). After the gelatine has dissolved, let cool the solution at room temperature. Add 0.25 g of chromium potassium sulfated. Mix and filter solution before use. Store at 4 °C.
3. Racks.

2.2 Tissue Preparation

1. Cryostat (Leica, Biosystem, Nussloch GmbH, Germany).

2.3 Assay Components

1. Liquid-repellent slide marker pen (PAP-PEN).
2. 1 M Tris–HCl, pH 7.4: Weight 157.6 g Tris hydrochloride and transfer to a glass beaker. Add water to a volume of 800 ml. Mix and adjust pH with 10 M NaOH. Make up to 1 l with water. Store at 4 °C (*see* **Note 1**).
3. 60 mM $MgCl_2$ stock solution: Add 600 µl of solution in 10 ml of water. Store at 4 °C.
4. 0.25 M EGTA stock solution: Dissolve 0.951 g in 10 ml of water. Store at 4 °C.
5. Bovine serum albumin (BSA): 0.5 % (wt/vol) in assay buffer. Lyophilized powder, essentially fatty acid free ≥96 %. Store at 4 °C.

6. 1.6 M NaCl stock solution: Dissolve 4.68 g in 50 ml of water. Store at 4 °C.
7. 1000 U Adenosine deaminase stock solution: Add 1 µl of adenosine deaminase in 999 µl of water, to prepare (fresh each time) a working solution.
8. Assay buffer: 1 M Tris–HCl, pH 7.4, 0.06 M $MgCl_2$, 0.25 M EGTA, 1.6 M NaCl, 0.5 % BSA, 1000 U adenosine deaminase. Mix all components and make up to a convenient volume with water at room temperature (*see* **Note 2**).
9. 300 mM Guanosine 5′-diphosphate disodium salt (GDP) stock solution: Dissolve 146 mg in 1 ml of assay buffer without 0.5 % BSA. Store at −20 °C.
10. Guanosine 5′-[^{35}S]triphosphate triethylammonium salt ([^{35}S]GTPγS, 1250 Ci/mmol in 250 µl): Add 2 µl of [^{35}S]GTPγS in 498 µl of 50 mM Tris–HCl, to prepare a 40 nM [^{35}S]GTPγS stock solution. Add 50 µl of 40 nM stock solution in 450 µl of 50 mM Tris–HCl, to prepare the working solution. Store in a safe location at −20 °C (*see* **Note 3**).
11. 1 mM CP-55,940 stock solution: Dissolve 3.86 mg in 10 ml of ethanol. Store at −20 °C.
12. Carestream® Kodak® BioMax® MR film (18 cm × 24 cm): Store at room temperature.
13. GE Healthcare Amersham™ Hypercassette™ Autoradiography Cassette.
14. Developer D-19 and fixer Kodak™ Processing chemicals: Developer: Dissolve 607 g in 3.8 l of hot water (38–52 °C), and stir until complete dissolution. Fixer: Dissolve 700 g in 3.8 l of water (18–25 °C), and stir until complete dissolution (*see* **Note 4**). It is also possible to use a developing machine.

3 Methods

3.1 Gelatine-Coated Slides

1. Place the slides into racks.
2. Dip the racks containing the slides three times (30 s each) into the gelatin solution.
3. Remove the excess solution from the racks. For better drainage, gently tap the racks against a piece of paper (*see* **Note 5**).
4. Cover each rack with an aluminum foil and dry overnight at 50 °C.
5. Put the dried slides into the boxes to protect from dust. Enumerate them before use.

3.2 Tissue Preparation

1. Rat or mouse brain is rapidly removed, frozen in liquid nitrogen, and stored at −80 °C until sectioning.
2. Cut 20 μm thick brain sections according to the rat or mouse brain atlas (e.g., Paxinos and Watson), at −20 °C using the cryostat. For each brain area of interest (according to the atlas coordinates), prepare a set of slides. Proceed collecting the first section for each slide, and then repeat the procedure collecting the second section for each slide. Each slide can contain till four rat brain sections or five mouse brain sections. In this way, each slide contains the area of interest in its whole extension and is comparable to the others (*see* **Note 6**).
3. Thaw-mount the sections onto gelatin-coated microscope slides and store at −80 °C until use (*see* **Note 7**).

3.3 Assay

The assay is composed of three consecutive incubations. All procedures must be carried out at room temperature (25 °C) and attention must be paid when handling and storing of radioisotopes. These must be used in the dedicated rooms.

1. Lead the slides at room temperature to thaw. For the binding assay two consecutive slides are needed (e.g., even and odd number) for each brain area of interest.
2. When the slides are dried, surround the sections with the liquid blocker PAP-PEN (*see* **Note 8**).
3. Prepare an appropriate volume of the assay buffer (400 μl per slide × 3 incubations) by mixing 50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.5 % BSA, 10 mU/ml adenosine deaminase, and deionized water in a graduate cylinder with the aid of a magnetic stirrer (*see* **Note 9**).
4. Preincubate slides in assay buffer for 10 min at room temperature by adding 400 μl of assay buffer and ensure that all the sections are covered.
5. Add 300 mM GDP to the remaining assay buffer to reach a final concentration of 3 mM GDP (1:100 dilution) (*see* **Note 10**).
6. Drain the slides and re-incubate in assay buffer supplemented with 3 mM GDP at room temperature for 15 min.
7. Under proper safe conditions, lead the working solution of [^{35}S]GTPγS at room temperature to thaw. Dilute the [^{35}S]GTPγS 1:100 in assay buffer containing 3 mM GDP. Add an appropriate volume of 4 nM [^{35}S]GTPγS in the assay buffer, and split equal volumes of the solution in two conical tubes. Add 1 mM CP-55,940 to one tube to reach a final concentration of 5 μM CP-55,940 (1:200 dilution) that will be used for the stimulation. The tube containing the buffer without agonist will be used to determine the basal signal.

8. Drain the slides. Then, place them on the bench dividing them into two groups:
   (a) Stimulated slides (e.g., odd numbers).
   (b) Basal slides (e.g., even numbers).
9. Incubate the slides in assay buffer containing 3 mM GDP and 0.04 nM [^{35}S]GTPγS with (stimulated) or without (basal) 5 μM of CP-55,940 at room temperature for 2 h (*see* **Note 11**).
10. To remove unbound radioactivity, transfer the slides into racks and rinse twice in 50 mM Tris–HCl at 4 °C for 5 min, and once in deionized water (*see* **Note 12**).
11. Rapidly dry the slides under a stream of cold air, arrange into an autoradiography cassette, and expose to Biomax MR Kodak film for 48 h (*see* **Note 13**).
12. After exposure, develop the film manually or in automated procedure (*see* **Note 14**).

3.4 Image Analysis

1. Scan the autoradiography film using a scanner connected to a PC running Microsoft Windows.
2. Analyze the images with image analyzer programs such as Image-Pro Plus 5.0 (MediaCybernetics, Silver Spring, USA) or NIH Image J (*see* **Note 15**).
3. Conduct all statistical analyses using GraphPad Prism® (La Jolla, CA, USA) for Microsoft Windows, or equivalent program.

4 Notes

1. When performing the experiment, check carefully the pH of the solution.
2. When used in the protocol, this buffer produces final concentrations in the incubation reaction mixture of 50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, and 10 mU/ml adenosine deaminase.
3. One inconvenient aspect of the assay is the short physical half-life (87.4 days) of [^{35}S]GTPγS. Check the fresh lot days on the website of the supplier.
4. These solutions must be prepared and stored in the dark.
5. The gelatine solution can be used for several times; store at 4 °C and bring at room temperature before use. Slides need to be coated with gelatine to enhance adhesion of the tissue and to prevent or reduce the loss of sections during the treatment. Coating must be done carefully to avoid air bubbles.
6. You can collect coronal and/or sagittal sections. We suggest to collect sagittal sections for an overview of CB_1 receptor-stimulated

GTPγS throughout the brain. Instead, to obtain more detailed information on specific brain regions or nuclei, use coronal sections.

7. Warning: To obtain optimal signal do not use brain sections older than 6 months, unless perfectly stored at −80 °C. Never use sections older than 1 year.
8. The PAP PEN creates a hydrophobic barrier when a circle is drawn around tissue sections. This barrier stops spreading and reduces waste. The target area drawn around the sections must be the same for all slides, 400 μl for 2.5 cm × 4 cm, to allow the same concentration of reagents.
9. Pay attention to $MgCl_2$ concentration in the assay buffer, since its change can affect the outcome of the assay.
10. This solution will be used for the next incubations (Fig. 1). This assay requires a large excess of GDP to ensure that G proteins are present in the inactive state. This is needed to suppress basal binding and is reached with 1–5 mM GDP. This will allow to obtain clear section images on the autoradiographic film. When sections are too dark, try to increase GDP concentration.
11. Pay attention! Slides must not dry during this incubation. Surround slides with wet paper to maintain humidity.
12. Prepare 200 ml of 50 mM Tris–HCl per wash.
13. Autoradiography film must be handled under proper safelight condition. Make sure that the slides are completely air-dried before exposure. The emulsion coating is on a single side of the film. Film must be positioned so that the emulsion side is in contact with the slide.
14. Automated processing: Place the film into automatic processor. See the manual for your instrument. Manual processing: transfer

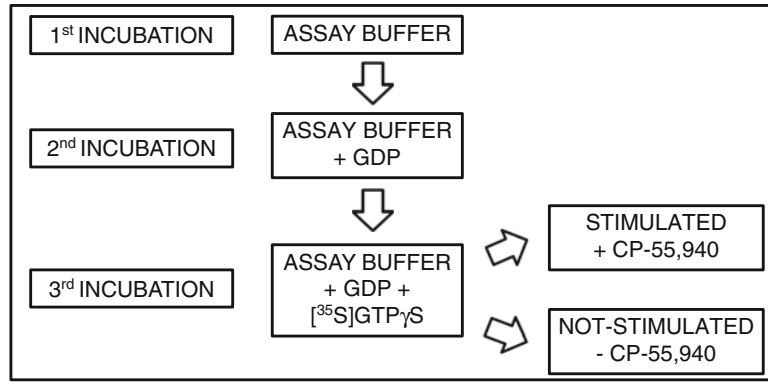

Fig. 1 Main steps of the GTPγS assay

the film in developer for 4 min with moderate agitation. Repeat this procedure with fixative and water. Dry the film.

15. Calibrate the software so that grey-level range is between 0 and 255. Trace each cerebral area with the mouse cursor control, and calculate the signal intensity (in terms of grey levels) for each traced area. For each slide also calculate the grey level of its background. Signal intensity for each region is then calculated by subtracting the value of background from each measured region. This grey level will be established within the linear range, determined by using ^{35}S standards made in the laboratory (or ^{14}C standards). For each pair of slide (stimulated/basal) calculate the percent of net stimulation as follows: (value of stimulated/value of basal) × 100.

References

1. Harrison C, Traynor JR (2003) The [^{35}S] GTPgammaS binding assay: approaches and applications in pharmacology. Life Sci 74: 489–508
2. Rosenbaum DM, Rasmussen SG, Kobilka BK (2009) The structure and function of G-protein-coupled receptors. Nature 459: 356–363
3. Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven-transmembrane receptors. Nat Rev Mol Cell Biol 3:639–650
4. Sóvágó J, Dupuis DS, Gulyás B et al (2001) An overview on functional receptor autoradiography using 35 [S]GTPgS. Brain Res Brain Res Rev 38:149–164
5. Zarbin MA, Palacio SJM, Wamsley K et al (1983) Axonal transport of b-adrenergic receptors. Antero- and retrogradely transported receptors differ in agonist affinity and nucleotide sensitivity. Mol Pharmacol 24:341–348
6. Gehlert DR, Wamsley JK (1986) In vitro autoradiographic localization of guanine nucleotide binding sites in sections of rat brain labeled with [3H]guanylyl-59-imidodiphosphate. Eur J Pharmacol 129:169–174
7. Sim LJ, Selley DE, Childers SR (1995) In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 59-[γ-[S]thio]-triphosphate binding. Proc Natl Acad Sci U S A 92:7242–7246

Chapter 11

Protocol to Study β-Arrestin Recruitment by CB_1 and CB_2 Cannabinoid Receptors

Marjolein Soethoudt, Noortje van Gils, Mario van der Stelt, and Laura H. Heitman

Abstract

Cannabinoid CB_1 and CB_2 receptors are G-protein-coupled receptors (GPCRs) that recruit β-arrestins upon activation by (partial) agonists. β-Arrestin recruitment is induced by phosphorylation of their C-terminal tails, and is associated with the termination of GPCR signaling; yet, it may also activate cellular signaling pathways independent of G-proteins. Here, we describe a detailed protocol to characterize the potency and efficacy of ligands to induce or inhibit β-arrestin recruitment to the human CB_1 and CB_2 receptors, by using the PathHunter® assay. The latter is a cellular assay that can be performed in plates with 384-wells. The PathHunter® assay makes use of β-galactosidase complementation, and has a chemiluminescent readout. We used this assay to characterize a set of reference ligands (both agonists and antagonists) on human CB_1 and CB_2 receptors.

Key words GPCRs, Receptor signaling, Internalization, β-Arrestin, DiscoveRx Pathunter®, Cannabinoid receptors, Biased signaling, Functional selectivity

1 Introduction

G-protein-coupled receptors (GPCRs) are responsible for the translation of extracellular signals into the intracellular environment, thereby modulating secondary signal transduction pathways, such as adenylate cyclase or phospholipase C. This enables the GPCR to control different cellular processes [1, 2]. Inactivation of GPCR signaling involves agonist-induced phosphorylation of the C-terminal tail of the receptor, followed by recruitment of β-arrestins [1]. The binding of β-arrestins to the GPCR may lead to inactivation of the receptor, due to desensitization and internalization, but may also result in G-protein-independent activation of signaling pathways, such as extracellular receptor kinases [3]. Some ligands activate multiple different signal transduction pathways with similar efficacies, while others preferentially modulate only one specific pathway [4]. The latter is referred to as "biased

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signaling," or functional selectivity [5]. It should be noted that ligand-induced specificity in intracellular signaling may offer new opportunities for drug discovery [6, 7].

Cannabinoid CB_1 and CB_2 receptors are members of the GPCR superfamily and important therapeutic targets, due to their physiological role in the control of energy metabolism [8] and inflammatory processes, respectively [9]. Both receptors couple to $G_{i/o}$ proteins, inhibit cAMP formation, induce ERK phosphorylation, inhibit ion channels, and recruit β-arrestin. To characterize the potency and efficacy of cannabinoid receptor ligands to recruit β-arrestins, we have employed the previously reported β-arrestin PathHunter® assay [12]. In this assay, β-arrestin activity is measured in live cells (DiscoveRx® Pathunter® cell lines) by using an enzyme complementation assay and a chemoluminescent readout (Fig. 1) [10]. The cell lines are overexpressing either the human CB_1 or CB_2 receptor, which are both tagged at the C-terminus by a ProLink™, i.e., a small fragment of β-galactosidase. Additionally, β-arrestin is fused to a catalytically inactive N-terminal deletion mutant of β-galactosidase, i.e., the enzyme acceptor (EA), which is stably co-expressed in the same cells [10, 11]. Activation of the CB_1 or CB_2 receptor stimulates binding of β-arrestin to the ProLink-tagged GPCR, which induces the complementation of the enzyme fragments ProLink™ and EA, and subsequently results in the formation of an active β-galactosidase enzyme [10, 12]. The

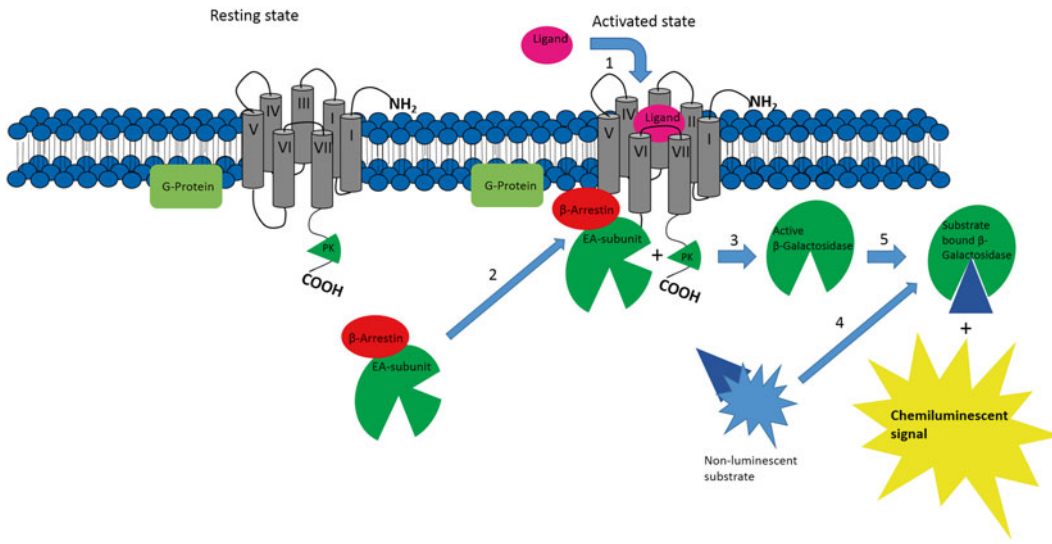

Fig. 1 Schematic representation of the β-arrestin recruitment assay. Upon activation of the receptor (1), the β-arrestin-EA complex is recruited to the receptor (2), which subsequently results in the complementation of the ProLink enzyme fragment with the free EA subunit to form an active β-galactosidase enzyme (3). The latter then reacts with the substrate in the PathHunter® detection reagent mixture (4), resulting in light emission that is proportional to the amount of β-arrestin recruited to the activated receptor (5). The complemented enzyme remains bound to the receptor, but this is not shown in the figure for the sake of clarity

latter is able to convert a substrate into a chemiluminescent product [11, 13, 14]. The light emission by the product is directly related to the activity of the β-galactosidase, and hence to the level of β-arrestin recruited to the receptor after ligand binding [10]. We successfully applied this assay to measure β-arrestin recruitment for a variety of cannabinoid receptor ligands.

2 Materials

All buffers and solutions are prepared using Millipore water (deionized using a MilliQ A10 Biocel™, with a 0.22 μm filter) and analytical grade reagents and solvents. Buffers are prepared at room temperature (RT) and stored at 4 °C, unless stated otherwise. Cannabinoid ligands were obtained from Hoffman-La Roche.

2.1 Cell Culture

1. Cells: PathHunter® CHO-K1 β-Arrestin Cell Line CNR1 or CNR2 (DiscoveRx), overexpressing CB_1 or CB_2 receptor. In this chapter, these cells are named CHOK1hCB_1_bgal or CHOK1hCB_2_bgal, respectively.
2. Phosphate-buffered saline (PBS: 1.9 mM KH_2PO_4, 136.9 mM NaCl, 8.0 mM $Na_2HPO_4{\cdot}2H_2O$, 3.4 mM KCl).
3. Trypsin solution (0.25 % in PBS, containing 0.44 mM EDTA).
4. Ham's F12 Nutrient Mixture (500 ml), supplemented with 50 ml of fetal calf serum (FCS), 5 ml of 200 mM Glutamax, 1 ml of 100 mg/ml P/S, 2 ml of 200 mg/ml G418, and 750 μl of 200 mg/ml hygromycin. The antibiotic stock solutions can be pre-made and stored at −20 °C.

   Once the medium is made, store at 4 °C, but warm to 37 °C before use.
5. Ten cm ø culture dishes.

2.2 Assay

1. Ligands to be tested are prepared as DMSO stock solutions prior to testing, with the exception of endocannabinoids (anandamide and 2-arachidonoylglycerol). Since these are unstable in DMSO, stock solutions are prepared in acetonitrile. Stock solutions of most ligands are stable at −20 °C, but one should always be cautious with using stock solutions for longer periods of time. Product stability and purity should be checked regularly.
2. Plates: Solid white-walled 384-well assay plates with low fluorescence background (*see* **Note 1**).
3. To count cells prior to seeding onto plates, an automatic cell counter can be used, such as a TC10™ automated cell counter (Bio-Rad).

2.3 Detection and Analysis

1. Detection is done with the PathHunter detection kit® (DiscoveRx). The detection mixture is prepared as follows (*see* **Note 2**): 19 parts of cell assay buffer, 5 parts of substrate reagent 1, and 1 part of substrate reagent 2, according to the manufacturer's protocol [15].
2. Measurement of the plates can be done by any multi-mode or luminescence plate reader, for example a Wallac EnVision™ 2104 Multilabel reader (Perkin Elmer).
3. Analysis of raw experimental data can be done by using the nonlinear regression curve fitting program GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

3 Methods

All procedures should be carried out at RT, unless specified otherwise.

3.1 Cell Culture

1. The CHOK1hCB_1_bgal or CHOK1hCB_2_bgal cells are cultured in cell culture medium in a humidified atmosphere at 37 °C and 5 % CO_2.
2. Cells are subcultured twice a week at a ratio of 1:20 on 10 cm ø plates by trypsinization. For trypsinization, add 1 ml of trypsin solution per dish and incubate for a maximum of 5 min at 37 °C. After incubation add 4 ml of medium to each dish. The cells are distributed equally over four plates and diluted 20× with cell culture medium. Each subculture step is one passage.
3. For the assay, cells should be passaged at least 3 times and no more than 24 times (max. 3 months in culture). Do not let the cells grow >90 % confluent prior to harvesting for the assay.

3.2 Ligands Under Investigation

1. High-throughput screening can be performed using a single high final concentration (often 10 μM) of each ligand. Instead, full dose-response curves of ligands are necessary for determination of their potency and efficacy.
2. Stock solutions of ligands under investigation are made in DMSO (or acetonitrile for endocannabinoids). These stock solutions should be diluted to the desired concentration in cell culture medium. Make sure that there is an equal amount of organic solvent (e.g., DMSO or acetonitrile) present in every dilution (*see* **Note 3**). For agonistic assays, compound dilutions should be five times the desired final concentration, while for antagonistic assays (and the EC_{80} of a reference agonist) the pre-made concentrations should be ten times the desired final concentration.

3. In all assays, also the E_{max} of a reference full agonist should be taken (e.g., CP55,940 at a final concentration of 10 μM).

3.3 PathHunter® Assay Protocol

1. Cells are harvested by adding 1 ml of Trypsol.
2. Incubate for a maximum of 5 min at 37 °C and 5 % CO_2.
3. Inactivate trypsin solution by addition of 4 ml of cell culture medium.
4. Transfer to Falcon tube and spin down (5 min at 1000 × *g*).
5. Resuspend pellet in 1 ml of cell culture medium, and count living cells by adding 10 μl of cell suspension to 10 μl of trypan blue.
6. Dilute the cells further with cell culture medium, to get a *density of 2.5× 105 cells/ml* (*=5000 cells/well*).
7. Add 20 μl of cell suspension per well in the 384-well plate. Make sure that you fill enough wells for background measurements, i.e., 3 wells containing only medium (negative control) and 3 wells containing unstimulated cells (basal activity). In case of antagonistic assay, include also 3 wells containing cells stimulated by EC_{80} of the reference agonist (positive control that is used as E_{max}).
8. Incubate cells overnight (between 16 and 18 h) at 37 °C and 5 % CO_2.

3.3.1 Plating Cells

3.3.2 Treatment of Cells and Incubation

Antagonistic assay:

1. Dilute antagonists of interest into cell culture medium to 10× the desired final concentration.
2. Add 2.5 μl of antagonist solution per well.
3. Incubate for 30 min at 37 °C and 5 % CO_2.
4. Add 2.5 μl of a 10× EC_{80} solution of CP55,940, also to wells for background measurements (EC_{80} of CP55,940 needs to be determined prior to the assay).
5. Make sure that there is an equal amount of DMSO or acetonitrile in all wells (≤1 % per well), including those for background measurements.
6. Incubate for 90 min at 37 °C and 5 % CO_2.

Agonistic assay:

1. Dilute agonists of interest into cell culture medium to 5× the desired final concentration.
2. Add 5 μl of agonist solution per well.
3. Make sure that there is an equal amount of DMSO or acetonitrile in all wells (≤1 % per well), including those for background measurements.
4. Incubate for 90 min at 37 °C and 5 % CO_2.

3.3.3 Detection and Measurements

1. Add 12 μl of detection mixture to each well that contains cells, as well as to 3 empty wells to measure background luminescence.
2. Incubate for 60 min *in the dark* at RT.
3. Measurement of the chemiluminescence of the plate can be done by any multi-mode or luminescence plate reader (*see* **Note 4**).

3.4 Data Analysis

Raw experimental data is analyzed by using the nonlinear regression curve fitting program GraphPad Prism. Potency, inhibitory potency, or efficacy values (EC_{50}, IC_{50}, or E_{max}, respectively) of ligands can be obtained by choosing the nonlinear regression option "log (agonist or inhibitor) vs. response." All data points are corrected for both background luminescence and negative control. The response of agonists in each sample is normalized to the effect of a reference agonist (e.g., 10 μM CP55,940), and the response of antagonists is normalized to the EC_{80} of a reference agonist (e.g., CP55,940). Basal activity of the cells is set at 0 %.

3.5 Results

We applied the protocol described here to characterize the well-known cannabinoid receptor reference ligands CP55,940, Rimonabant, SR144528, JWH133, and WIN55212-2 (*see* Fig. 2 and Table 1). Our findings correlate well with literature data [6, 13, 16].

3.6 Conclusions

The Pathunter® β-arrestin recruitment assay is easy to use and delivers reproducible results. In addition, its 384-well format enables high-throughput screening, a useful feature in early drug discovery research. We successfully applied this assay to characterize a set of cannabinoid receptor reference ligands, and would therefore recommend its use to other researchers in the field.

4 Notes

1. Perkin Elmer's white-walled 384-well assay plates are delivered in a sterile environment, and they should be kept that way by opening them only in an appropriate flow cabinet. Do not touch the plates at the bottom, because greasy fingerprints can give extra background and/or decrease reading efficiency of the plate reader. Also make sure that there is no dust in the wells, because this will give extra background and will increase well-to-well variation.
2. All PathHunter™ detection reagents should be aliquoted in Eppendorf tubes and stored at −20 °C upon receipt. Reagents can only be thawed and refrozen two times. When the reagents

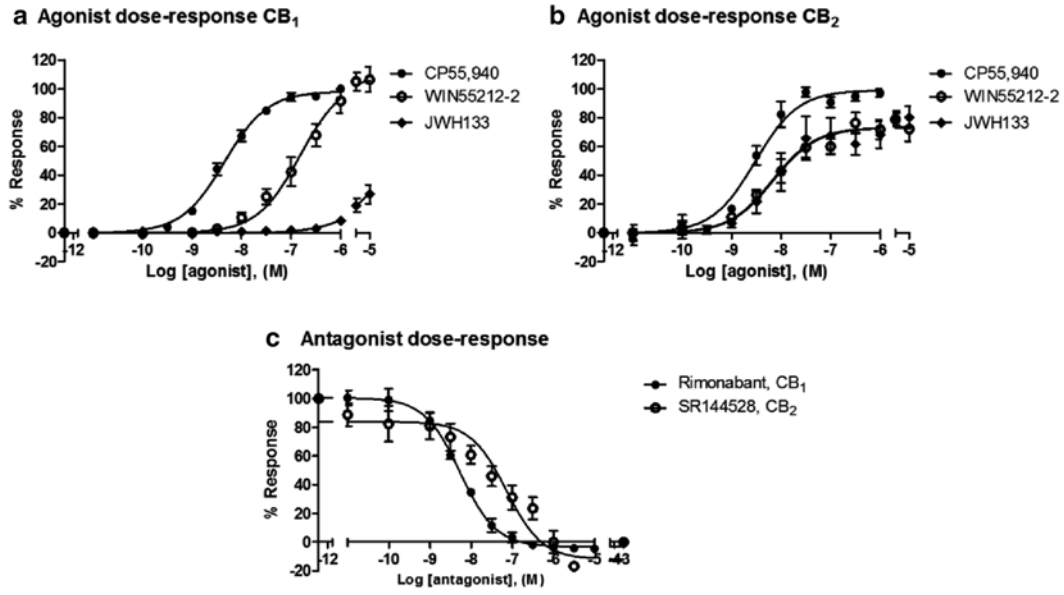

Fig. 2 Dose-response curves of cannabinoid ligands on β-arrestin recruitment in PathHunter® CHOK1hCB_1_bgal or CHOK1hCB_2_bgal cells. (**a** and **b**) Basal activity of the cells was set at 0 %, and the efficacy was calculated as a percentage of the maximum effect induced by 10 μM CP55,940 (set at 100 %). (**c**) Cells were pre-incubated with the EC_{80} concentration of CP55,940 (25 nM or 46 nM for the CB_1 or CB_2 receptor, respectively) for 30 min, and then they were treated with varying antagonist concentrations. The potency of antagonists was calculated as a percentage of the maximum effect induced by the EC_{80} concentration of CP55,940 (set at 100 %). The present results were obtained from three or four (with CP55,940) independent experiments, performed in duplicate

Table 1
Potency and efficacy data of cannabinoid receptor ligands obtained with the PathHunter® β-arrestin recruitment assay

	hCB_2		hCB_1	
Agonists	**pEC_{50} ± SEM**	**E_{max} ± SEM**	**pEC_{50} ± SEM**	**E_{max} ± SEM**
WIN55212-2	7.95 ± 0.41	82 ± 8	6.80 ± 0.21	109 ± 7
CP55,940	8.54 ± 0.11	100 ± 1	8.25 ± 0.2	99 ± 1
JWH133	7.97 ± 0.16	73 ± 3	5.56 ± 0.05	33 ± 3
Antagonists	pIC_{50} ± SEM	Max inhibition ± SEM	pIC_{50} ± SEM	Max inhibition ± SEM
Rimonabant	5.36 ± 0.04	−89 ± 3	8.26 ± 0.04	−105 ± 1
SR144528	7.11 ± 0.18	−133 ± 5	5.56 ± 0.13	−82 ± 5

The values shown are obtained from three or four (CP55,940) independent experiments performed in duplicate

are used for the detection mixture, they are thawed to RT *in the dark*, and then are mixed in the abovementioned ratio. Once the detection mixture is made, it is stable for 24 h at RT *in the dark*.

3. Stock solutions of ligands under investigation are made in DMSO or acetonitrile. Always make sure that there is an equal amount of organic solvent present in every dilution (≤1 %), in order to avoid disturbances due to the solvent. To obtain an equal concentration of organic solvent in every dilution, a dilution series in 100 % DMSO or acetonitrile should be made that is no less than 100× (agonist) or 200× (EC_{80} reference agonist or antagonist) the desired final concentration. Dilute this series accordingly in cell culture medium to 5× or 10× the desired final concentration, depending on whether an agonistic or antagonistic assay is performed.
4. The exposure time to read samples can depend on the instrument used. For example, the settings for a Wallac EnVision™ 2104 Multilabel reader should be:
   (a) Instrument settings: EnVision Single emission, with single emission mirror block.
   (b) Filters:
      - Emission filter Luminescence 700.
      - Mirror module Luminescence.
   (c) Install the emission filter in the Emission Filter Slides correctly. The filters must occupy *adjacent* slots in the Emission Filter Slide.
   (d) The mirror needs to be manually changed to the top position within the machine, and selected in the software before use.
   (e) Allow the lamp to warm up for at least 10 min.
   (f) Use protocol LUM Single (1.0 s read).

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References

1. Rang HP, Dale MM, Ritter JM et al (2008) Rang and Dale's pharmacology, 6th edn. Elsevier, Edinburgh
2. Hamm HE (1998) The many faces of G protein signaling. J Biol Chem 273:669–672
3. Shenoy SK, Drake MT, Nelson CD et al (2006) Beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. J Biol Chem 281:1261–1273
4. Whalen EJ, Rajagopal S, Lefkowitz RJ (2011) Therapeutic potential of β-arrestin- and G protein-biased agonists. Trends Mol Med 17:126–139
5. Violin JD, Lefkowitz RJ (2007) Beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci 28:416–422
6. Atwood BK, Wager-Miller J, Haskins C et al (2012) Functional selectivity in CB2 cannabinoid receptor signaling and regulation: implications for the therapeutic potential of CB2 ligands. Mol Pharmacol 81:250–263
7. Bosier B, Muccioli GG, Hermans E et al (2010) Functionally selective cannabinoid receptor signalling: therapeutic implications and opportunities. Biochem Pharmacol 80:1–12
8. Price MR (2005) Allosteric modulation of the cannabinoid CB1 receptor. Mol Pharmacol 68:1484–1495
9. Han S, Thatte J, Buzard DJ et al (2013) Therapeutic utility of cannabinoid receptor type 2 (CB2) selective agonists. J Med Chem 56:8224–8256
10. DiscoveRx. http://www.discoverx.com/technologies-platforms/enzyme-fragment-complementation-technology/pathhunter-efc-cell-based-assay-platform/protein-protein-interactions/gpcrs-b-arrestin
11. Zhang R, Xie X (2012) Tools for GPCR drug discovery. Acta Pharmacol Sin 33:372–384
12. Patel A, Murray J, McElwee-Whitmer S et al (2009) A combination of ultrahigh throughput PathHunter and cytokine secretion assays to identify glucocorticoid receptor agonists. Anal Biochem 385:286–292
13. Van der Lee MMC, Blomenröhr M, van der Doelen AA et al (2009) Pharmacological characterization of receptor redistribution and beta-arrestin recruitment assays for the cannabinoid receptor 1. J Biomol Screen 14:811–823
14. Van Der Lee MMC, Bras M, van Koppen CJ et al (2008) beta-Arrestin recruitment assay for the identification of agonists of the sphingosine 1-phosphate receptor EDG1. J Biomol Screen 13:986–998
15. DiscoveRx. http://www.discoverx.com/DiscoveRx/media/ContentFiles/DataSheets/93-0001L.pdf
16. Yin H, Chu A, Li W et al (2009) Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay. J Biol Chem 284:12328–12338

Chapter 12

Assay of NAT Activity

Toru Uyama and Natsuo Ueda

Abstract

In animal tissues, *N*-acyltransferase (NAT) catalyzes the first reaction in the biosynthetic pathway of bioactive *N*-acylethanolamines, in which an acyl chain is transferred from the *sn*-1 position of the donor phospholipid, such as phosphatidylcholine, to the amino group of phosphatidylethanolamine, resulting in the formation of *N*-acylphosphatidylethanolamine. NAT has long been known to be stimulated by Ca^{2+}, and hence it has been referred to as Ca^{2+}-dependent NAT. On the other hand, members of the phospholipase A/acyltransferase (PLA/AT) family (also known as HRAS-like suppressor family) show Ca^{2+}-independent NAT activity. In this chapter, we describe (1) partial purification of Ca^{2+}-dependent NAT from rat brain, (2) purification of recombinant PLA/AT-2, and (3) NAT assay using radiolabeled substrate.

Key words *N*-acylphosphatidylethanolamine, NAPE, *N*-acyltransferase, Ca-NAT, COS-7 cell, HRASLS family, PLA/AT family, Rat brain, Radioisotope, Thin-layer chromatography

1 Introduction

In animal tissues, bioactive *N*-acylethanolamines are formed from glycerophospholipids via *N*-acylphosphatidylethanolamines (NAPEs), a unique class of glycerophospholipid species possessing three long fatty acyl chains per one molecule [1]. The formation of NAPE is catalyzed by *N*-acyltransferase (NAT), which transfers an acyl chain from the *sn*-1 position of the donor phospholipid, such as phosphatidylcholine (PC), to the amino group of phosphatidylethanolamine (PE) (Fig. 1).

NAT has long been known, and exists in mammalian tissues such as brain and testis as a membrane-associated protein; it is stimulated by Ca^{2+} [2–4]. However, this enzyme has not been highly purified or cloned to date. In this chapter, we refer to this enzyme as Ca^{2+}-dependent NAT (Ca-NAT). Alternatively, we found that HRASLS-5, a protein belonging to the HRAS-like suppressor (HRASLS) family, has a NAPE-forming NAT activity [5]. Later, we revealed that all members of this family (HRASLS1–5) show NAT activity as well as phospholipase A_1/A_2 activity [1]. Based on these findings, we proposed to call HRASLS1–5 as phospholipase A/acyl-

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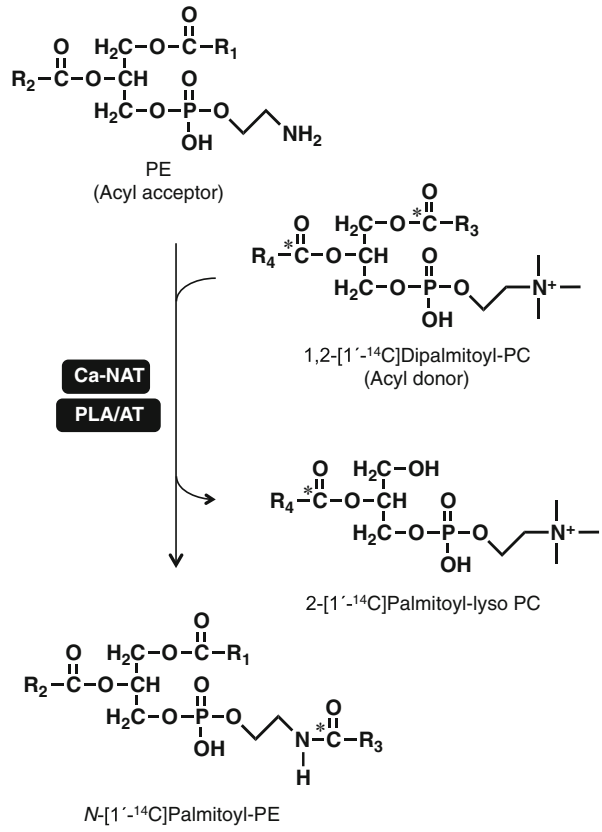

Fig 1 Formation of *N*-[^{14}C]palmitoyl-PE by NAT. *Asterisks* show the positions of the radiolabel

transferase (PLA/AT)-1–5, respectively [6]. Largely different from Ca-NAT, PLA/AT family proteins exist in both membrane and cytosol, and their NAT activity is not stimulated by Ca^{2+} [5–9].

In this chapter, we first describe partial purification of Ca-NAT from rat brain [5], as well as purification of recombinant PLA/AT-2, a PLA/AT family member exhibiting a relatively high NAT activity, from mammalian cells [10]. We then introduce our assay method to detect NAT activity with radioactive substrate. As an alternative method to detect NAPE produced by recombinant PLA/AT family proteins, we [10] and Golczak and colleagues [11] reported tandem mass spectrometry.

2 Materials

2.1 Partial Purification of Ca-NAT from Rat Brain

1. Pregnant Wistar/ST rats.
2. HiTrap Q anion-exchange column (bed volume, 5 ml) (GE Healthcare, Piscataway, NJ, USA).

3. Buffer A: 20 mM Tris–HCl (pH 7.4), 0.32 M sucrose.
4. Buffer B-1: 20 mM Tris–HCl (pH 7.4), 0.5 % (w/v) Nonidet P-40.
5. Buffer B-2: 20 mM Tris–HCl (pH 7.4), 0.5 % (w/v) Nonidet P-40, 100 mM NaCl.
6. Buffer B-3: 20 mM Tris–HCl (pH 7.4), 0.5 % (w/v) Nonidet P-40, 200 mM NaCl.
7. Buffer B-4: 20 mM Tris–HCl (pH 7.4), 0.5 % (w/v) Nonidet P-40, 500 mM NaCl.
8. Protein assay dye reagent concentrate for Bradford protein assay (Bio-Rad, Hercules, CA, USA), and bovine serum albumin (BSA) as a standard.
9. Motor-driven Polytron homogenizer with a stainless-steel generator shaft of 12 mm in diameter (Kinematica, Littau, Switzerland).
10. Potter-type Teflon-glass grinder.

2.2 Purification of Recombinant PLA/AT-2

1. COS-7 cells.
2. Culture medium for COS-7 cells: Dulbecco's modified Eagle medium (DMEM) with 10 % fetal calf serum and 0.1 mM nonessential amino acids.
3. 0.05 % Trypsin/0.02 % ethylenediaminetetraacetic acid (EDTA).
4. Phosphate-buffered saline (PBS).
5. Lipofectamine 2000.
6. pEF6/Myc-His vector harboring C-terminally FLAG-tagged human PLA/AT-2 cDNA [9] (*see* **Note 1**).
7. Branson Sonifier model 250 equipped with a tapered microtip (end diameter of 1/8 in.) (Branson, Danbury, CT, USA).
8. Buffer C: 20 mM Tris–HCl (pH 7.4).
9. Buffer D: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.05 % (w/v) Nonidet P-40.
10. Anti-FLAG M2 affinity gel and FLAG peptide.

2.3 Enzyme Assay

1. 1,2-[1′-^{14}C]dipalmitoyl-PC (45,000 cpm/2 μl in ethanol): Dilute 1,2-[1′-^{14}C]dipalmitoyl-PC (toluene/ethanol (1:1) solution, 1.11–2.22 TBq/mmol, 37 MBq/ml) (PerkinElmer Life Science, Boston, MA, USA) with ethanol.
2. 4 mM 1,2-Dipalmitoyl-PC in chloroform/methanol (2:1, by vol.).
3. 7.5 mM 1,2-Dioleoyl-PE in chloroform/methanol (2:1, by vol.).
4. 50 mM Dithiothreitol.
5. 0.5 M Glycine-NaOH (pH 9.0).

6. 10 % (w/v) Nonidet P-40.
7. 0.1 M $CaCl_2$.
8. Round-bottom 5 ml glass test tubes (12 mm diameter × 90 mm length).
9. Nitrogen gas cylinder with a regulator.
10. Water bath-type sonicator.
11. Stop solution: Chloroform/methanol (2:1, by vol.).
12. Calibrated 100 μl glass micropipets and a rubber aspirator tube (Drummond Scientific, Broomall, PA, USA) (*see* **Note 2**).
13. Conventional hair dryer held by a clamp and a stand.
14. Precoated silica gel 60 F_{254} aluminum sheet for thin-layer chromatography (TLC) (20 × 20 cm, 0.2 mm thickness): Cut it into equal halves (20 × 10 cm each) by scissors or a guillotine paper cutter.
15. TLC solvent: Chloroform/methanol/28 % ammonium hydroxide (80:20:2, by vol.). Mix 400 ml of chloroform, 100 ml of methanol, and 10 ml of 28 % ammonium hydroxide in a 500 ml glass bottle, and shake it well. Store at 4 °C.
16. Rectangular glass TLC tank (inside dimension: 235 × 105 × 210 mm) with a glass lid: Attach Whatman 3MM filter paper to the inner wall and pour the TLC solvent (120–150 ml) into the tank (*see* **Note 3**). Place the tank in cold room (4 °C).
17. Imaging analyzer FLA-7000 with an Imaging Plate (20 × 40 cm) and a cassette (Fujifilm, Tokyo, Japan).

3 Methods

3.1 Partial Purification of Ca-NAT from Rat Brain

1. Keep pregnant Wistar/ST rats in an animal facility and obtain their infants (*see* **Note 4**).
2. Anesthetize 2-day-old infants, and sacrifice them by decapitation. Isolate the brains from infants, and wash them with ice-cold PBS (*see* **Note 5**). Weigh the brains in a glass beaker (*see* **Note 6**).
3. Keeping the glass beaker on ice, mince the brains with scissors, and add five times volume (ml/g of tissue weight) of ice-cold Buffer A.
4. Homogenize the tissues using a Polytron homogenizer at setting 7 in an ice-water bath three times for 20 s each (*see* **Note 7**).
5. Centrifuge homogenates at 800 × *g* for 15 min at 4 °C, and further centrifuge the resultant supernatant at 105,000 × *g* for 55 min at 4 °C.
6. Transfer the pellet (membrane fraction) to a precooled chamber of a Teflon-glass grinder with a spatula. Add twice a volume (ml/g of tissue weight) of ice-cold Buffer A to the

chamber in the ice-water bath, and suspend the pellet with the aid of the grinder.

7. Transfer the prepared membrane fraction to a polypropylene tube and freeze it in a −80 °C freezer. After thawing the membrane fraction, perform another centrifugation at 105,000 × *g* for 55 min at 4 °C.
8. To solubilize Ca-NAT with the nonionic detergent Nonidet P-40, resuspend the pellet in Buffer B-1 with a Potter-type Teflon-glass grinder as described in **step 6**, and centrifuge it again at 105,000 × *g* for 55 min at 4 °C.
9. The resultant clear supernatant contains the solubilized Ca-NAT. Determine the protein concentration, according to the manufacturer's instructions for protein assay dye reagent concentrate for Bradford protein assay. Use 0.25–6 μg of BSA in 250 μl volume of reactions to plot a standard curve. Freeze the supernatant at −80 °C until use.
10. Equilibrate a HiTrap Q anion-exchange column with at least 15 ml of Buffer B-1 (*see* **Note 8**).
11. Slowly thaw the solubilized Ca-NAT (50 mg of protein), which was frozen in **step 9**, and load it onto the pre-equilibrated HiTrap Q anion-exchange column.
12. Wash the column with 15 ml of Buffer B-2, followed by 20 ml of Buffer B-3.
13. Elute Ca-NAT with 6 ml of Buffer B-4. Use this eluate as partially purified Ca-NAT.
14. Determine protein concentration as described in **step 9**. Store at −80 °C until use.

3.2 Purification of Recombinant PLA/AT-2

1. Culture COS-7 cells at 37 °C to 90 % confluency in three 150 mm dishes, containing DMEM with 10 % fetal calf serum in a humidified 5 % CO_2 and 95 % air incubator (*see* **Note 9**).
2. Introduce 25 μg/dish of the expression vector pEF6/Myc-His harboring cDNA of C-terminally FLAG-tagged human PLA/AT-2 into COS-7 cells, using 75 μl/dish of Lipofectamine 2000 according to the manufacturer's instructions and culture the cells for 48 h (*see* **Note 10**).
3. Treat the cells with 0.05 % trypsin/0.02 % EDTA for 2 min and harvest the suspended cells from three dishes into a 50 ml polypropylene tube. Centrifuge the cell suspension to collect cells at 4 °C. Wash the cells with 10 ml of ice-cold PBS and centrifuge at 4 °C. Perform this step again with a total of two washings.
4. Suspend the cell pellet with 4 ml of Buffer C in the same tube and sonicate the cells using a Branson Sonifier at the amplitude 3 three times for 3 s each. Add 20 μl of 10 % Nonidet P-40 to the resultant cell homogenate. Centrifuge the cell homogenate

at 105,000 × g for 55 min at 4 °C. Use the supernatant as the cytosolic fraction (*see* **Note 11**).

5. To pre-equilibrate anti-FLAG M2 affinity gel, add 4 ml of Buffer D and 1 ml of the gel (50 % slurry) in a 5 ml polypropylene tube. Centrifuge the gel at 3000 × *g* for 5 min at 4 °C. Discard the supernatant, add 5 ml of Buffer D to the tube, suspend the gel by gentle mixing, and centrifuge it under the same conditions. Perform this washing step by centrifuging three times.
6. Add the cytosolic fraction (about 4 ml) to the tube containing 0.5 ml of anti-FLAG M2 affinity gel pre-equilibrated with Buffer D, and incubate it overnight at 4 °C under gentle mixing.
7. Pack the gel into a column with a filter disc and wash it three times each with 12 ml of Buffer D.
8. Elute the FLAG-tagged PLA/AT-2 protein with Buffer D containing 0.1 mg/ml of FLAG peptide. Collect every 0.5 ml fraction. Use active fractions as purified PLA/AT-2 (*see* **Note 12**).
9. Determine protein concentration of active fractions as described in Subheading 3.1, **step 9.** Store at –80 °C until use.

3.3 Enzyme Assay

1. The reaction mixture necessary for one assay (80 μl) can be prepared with 10 μl of 0.5 M glycine-NaOH (pH 9.0), 4 μl of 50 mM dithiothreitol, 0.5 μl of 10 % Nonidet P-40, and 65.5 μl of water (*see* **Note 13**). When Ca-NAT is used as NAT, 5 μl of 0.1 M $CaCl_2$ is also included and the same amount of water is reduced, so that the reaction mixture remains 80 μl. Prepare the required amount of this reaction mixture in a glass tube, according to total number of samples to be assayed at a time.
2. The substrate mixture necessary for one assay can be prepared with 1 μl of 4 mM 1,2-dipalmitoyl-PC, 1 μl of 7.5 mM 1,2-dioleoyl-PE and 2 μl of 1,2-[1′-^{14}C]dipalmitoyl-PC (45,000 cpm/2 μl in ethanol) (*see* **Note 14**). According to the total number of samples to be assayed at a time, prepare the required amount of this substrate mixture in a 5 ml glass test tube, and evaporate it to dryness under the stream of nitrogen gas (*see* **Note 15**).
3. Transfer the reaction mixture prepared in **step 1** into this glass tube. Sonicate the solution for 20 s in a water bath-type sonicator and vigorously mix it by vortex. Perform this sonication and mix three times in total.
4. Dispense 80 μl of the mixture to each glass test tube.
5. Start the enzymatic reaction by adding 20 μl of the enzyme source (Ca-NAT, 23 μg protein or PLA/AT-2, 0.15 μg protein) to the tube and vortex it briefly (*see* **Note 16**). Allow the enzymatic reaction to proceed for 30 min by incubating the tube at 37 °C in the water bath.

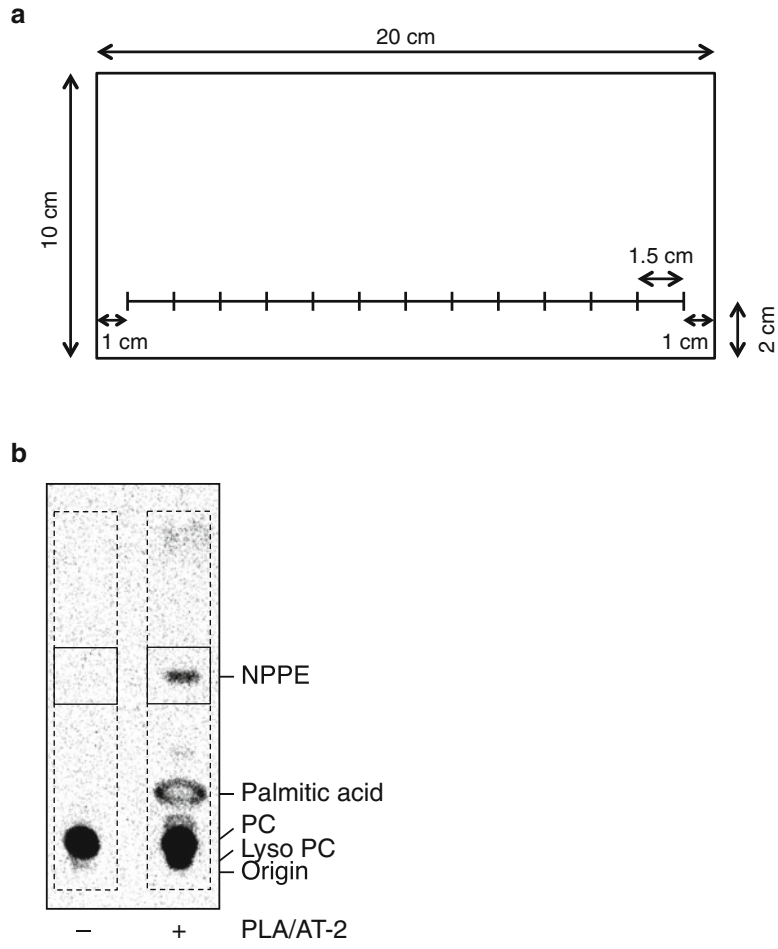

Fig 2 Marking of TLC plate (a) and an example of thin-layer chromatograms (b) in NAT assay using the purified human PLA/AT-2. *NPPE N*-palmitoyl-PE

6. Terminate the reaction by adding 320 μl of stop solution (*see* **Note 16**). Mix it vigorously by vortex.
7. Centrifuge the tube at 2000 × *g* for 5 min at 4 °C.
8. Draw an 18 cm horizontal line at 2 cm apart from the bottom edge on an aluminum TLC sheet by a soft pencil, leaving 1 cm blanks at both side edges. Draw short vertical lines at 1.5 cm interval across the horizontal line to form 12 lanes per sheet (Fig. 2a).
9. Spot 100 μl of the lower chloroform layer on each lane along the horizontal line (*see* **Note 17**). Use glass micropipet, which is connected to a rubber aspirator tube, under airflow by a hair dryer (*see* **Note 18**). After spotting all the samples, leave the plate under airflow for a few minutes to evaporate the solvent completely.
10. Immerse the lower edge (<2 cm) of the TLC sheet in TLC solvent placed in the tank. Develop the sheet at 4 °C for

20 min. Take the sheet out of the tank, and completely dry it up under airflow (*see* **Note 18**).

11. Wrap the TLC sheet with a plastic film, and expose an imaging plate to the wrapped plate in a cassette for 6–18 h.
12. Scan the plate by an imaging analyzer FLA-7000 to see the distribution of the radioactivity (Fig. 2b) (*see* **Note 19**). Quantify total radioactivity (a) on the lane (radioactivity in the dashed box in Fig. 2b) and radioactivity of the produced *N*-[1′-^{14}C]palmitoyl-PE (b) (radioactivity in the solid box), and calculate the conversion rate ($c = b/a$), as well as the background value obtained with an enzyme-free sample ($c' = b'/a'$). Since one PC molecule used as a substrate contains two atoms of ^{14}C at positions 1′ of *sn*-1 and *sn*-2 palmitoyl chains, and since the produced *N*-[1′-^{14}C]palmitoyl-PE and [^{14}C]lyso PC contain one ^{14}C atom per molecule, respectively (Fig. 1), the increase in radioactivity of *N*-[^{14}C]palmitoyl-PE during the reaction is half of the decrease in radioactivity of [^{14}C]PC. Thus, the conversion rate (c and c') should be doubled, and the NAPE-forming NAT activity is expressed as $4 \times 2 \times (c - c')/30$ (nmol/min).

4 Notes

1. The purification procedure utilizing the FLAG tag can also be applied for recombinant proteins of other PLA/AT family members [5–9].
2. The rubber aspirator tube is supplied as an accessory of the glass micropipets.
3. At least 1–2 h is necessary to saturate the TLC tank with solvent vapors. In order to minimize evaporation of the solvent and change in its composition, put a 1 kg weight on the lid of the tank.
4. Follow institutional guidelines for care and use of animals.
5. NAT activity in rat brain decreases age dependently [12].
6. Brains from twenty 2-day-old infants are around 6.5 g in weight. The capacity of the glass beaker should be 2–3 times larger than the volume of the homogenization buffer added in **step 3**.
7. To prevent temperature rising, take a pause (at least 30 s) between each homogenization or sonication with gentle mixing in the ice-water bath.
8. To load buffer solution and the solubilized Ca-NAT onto the column, use a disposable syringe or a peristaltic pump.
9. The cells may be maintained in the presence of 100 U/ml penicillin and 0.1 mg/ml streptomycin. However, these antibiotics should be removed before starting lipofection in **step 2**.
10. The medium may be replaced with fresh medium without Lipofectamine 2000 at 24 h from transfection.

11. The pellet obtained by ultracentrifugation also contains recombinant PLA/AT-2 protein to a certain extent. This pellet can be suspended in Buffer C by being passed through a 25-gauge syringe and used for enzyme assay as membrane-bound PLA/AT-2.
12. FLAG-tagged recombinant protein is usually eluted in Fractions No. 2–4. Purity and abundance of the recombinant protein can be confirmed by SDS-polyacrylamide gel electrophoresis, followed by Coomassie Brilliant Blue staining. Western blotting can also be carried out with anti-FLAG monoclonal antibody M2.
13. The final concentrations of each component are as follows: 50 mM glycine-NaOH (pH 9.0), 2 mM DTT, and 0.05 % Nonidet P-40. For Ca-NAT assay, 5 mM $CaCl_2$ is also contained.
14. The final concentrations of each substrate are as follows: 40 μM 1,2-dipalmitoyl-PC and 75 μM 1,2-dioleoyl-PE. The amount of 1,2-[^{14}C]dipalmitoyl-PC is about 0.025 % of non-radiolabeled 1,2-dipalmitoyl-PC and is negligible.
15. Connect a Pasteur pipette to a regulator of a nitrogen gas cylinder via a silicon tube and carefully evaporate organic solvent under the stream of nitrogen gas.
16. For multiple samples, each reaction should be started every 15 or 20 s by adding the enzyme source and terminated at the same time interval by the stop solution.
17. Synthetic [^{14}C]NAPE may be used as the authentic positive standard for TLC. *N*-[^{14}C]palmitoyl-PE is prepared from [1-^{14}C]palmitic acid and 1,2-dioleoyl-PE essentially according to the method by Schmid and colleagues [13]. Briefly, dilute 50 μCi (5×10^6 cpm) of [l-^{14}C]palmitic acid with unlabeled palmitic acid (1.3 mg, 5.0 μmol) to a specific activity of 1.0×10^3 cpm/nmol and allow it to react with 4.9 μmol of carbonyldiimidazole in 1 ml of dry benzene at 40 °C for 4 h. Then, add a 50 % molar excess of 1,2-dioleoyl-PE in 0.5 ml of benzene, along with 1 mg of *N*,*N*-dimethyl-4-aminopyridine and 0.1 ml of pyridine. After overnight incubation at 40 °C, evaporate the solvent of the mixture under a stream of nitrogen gas, and purify the resultant *N*-[^{14}C]palmitoyl-PE by TLC on a precoated silica gel 60 F_{254} glass plate (20×20 cm, 0.2 mm thickness) with a mixture of chloroform/methanol/28 % ammonium hydroxide (80:20:2, by vol.).
18. Use normal-temperature airflow from a hair dryer held by a clamp and a stand. Hot airflow should be avoided.
19. Since PLA/AT-2 also shows phospholipase A_1/A_2 activity toward [^{14}C]PC, some [^{14}C]free palmitic acid is concomitantly produced. We can separate NAT activity from phospholipase A_1/A_2 activity because we calculate NAT activity based on radioactivity of the produced *N*-[1′-^{14}C]palmitoyl-PE.

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References

1. Ueda N, Tsuboi K, Uyama T (2013) Metabolism of endocannabinoids and related *N*-acylethanolamines: canonical and alternative pathways. FEBS J 280:1874–1894
2. Di Marzo V, Fontana A, Cadas H et al (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372:686–691
3. Cadas H, di Tomaso E, Piomelli D (1997) Occurrence and biosynthesis of endogenous cannabinoid precursor, *N*-arachidonoyl phosphatidylethanolamine, in rat brain. J Neurosci 17:1226–1242
4. Hansen HS, Moesgaard B, Hansen HH et al (2000) *N*-Acylethanolamines and precursor phospholipids—relation to cell injury. Chem Phys Lipids 108:135–150
5. Jin X-H, Okamoto Y, Morishita J et al (2007) Discovery and characterization of a Ca^{2+}-independent phosphatidylethanolamine *N*-acyltransferase generating the anandamide precursor and its congeners. J Biol Chem 282:3614–3623
6. Shinohara N, Uyama T, Jin X-H et al (2011) Enzymological analysis of the tumor suppressor A-C1 reveals a novel group of phospholipid-metabolizing enzymes. J Lipid Res 52:1927–1935
7. Jin X-H, Uyama T, Wang J et al (2009) cDNA cloning and characterization of human and mouse Ca^{2+}-independent phosphatidylethanolamine *N*-acyltransferases. Biochim Biophys Acta 1791:32–38
8. Uyama T, Morishita J, Okamoto Y et al (2009) The tumor suppressor gene H-Rev107 functions as a novel Ca^{2+}-independent cytosolic phospholipase $A_{1/2}$ of the thiol hydrolase-type. J Lipid Res 50:685–693
9. Uyama T, Jin X-H, Tsuboi K et al (2009) Characterization of the human tumor suppressors TIG3 and HRASLS2 as phospholipid-metabolizing enzymes. Biochim Biophys Acta 1791:1114–1124
10. Uyama T, Ikematsu N, Inoue M et al (2012) Generation of *N*-acylphosphatidylethanolamine by members of the phospholipase A/acyltransferase (PLA/AT) family. J Biol Chem 287:31905–31919
11. Golczak M, Kiser PD, Sears AE et al (2012) Structural basis for the acyltransferase activity of lecithin:retinol acyltransferase-like proteins. J Biol Chem 287:23790–23807
12. Natarajan V, Schmid PC, Schmid HHO (1986) *N*-Acylethanolamine phospholipid metabolism in normal and ischemic rat brain. Biochim Biophys Acta 878:32–41
13. Schmid PC, Reddy PV, Natarajan V et al (1983) Metabolism of *N*-acylethanolamine phospholipids by a mammalian phosphodiesterase of the phospholipase D type. J Biol Chem 258:9302–9306

Chapter 13

Assay of NAPE-PLD Activity

Filomena Fezza, Nicolina Mastrangelo, and Mauro Maccarrone

Abstract

N-acyl-phosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D (NAPE-PLD) is a prominent enzyme involved in the biosynthesis of fatty acid amides (FAAs), a family of bioactive lipids including anandamide (AEA) as the prototypical member. Here, we describe a NAPE-PLD assay based on radioactive substrates and product separation by thin-layer chromatography (TLC).

Key words *N*-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D, Fatty acid amides, Anandamide, *N*-palmitoylethanolamine, *N*-acyl-phosphatidylethanolamines, Thin-layer chromatography, Radioactive assay

1 Introduction

N-acyl-phosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D (NAPE-PLD; EC 3.1.4.4) belongs to the zinc metallo-β-lactamase protein fold family, has no homology with the classical PLDs, and is highly conserved from rodents (396 amino acids) to human (393 amino acids) [1]. It is responsible for the release of fatty acid amides (FAAs, also called *N*-acylethanolamines) from membrane phospholipids, and therefore its activity is very important in determining the in vivo concentrations of these important bioactive lipids. *N*-acyl-phosphatidylethanolamines (NAPE) represent the natural substrates of NAPE-PLD, so that the C20:4-NAPE (called NArPE) is the precursor of *N*-arachidonoylethanolamine (anandamide, AEA).

Changes in FAA levels have been documented under different physiological and pathological conditions [2]; thus it appears of paramount importance to better understand the contribution of NAPE-PLD activity to the regulation of the endogenous tone of these compounds (in particular of AEA).

The activity of NAPE-PLD is not so easy to measure, because it requires a radiochromatographic approach based on reversed-phase high-performance liquid chromatography (HPLC) coupled to online scintillation counting [3].

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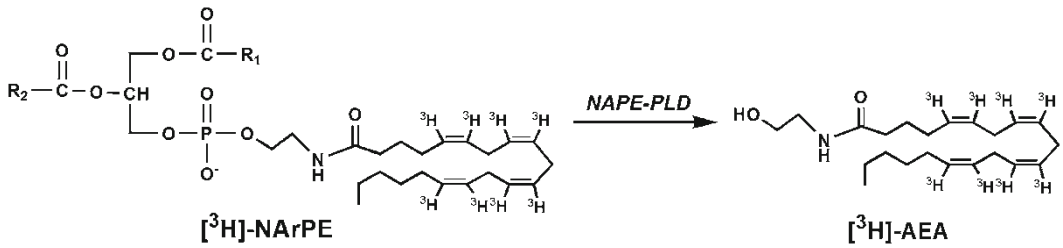

Fig. 1 NAPE-PLD activity using [^{3}H]-NArPE as a substrate

Here, we report a simple method that works well with both tissues and cell lines, and that is based on a radiolabeled NAPE. The latter leads to the release of radiolabeled FAA upon incubation with a biological extract that contains NAPE-PLD (Fig. 1). To separate the reaction products, we use a simple TLC that allows separation and quantitative determination of many samples at once.

2 Materials

Prepare all buffers and solutions by using ultrapure water and analytical grade reagents. Prepare and store all reagents at –20 °C (unless indicated otherwise).

2.1 Radiolabeled Components

1. Phosphatidylethanolamine, *N*-arachidonyl [arachidonyl-5,6,8,9,11,12,14,15-^{3}H(*N*)] ([^{3}H]-NArPE) (ARC, American Radiolabeled Chemicals, Inc, Saint Louis, MO, USA).
2. *N*-palmitoyl [1-^{14}C] phosphatidyl ethanolamine ([^{14}C]-NPPE) (ARC, American Radiolabeled Chemicals, Inc, Saint Louis, MO, USA).

2.2 Unlabeled Components

1. Buffer 1: Phosphate-buffered saline (PBS), 0.32 M sucrose. Make it fresh as required.
2. Buffer 2: 50 mM Tris–HCl, 0.1 % Triton, pH 7.4.
3. Chloroform/methanol (2:1, vol/vol).
4. Mobile phase: Methanol/chloroform/NH_4OH (85:15:1, vol/vol/vol).
5. Bromothymol blue solution (BBS): 0.1 % Bromothymol blue in 10 % aqueous ethanol, made just alkaline with NH_4OH (*see* **Note 1**).
6. *N*-arachidonoyl-phosphatidylethanolamine (NArPE) (*see* **Note 2**).
7. *N*-palmitoyl-phosphatidylethanolamine (NPPE) (*see* **Note 2**).
8. Anandamide (AEA) (*see* **Note 2**).

9. *N*-palmitoylethanolamine (PEA) (*see* **Note 2**).
10. URB597 (inhibitor of AEA-hydrolyzing enzyme fatty acid amide hydrolase).
11. Iodine (*see* **Note 3**).
12. Liquid scintillation cocktail (Ultima Gold™ XR).

2.3 Equipment

1. Aluminum TLC plates (20 × 20).
2. 2 mL Eppendorf microtubes.
3. TLC developing chamber.
4. Iodine vapor chamber.
5. Teflon-glass homogenizer.
6. Vortex mixer.
7. UltraTurrax T25.
8. Vacuum concentrator.
9. Centrifuge.
10. Thermoblock or water bath (37 °C).
11. Scintillation vials (20 mL).
12. Liquid scintillation β-counter (LCS).

3 Methods

All procedures need to be performed on ice, and all solutions should be prepared fresh just before processing biological samples.

3.1 Substrate Preparation

To prepare the substrate solution, it is necessary to add unlabeled (cold) NArPE or NPPE (*see* **Note 4**) to [^{3}H]-NArPE or [^{14}C]-NPPE, respectively, in order to bring the molar concentration up to the needed value (*see* **Note 5**). The final concentration is 100 μM/0.045 μCi (or 100,000 dpm, disintegrations per min; 1 μCi = 2.22 × 10^6 dpm) for each experimental point (*see* **Note 5**).

3.2 Preparation of Tissue and Cell Samples

1. Homogenize tissue (fresh weight/volume ratio = 1/10, g/mL) (*see* **Note 6**) or cell samples (30 × 10^6 cells/mL) with a glass/Teflon Potter homogenizer in buffer 1, precooled at 4 °C (*see* **Note 7**).
2. Centrifuge the homogenate at 1000 × *g* and 4 °C for 5 min.
3. Collect supernatant and determine protein concentration with any commercially available assay (i.e., Bradford colorimetric assay).

3.3 Enzyme Assay

NAPE-PLD assay can be performed by using [^{3}H]-NArPE or [^{14}C]-NPPE as substrate, and by separating the corresponding

products (AEA or PEA, respectively) under the same experimental conditions.

1. In 2 mL microtubes (*see* **Note 8**), preincubate for 10 min tissue or cell homogenates (*see* **Note 9**) with 0.1 nM URB597 (*see* **Note 10**), in a final volume of 200 μL of pre-warmed buffer 2.
2. Add [^{3}H]-NArPE or [^{14}C]-NPPE (each at the final concentration of 100 μM), to start the reaction.
3. Incubate for 30 min at 37 °C.
4. Stop the reaction by adding 600 μL of ice-cold chloroform/methanol, and shake.
5. Centrifuge the mixture at 3000 × *g* for 5 min (*see* **Note 11**).
6. Remove the upper aqueous layer by suction, and dry the lower organic phase (*see* **Note 12**).

3.4 TLC

1. Put 100 mL of mobile phase in TLC developing chamber (*see* **Note 13**).
2. Draw the TLC (*see* **Note 14** and Fig. 2a).
3. Load standards (AEA and NArPE, or PEA and NPPE), at the origin of the TLC (*see* **Note 15** and Fig. 2a).
4. Dissolve the dried lipids into 30 μL of chloroform/methanol, shake, centrifuge, and load on the TLC to the corresponding line (Fig. 2a).
5. Repeat **step 4** with additional 25 μL of chloroform/methanol.
6. Wait until the solvent is dried out, and put the TLC in the developing chamber.
7. Remove the TLC from the chamber when the solvent reaches approximately 1 cm below the top (Fig. 2b), and immediately draw with a pencil a line across the solvent front.
8. Once dried out, develop the TLC with BBS (*see* **Note 16**), and immediately circle the standards with a pencil (Fig. 2b).
9. Draw and cut out the TLC (*see* **Note 17** and Fig. 2c).
10. Place the clippings in the corresponding scintillation vials, and add 3 mL of methanol and 15 mL of scintillation cocktail (*see* **Note 18**).
11. Measure radioactivity in a liquid scintillation counter (*see* **Note 19**).

4 Notes

1. BBS is a stain used for the detection of lipids and phospholipids. When sprayed on the dried plate, (phospho)lipids give blue-green colors.
2. To make stock solutions (10 mM AEA, 2 mM NArPE, and 5 mM NPPE), aliquot them (10–50 μL) and store at −20 °C.

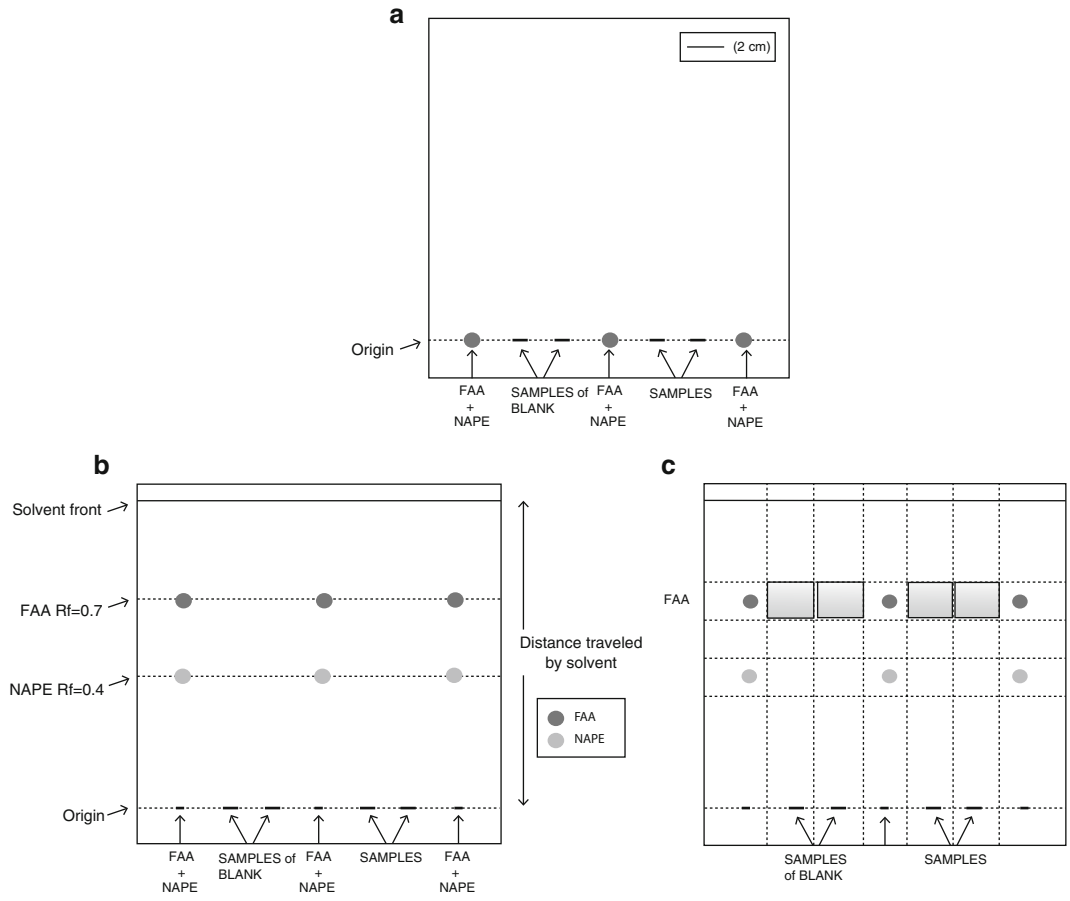

Fig. 2 Scheme of TLC preparation (**a**), standards and samples loading at baseline (**b**), TLC after the run and Rf corresponding to NAPE and FAA standards (**c**)

3. Iodine vapor is a universal and (relatively) unspecific reagent for many organic compounds. Put some crystals of iodine into the chamber, and then place the developed, dried chromatogram in iodine vapor. Spots will turn tan-brown in color.
4. To evaluate NAPE-PLD activity, it is possible to use either NArPE or NPPE as substrates, but in tissues or cell lines that express low amounts of enzyme it is better to use the latter compound. Indeed, NAPE-PLD prefers *N*-palmitoyl over *N*-arachidonoyl moiety as a substrate [5].
5. The "appropriate" concentration should be obtained from Michaelis–Menten kinetics. In our procedure, 100 μM NArPE (or NAPPE) works well [1, 5]. It should be stressed that radioactive compounds usually have high specific activity, and yet they are at very low concentration. Since, for enzymatic assay, higher substrate concentrations are required, it is necessary to add "cold" compound to prepare an appropriate substrate solution. Therefore, in order to obtain 100 μM NArPE (or NPPE)

in 0.2 mL, 0.02 μmol NArPE (or NPPE) is needed for each experimental point. Of course, addition of cold compound will change the specific activity; therefore, the specific activity of substrate solution has to be recalculated. To estimate the corrected specific activity, you have to make a ratio between the total amount of radioactive compound and the total amount of cold compound added in solution. An example of the calculation of [^{3}H]-NArPE molar concentration follows.

Radioactive concentration (r.c.): 1 Ci/L (indicated by the supplier).

Specific activity (s.a.): 200 Ci/mmol (indicated by the supplier).

[3H-NArPE] = r.c./s.a. = 1 Ci/L/200 Ci/mmol = 0.005 mM.

An example of substrate solution preparation for ten experimental points follows.

Total volume of substrate solution: 2.5 μL × 10 = 25 μL – volume of cold and radiolabeled substrate.

Total amount of NArPE moles: 0.02 × 10 = 0.2 μmol.

Total amount of radiolabeled [^{3}H]-NArPE: 0.045 × 10 = 0.45 μCi.

Calculation of specific activity of the substrate solution: 0.45 μCi/0.2 μmol = 2.25 μCi/μmol.

6. With tissues and other fibrous materials, it is more appropriate to use UltraTurrax T 25 to facilitate homogenization before using the glass/Teflon Potter homogenizer. An alternative procedure to prepare cell homogenates is via a Vibracell sonifier, which provides three cycles of sonication for 10 s each, interspersed by 10s breaks.
7. The best results are obtained with fresh cells and tissues.
8. Prepare also 2 mL microtubes with the same buffer used for the samples, but without proteins (blank). Prepare a suitable number of blanks, based on the number of TLC that you have to load.
9. For unknown biological samples it is appropriate to conduct a dose-response curve. The effect of protein content on NAPE-PLD assay in mouse brain is shown in Fig. 3.
10. URB597 is an inhibitor of fatty acid amide hydrolase (FAAH), a major FAAs-hydrolyzing enzyme [4]. Addition of this compound is important to prevent hydrolysis of FAAs generated by NAPE-PLD.
11. The centrifuge must be programmed in order to exclude the brake.
12. Use a vacuum concentrator for 30 min at 37 °C, or a nitrogen flow.
13. The chamber should contain enough solvent to simply cover the bottom.

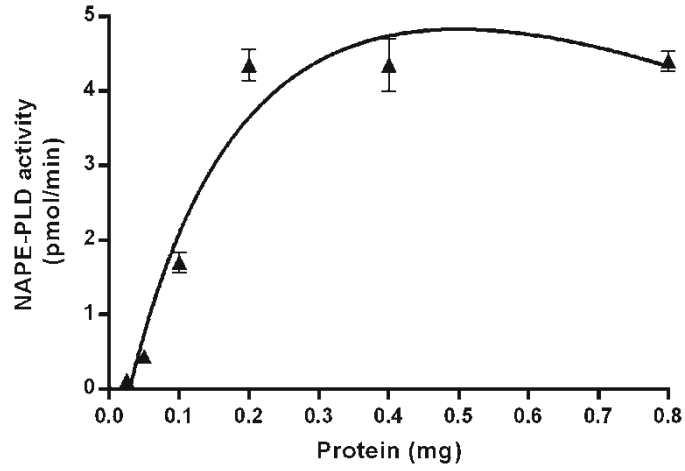

Fig. 3 Dependence of NAPE-PLD activity on mouse brain protein concentration

14. Use a pencil (never use a pen!), and leave at least 2 cm from the bottom and side edges.
15. Make sure that a sufficient amount of standard is spotted on the plate (20–40 nmol for each compound).
16. For NArPE and AEA, but not for NPPE and PEA, an alternative way for visualization can be the use of iodine vapor.
17. Count only the boxes that correspond to FAA spots (squares in gray in Fig. 2c).
18. The optimal methanol/scintillation cocktail ratio depends on the characteristics of the scintillation liquid (i.e., ULTIMA Gold XR is a liquid scintillation cocktail with very high sample load capacity and an extended range of sample holding capacity, up to 50 %).
19. To convert cpm (counts per min) into dpm, you need to know the efficiency of your β-counter for the given radioisotope ([^{3}H] or [^{14}C]), subtract the average of the blanks from each sample and transform dpm to moles based on the substrate-specific activity. To calculate NAPE-PLD-specific activity, divide the moles of product by the reaction time (min) and the protein content of the reaction mixture (mg of protein), as moles/(min × mg of protein).

    Example:

    dpm obtained – dpm average blanks:
    3000 dpm = 9.09×10^{-4} μCi (1 μCi = 2.22×10^{6} dpm).
    μmol formed: 9.09×10^{-4} μCi/2.25 μCi/
    μmol = 4.04×10^{-4} μmol.
    NAPE-PLD-specific activity = 4.04×10^{-4} μmol/30 min × 0.1
    mg = 1.34×10^{-4} μmol/(min × mg of protein).

Acknowledgements

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References

1. Okamoto Y, Morishita J, Tsuboi K et al (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. J Biol Chem 279:5298–5305
2. Balvers MG, Verhoeckx KC, Meijerink J et al (2013) Measurement of palmitoylethanolamide and other *N*-acylethanolamines during physiological and pathological conditions. CNS Neurol Disord Drug Targets 12:23–33
3. Fezza F, Gasperi V, Mazzei C et al (2005) Radiochromatographic assay of N-acyl-phosphatidylethanolamine-specific phospholipase D activity. Anal Biochem 339:113–120
4. Bisogno T, Maccarrone M (2013) Latest advances in the discovery of fatty acid amide hydrolase inhibitors. Expert Opin Drug Discov 8:509–522
5. Tsuboi K, Okamoto Y, Ikematsu N et al (2011) Enzymatic formation of *N*-acylethanolamines from *N*-acylethanolamine plasmalogen through *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D-dependent and -independent pathways. Biochim Biophys Acta 1811:565–577

Chapter 14

Assay of FAAH Activity

Monica Bari, Monica Feole, and Mauro Maccarrone

Abstract

Fatty acid amide hydrolase (FAAH) is an intracellular enzyme responsible for the hydrolysis of endogenous anandamide (AEA), a reaction that terminates the biological effects of this lipid mediator. The final products of this reaction are arachidonic acid and ethanolamine. In the method described herein, FAAH activity is measured through the use of a radioactive substrate by quantification of reaction products, that is, [^{14}C]-ethanolamine from [^{14}C-ethanolamine]-AEA.

Key words Arachidonic acid, AEA, Bioactive lipids, Ethanolamine, Endocannabinoids, Enzyme, FAAH

1 Introduction

In vivo levels of AEA are regulated by a tight balance between biosynthetic and degradative enzymes, and FAAH (E.C.3.5.1.4) plays a pivotal role in this fine regulation [1–5]. Indeed, FAAH terminates AEA signaling by a hydrolytic reaction which generates arachidonic acid (AA) and ethanolamine (Fig. 1), and represents a promising target to treat several pathological conditions [6, 7].

The study of FAAH activity is relevant to better understand how cells or tissues can terminate AEA signaling upon different stimuli [8, 9]. Here, we describe a method based on radiolabeled AEA and aimed at quantifying the time-dependent formation of the radiolabeled product of reaction, ethanolamine. At variance with previous radiometric protocols [10, 11], we use a substrate labeled on the ethanolamine moiety [12] that makes the assay simpler and less expensive. The present method is suitable for cells and tissues of different types and origins.

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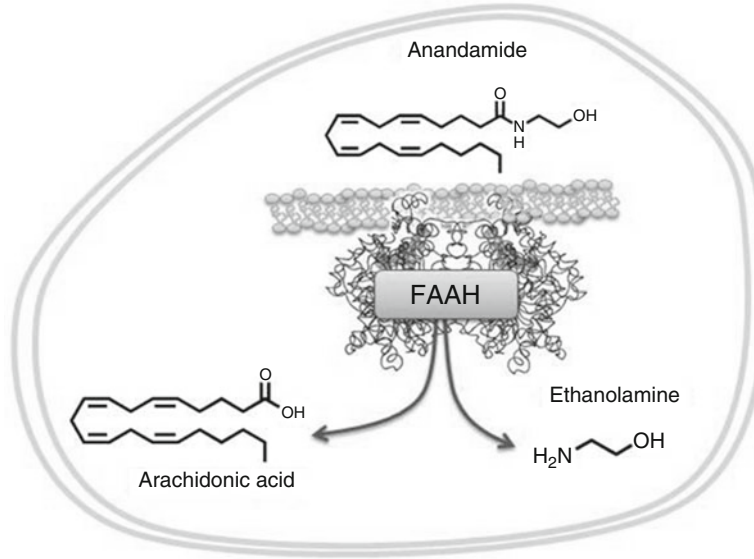

Fig. 1 Schematic representation of AEA hydrolysis catalyzed by FAAH

2 Materials

2.1 Chemicals

All reagents must be of the purest analytical grade.

1. Lysis buffer: 50 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4.
2. Reaction buffer: 50 mM Tris–HCl, pH 9.0 (*see* **Note 1**).
3. Methanol/chloroform (2:1, vol/vol).
4. "Cold" anandamide.
5. "Hot" [^{14}C-ethanolamine]-AEA (*see* **Note 2**).

2.2 Equipment

1. Teflon-glass homogenizer.
2. Ultra Turrax T25.
3. Vortex.
4. Centrifuge for Eppendorf tubes.
5. Spectrophotometer.
6. Cuvettes.
7. Thermoblock.
8. Scintillation vials (6 mL volume).
9. Liquid scintillation cocktail (Ultima Gold XR).
10. Liquid scintillation β-counter.

3 Methods

3.1 Sample Preparation

All steps must be carried out on ice.

1. Homogenize tissue or cell samples (*see* **Note 3**) in lysis buffer by using a Teflon potter, at a weight/volume (g/mL) ratio of 1:10.
2. Centrifuge the homogenate at 800 × *g* for 5 min at 4 °C, in order to remove tissue fragments.
3. Recover the supernatant in a new vial, measure protein content with any commercially available assay, and bring the sample to a protein concentration of 2 mg/mL (*see* **Note 4**).

3.2 Substrate Mix Preparation

Prepare a mixture of unlabeled and radiolabeled AEA (*see* **Note 5**) by mixing AEA/[^{14}C]-AEA (10 μM/0.009 μCi for each experimental point).

3.3 Enzymatic Reaction

1. Incubate, in a 2 ml Eppendorf tube, 40 μg of sample homogenates (*see* **Note 6**) with 5 μL of [^{14}C-ethanolamine]-AEA mix (*see* **Note 7**) for 15 min at 37 °C, in 500 μL of pre-warmed reaction buffer.
2. During the reaction time, prepare enough scintillation vials for collection of all samples, and fill them with the appropriate volume of scintillation cocktail (*see* **Note 8**).
3. Stop the reaction by adding 800 μL of ice-cold methanol/chloroform, with vortexing.
4. Centrifuge the mixture at 3000 × *g* for 5 min without break, at room temperature (*see* **Note 9**).
5. Put the upper aqueous layer in a scintillation vial containing liquid scintillation cocktail.
6. Quantify radioactivity in a β-counter (*see* **Note 10**).

4 Notes

1. FAAH shows an optimal activity at pH 9.0, but if you need to assay it under physiological conditions, the same buffer can be adjusted at pH 7.4. At this value FAAH activity will be about half of that recorded at pH 9.0.
2. Commercially available radioactive AEA can be [^{3}H]-labeled on arachidonic acid or [^{14}C]-labeled on ethanolamine. The latter [^{14}C]-AEA is more stable, a feature that reduces background and is critical when working with samples containing little FAAH activity.

3. The best FAAH assay is performed on fresh tissues and cells. However, it can be performed also on stored samples, if the latter are collected and immediately frozen and stored at −80 °C. At any rate, sample storage should never last longer than 1 month. Homogenization with a Teflon potter is usually preferred, while Turrax is recommended when samples are very fibrous (i.e., skeletal muscles).
4. The most common method to evaluate protein concentration is a colorimetric assay based on the binding of Coomassie dye under acidic conditions, which results in a color change from brown to blue (visible at 595 nm in any spectrophotometer). The final concentration should be adjusted around 1–2 mg of protein/ml, paying attention to avoid excessive sample dilution.
5. The best substrate concentration ([S]) for a single-point assay is defined from the Michaelis–Menten constant (K_m) that should be calculated from a saturation curve with different amounts of substrate (Fig. 2).

   The concentration of substrate involved in the enzyme reaction should be at saturating levels; therefore $[S] > K_m$ is advisable. In our assay with mouse brain, K_m is approximately 3.5 μM; thus we suggest a final AEA concentration of 10 μM. It should be noted that radiolabeled substrate often has a very low concentration that can be calculated from the specific activity (reported in the data sheet) as the ratio between radioactivity and amount (Ci/mmoles). Therefore, it is necessary to adjust the final substrate concentration by adding unlabeled AEA.

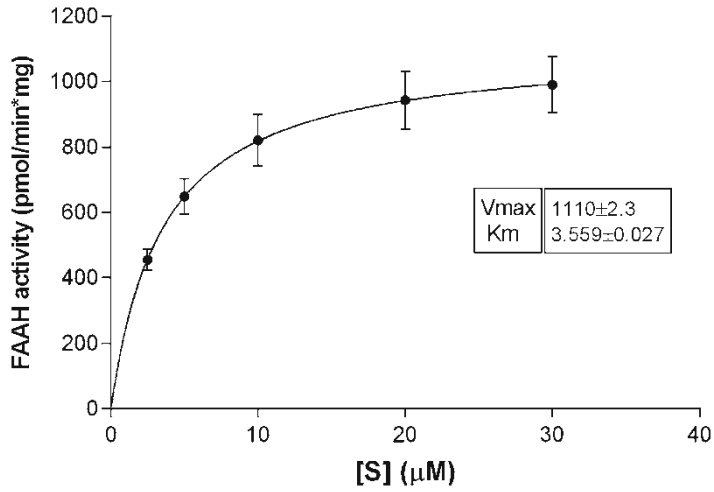

Fig. 2 Michaelis–Menten curves of mouse brain FAAH activity, determined by using 40 μg of proteins and different concentrations of substrate ([^{14}C-ethanolamine]AEA, 0–20 μM range). Nonlinear regression analysis of the kinetic data allowed to calculate apparent K_m (μM) and maximum velocity (V_{max}, expressed as pmol/(min mg of protein)) for [^{14}C]-AEA hydrolysis

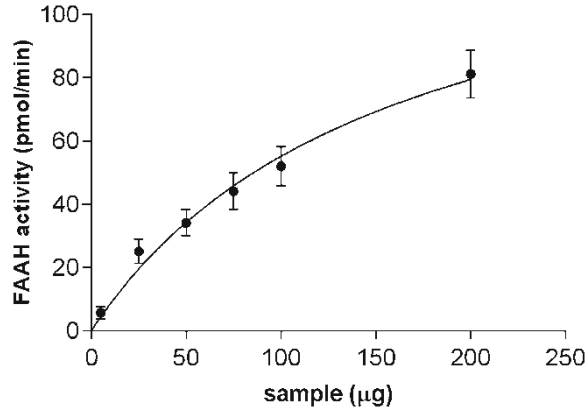

Fig. 3 FAAH activity in a dose-response curve for mouse brain sample. Range of protein concentration was 5–200 μg

6. The optimal protein concentration for FAAH assay depends on the amount of enzyme present in the sample. In the present protocol FAAH concentration refers to mouse brain. In general, a dose-dependence curve of FAAH activity should be built for every new sample, in order to ascertain the optimal protein concentration to be used (Fig. 3).
7. If you have an [^{14}C]-AEA batch with a specific activity of 55 mCi/nmoL and a concentration of 0.1 mCi/mL, and a 2 M stock solution of unlabeled AEA, the following is an example of mix composition for 100 reactions (500 μl), each performed in a 1.5 mL Eppendorf tube. Note that all steps must be performed on ice:
   (a) [^{14}C]-AEA = 0.009 μCi × 100 reactions = 0.9 μCi (corresponding to 9 μL from batch).
   (b) Cold AEA 100× (i.e., 1 mM) = 5 nmoL × 100 = 500 nmoL of AEA (corresponding to 50 μL from 10 mM AEA stock solution).

   Solutions (a) and (b) will be diluted with ethanol to a final volume of 500 μL (5 μL× 100 reactions). Note that it is recommended to prepare an excess of mix, compared to the planned experimental activities.
8. For a better quantitation, it is recommended to recover the same volume of the aqueous phase from all samples, even if it does not correspond to the whole volume of the phase. The scintillation liquid is added to the aqueous phase in a 7:1 ratio (scintillation liquid/aqueous phase, vol/vol). We usually use a 3.5 mL:0.5 mL ratio.
9. At the end of sample centrifugation, two phases will be observed: the lower is the organic phase, and the upper is the aqueous phase containing labeled ethanolamine.

10. Radioactivity of [^{14}C]-ethanolamine is expressed as disintegrations per minute (dpm) that must be transformed in moles by using the specific activity of the substrate mix (Ci/mmol), and keeping in mind that 1 μCi = 2.22×10^6 dpm. The resulting moles of ethanolamine will be divided by the reaction time (min) and the protein content (mg) in the reaction mixture, to obtain the specific activity of FAAH as moles/(min mg of protein).

References

1. Maccarrone M, Bab I, Bíró T et al (2015) Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci 36:277–296
2. Fezza F, Bari M, Florio R et al (2014) Endocannabinoids, related compounds and their metabolic routes. Molecules 19:17078–17106
3. Deutsch DG, Ueda N, Yamamoto S (2002) The fatty acid amide hydrolase (FAAH). Prostaglandins Leukot Essent Fatty Acids 66:201–210
4. Cravatt BF, Giang DK, Mayfield SP et al (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384:83–87
5. Giuffrida A, Beltramo M, Piomelli D (2001) Mechanisms of endocannabinoid inactivation: biochemistry and pharmacology. J Pharmacol Exp Ther 298:7–14
6. Ramos JA, González S, Sagredo O et al (2005) Therapeutic potential of the endocannabinoid system in the brain. Mini Rev Med Chem 5:609–617
7. Bedse G, Romano A, Lavecchia AM et al (2015) The role of endocannabinoid signaling in the molecular mechanisms of neurodegeneration in Alzheimer's disease. J Alzheimer's Dis 43:1115–1136
8. Fezza F, De Simone C, Amadio D et al (2008) Fatty acid amide hydrolase: a gate-keeper of the endocannabinoid system. Subcell Biochem 49:101–132
9. Bari M, Battista N, Fezza F et al (2006) New insights into endocannabinoid degradation and its therapeutic potential. Mini Rev Med Chem 6:257–268
10. Fezza F, Battista N, Bari M et al (2006) Methods to assay anandamide hydrolysis and transport in synaptosomes. Methods Mol Med 123:163–168
11. Maccarrone M, Bari M, Finazzi AA (1999) A sensitive and specific radiochromatographic assay of fatty acid amide hydrolase activity. Anal Biochem 267:314–318
12. Gattinoni S, De Simone C, Dallavalle S et al (2010) Enol carbamates as inhibitors of fatty acid amide hydrolase (FAAH) endowed with high selectivity for FAAH over the other targets of the endocannabinoid system. ChemMedChem 5:357–360

Chapter 15

Assay of NAAA Activity

Kazuhito Tsuboi and Natsuo Ueda

Abstract

N-acylethanolamine-hydrolyzing acid amidase (NAAA) is a lysosomal hydrolase degrading various *N*-acylethanolamines at acidic pH. Since NAAA prefers anti-inflammatory and analgesic palmitoylethanolamide to other *N*-acylethanolamines as a substrate, its specific inhibitors are expected as a new class of anti-inflammatory and analgesic agents. Here, we introduce an NAAA assay system, using [^{14}C]palmitoylethanolamide and thin-layer chromatography. The preparation of NAAA enzyme from native and recombinant sources as well as the chemical synthesis of *N*-[1′-^{14}C]palmitoyl-ethanolamine is also described.

Key words NAAA, *N*-acylethanolamine-hydrolyzing acid amidase, Palmitoylethanolamide, Anti-inflammatory agent, Rat lung, HEK293 cell, Thin-layer chromatography, Radioisotope

1 Introduction

The major degradative pathway for bioactive *N*-acylethanolamines, including palmitoylethanolamide, oleoylethanolamide, and arachidonoylethanolamide (anandamide), is hydrolysis to free fatty acids and ethanolamine catalyzed by fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) [1]. NAAA is a lysosomal cysteine hydrolase acting at acidic pH and prefers palmitoylethanolamide, which shows anti-inflammatory and analgesic actions, to other *N*-acylethanolamines in in vitro cell-free assay systems [2]. NAAA is abundantly expressed in macrophages [3, 4], and specific NAAA inhibitors have been reported to increase local levels of endogenous palmitoylethanolamide in inflammation models, and to exhibit anti-inflammatory and analgesic effects [5]. Thus, the NAAA assay is considered an important method for screening and developing new anti-inflammatory and analgesic agents.

Here, we introduce our NAAA assay system, using *N*-[1′-^{14}C] palmitoyl-ethanolamine ([^{14}C]palmitoylethanolamide) as a substrate (Fig. 1). In this system, thin-layer chromatography (TLC) is used to separate the produced [^{14}C]palmitic acid from the remaining substrate. We also refer to the preparation of NAAA enzyme

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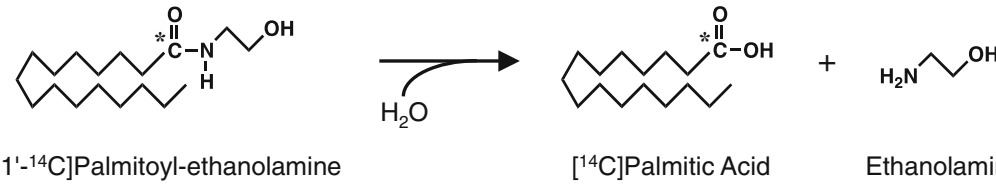

Fig. 1 Hydrolysis of *N*-[1′-^{14}C]palmitoyl-ethanolamine by NAAA. *Asterisks* show the positions of the radiolabel

from both native [6] and recombinant [2] sources, as well as the chemical synthesis of *N*-[1′-^{14}C]palmitoyl-ethanolamine. Another radioactive palmitoylethanolamide reported is [1,2-^{14}C] *N*-palmitoylethanolamine [7]. In the latter case, the radioactive product, [1,2-^{14}C]ethanolamine, is water soluble and remains in the aqueous phase after the extraction of [^{14}C]palmitoylethanolamide by chloroform/methanol (1:1, v/v). The radioactivity of the product can be then measured by a liquid scintillation counter. In addition, NAAA assay can be performed by mass spectrometry [8], or fluorometrically with *N*-(4-methylcoumarin)palmitamide, a fluorescent analog of palmitoylethanolamide, as a substrate [9].

2 Materials

2.1 Preparation of NAAA from Rat Lung

1. Wistar rats (male) (*see* **Note 1**).
2. Homogenization buffer: 20 mM Tris–HCl (pH 7.4), 0.32 M sucrose.
3. Motor-driven Polytron homogenizer with a stainless-steel generator shaft of 12 mm in diameter (Kinematica, Littau, Switzerland).
4. Phosphate-buffered saline (PBS).
5. Potter-type Teflon-glass grinder.
6. Protein assay dye reagent concentrate for Bradford protein assay (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as a standard.

2.2 Preparation of Recombinant NAAA

1. Human embryonic kidney 293 (HEK293) cells.
2. Culture medium for HEK293 cells: Dulbecco's modified Eagle medium (DMEM) with 10 % fetal calf serum and 0.1 mM nonessential amino acids.
3. Poly-L-lysine-coated 100 mm plastic cell culture dishes: Dissolve 5 mg of poly-L-lysine hydrobromide (molecular weight 30,000–70,000) in 50 ml of sterile water. Coat the culture surface with 2–3 ml of the poly-L-lysine solution for 10 min. Remove the solution (*see* **Note 2**), and rinse the culture surface with some sterile water. Aspirate off the water, and air-dry the surface under ultraviolet light in the clean bench.

4. pcDNA3.1(+) mammalian expression vector harboring human NAAA cDNA [2] (*see* **Note 3**).
5. Lipofectamine 2000.
6. 0.05 % Trypsin/0.02 % ethylenediaminetetraacetic acid (EDTA).
7. PBS.
8. Branson Sonifier model 250 equipped with a tapered microtip (end diameter of 1/8 in.) (Branson, Danbury, CT, USA).
9. Protein assay dye reagent concentrate for Bradford protein assay (Bio-Rad).

2.3 Synthesis of Radiolabeled Substrate

1. [1-^{14}C]Palmitic acid (ethanol solution, 1.48–2.22 GBq/mmol, 3.7 MBq/ml) (PerkinElmer Life Science, Boston, MA, USA).
2. Liquid scintillation counter.
3. Liquid scintillation mixture with a bottle-top dispenser.
4. Polyethylene 6 ml vials and glass vials.
5. 2-Aminoethanol (monoethanolamine).
6. Oxalyl chloride (*see* **Note 4**).
7. Methylene chloride.
8. Conical 10ml glass tubes with a screw cap.
9. Diethyl ether.
10. Nitrogen gas cylinder with a regulator.
11. Ethanol.
12. Conventional hair dryer held by a clamp and a stand.
13. Precoated silica gel 60 F_{254} glass plate for TLC (20 × 20 cm, 0.2 mm thickness).
14. TLC solvent A: The organic phase of isooctane/ethyl acetate/water/acetic acid (5:11:10:2, by vol.). Mix 100 ml of isooctane, 220 ml of ethyl acetate, 200 ml of deionized water, and 40 ml of acetic acid in a 500 ml glass bottle, and shake it well. After standing, transfer the upper organic phase to another bottle with a glass pipette. Store at room temperature (r.t.).
15. Rectangular glass TLC tank (inside dimension: 235 × 105 × 210 mm) with a glass lid: Attach Whatman 3MM filter paper to the inner walls, and pour TLC solvent A (120–150 ml) into the tank (*see* **Note 5**).
16. Imaging analyzer FLA-7000 with an imaging plate (20 × 40 cm) and a cassette (Fujifilm, Tokyo, Japan).
17. Chisel (12 mm blade in width) and microspatula.
18. Paraffin paper (15 × 15 cm).
19. Glass Buchner funnel with a G1 filter disc at the bottom.
20. Methanol.

21. Rubber bulb with a reservoir: Connect the bulb to a glass Buchner funnel via a glass tube penetrating a silicone stopper.
22. 40 mM Non-radiolabeled palmitoylethanolamide: Dissolve 10 mg of palmitoylethanolamide in 835 μl of ethanol (*see* **Note 6**).
23. Dimethyl sulfoxide.

2.4 Enzyme Assay

1. 0.5 M Citrate–sodium phosphate buffer (pH 4.5): Weigh 5.25 g of citric acid monohydrate, and add water to make 50 ml of 0.5 M citric acid. Weigh 8.95 g of $Na_2HPO_4 \cdot 12H_2O$, and add water to prepare 50 ml of 0.5 M Na_2HPO_4. Mix the solutions in a ratio that results in a pH value of 4.5.
2. 60 mM Dithiothreitol: Dissolve 185 mg of dithiothreitol in 20 ml of water. Dispense into 1 ml aliquots. Store at −20 °C. A fresh aliquot should be used every time before assay.
3. 4 % (w/v) Nonidet P-40: Weigh 1.6 g of Nonidet P-40 in a 50 ml plastic tube. Add about 35 ml of water, and rotate the tube for several hours or overnight at 4 °C. Adjust the volume to 40 ml with water. Store at 4 °C.
4. 1 % BSA: Dissolve 0.2 g of BSA (Cohn Fraction V) in 20 ml of water. Dispense the solution in 1.5 ml plastic tubes, and store at −20 °C.
5. 3 M NaCl: Dissolve 8.77 g of sodium chloride in water to make 50 ml solution.
6. [^{14}C]Palmitoylethanolamide (10,000 cpm/10 nmol/5 μl of dimethyl sulfoxide): Prepare as described in Subheading 3.3.
7. Round-bottom 5 ml glass test tubes (12 mm diameter × 90 mm length).
8. Stop solution: Diethyl ether/methanol/1 M citric acid (30:4:1, by vol.) (*see* **Note 7**). Store at −20 °C.
9. Calibrated 100 μl glass micropipets and a rubber aspirator tube (*see* **Note 8**).
10. Conventional hair dryer held by a clamp and a stand.
11. Precoated silica gel 60 F_{254} aluminum sheet for TLC (20 × 20 cm, 0.2 mm thickness): Cut it into equal halves (20 × 10 cm each) by scissors or a guillotine paper cutter.
12. TLC solvent B: Chloroform/methanol/28 % ammonium hydroxide (40:10:1, by vol.). Mix 400 ml of chloroform, 100 ml of methanol, and 10 ml of 28 % ammonium hydroxide in a 500 ml bottle and shake it well. Store at 4 °C.
13. Rectangular glass TLC tank (inside dimension: 235 × 105 × 210 mm) with a glass lid: Attach Whatman 3MM filter paper to the inner walls, and pour TLC solvent B (120–150 ml) into the tank (*see* **Note 5**).
14. Imaging analyzer FLA-7000 with an imaging plate (20 × 40 cm) and a cassette (Fujifilm).

3 Methods

3.1 Preparation of NAAA from Rat Lung

1. Anesthetize male Wistar rats, and sacrifice them by decapitation (*see* **Note 9**). Isolate lungs, and wash them with ice-cold PBS. Weigh the tissues in a glass beaker (*see* **Note 10**).
2. Keeping the glass beaker on ice, mince the lungs with scissors and add nine times volume (ml/g of tissue weight) of ice-cold homogenization buffer. Homogenize the tissues using a Polytron homogenizer at setting 7 in an ice-water bath three times for 20 s each (*see* **Note 11**).
3. Centrifuge the homogenate at 800 × *g* for 15 min at 4 °C. Transfer the supernatant to another centrifuge tube.
4. Centrifuge the supernatant at 12,000 × *g* for 30 min at 4 °C. Discard the resultant supernatant, and transfer the pellet to a precooled chamber of a Teflon-glass grinder with a spatula. Add one-seventh of the homogenate volume of ice-cold PBS to the chamber in an ice-water bath (*see* **Note 12**), and suspend the pellet with the aid of the grinder.
5. Transfer the suspended pellet to a polypropylene tube, and freeze it in a −80 °C freezer. Thaw the sample in a water bath at r.t., and freeze it again at −80 °C (*see* **Note 13**). These two cycles of freezing and thawing result in the solubilization of NAAA from lysosomes.
6. After thawing, ultracentrifuge the sample at 105,000 × *g* for 50 min at 4 °C. The solubilized NAAA is recovered in the supernatant.
7. Transfer the supernatant into a new tube. Determine the protein concentration (typically 2–3 mg/ml), according to the manufacturer's instruction for protein assay dye reagent concentrate for Bradford protein assay. Use 0.25–4 μg of BSA as a standard in 250 μl volume of reactions. Dispense the NAAA preparation into aliquots, and store at −80 °C until use.

3.2 Preparation of Recombinant NAAA

1. Culture HEK293 cells at 37 °C to 90 % confluency in a poly-L-lysine-coated 100 mm dish containing the culture medium in a humidified 5 % CO_2/95 % air incubator (*see* **Note 14**).
2. Introduce 8 μg of the expression vector pcDNA3.1(+) harboring cDNA of human NAAA into the cells using 36 μl of Lipofectamine 2000 according to the manufacturer's instruction and culture the cells for 48 h.
3. Suspend the cells with the aid of 0.05 % trypsin/0.02 % EDTA. Transfer the cell suspension to a 50 ml polypropylene conical tube, and centrifuge it to collect the cells. Wash the cells with 10 ml of PBS, followed by centrifugation. Discard the supernatant, suspend the cells again with 1 ml of PBS, and transfer the suspension of cells to a 1.5 ml tube.

4. After another centrifugation, resuspend the cells with 1 ml of PBS, and sonicate the cells using a Branson Sonifier at amplitude 3 in an ice-water bath three times for 3 s each (*see* **Note 11**).
5. Store the resultant cell homogenate at −80 °C until use as NAAA preparation. Determine the protein concentration (expected to be around 3 mg/ml), according to the manufacturer's instruction for protein assay dye reagent concentrate for Bradford protein assay. Use 0.25–4 μg of BSA as a standard in 250 μl volume of reactions.

3.3 Synthesis of Radiolabeled Substrate

1. To measure the radioactivity of [1-^{14}C]palmitic acid, dilute 5 μl of original [1-^{14}C]palmitic acid solution with 120 μl of ethanol (*see* **Note 15**). Add 5 μl of the diluted [1-^{14}C]palmitic acid solution into a polyethylene 6 ml vial containing 3 ml of liquid scintillation mixture (in triplicate). To subtract background radioactivity, add 5 μl of ethanol into another vial containing 3 ml of the scintillation mixture. Vortex all the vials, put them in bigger glass vials, and count the radioactivity with a liquid scintillation counter (*see* **Note 16**).
2. Based on the measured value of radioactivity, transfer [1-^{14}C] palmitic acid solution, corresponding to 1×10^8 cpm, to a conical 10 ml glass tube with a screw cap. Evaporate the solvent ethanol completely by nitrogen gas stream (*see* **Note 17**).
3. Add 200 μl of methylene chloride into the glass tube, and vortex it well.
4. In the safety cabinet, add 10 μl of oxalyl chloride into the tube. Close the cap tightly, and keep the solution at r.t. for 2 h. Vortex the tube at 0, 1, and 2 h. [^{14}C]Palmitoyl chloride is now produced.
5. Weigh 0.2 g of 2-aminoethanol in another conical 10 ml glass tube, and add 2 ml of methylene chloride into the tube. Vortex the solution, and place the tube on ice.
6. Open the tube containing [^{14}C]palmitoyl chloride (from **step 4**), and evaporate the solvent completely by nitrogen gas stream (*see* **Note 17**). Add 200 μl of 2-aminoethanol solution (prepared in **step 5**) into the tube. Close the cap and vortex the mixture.
7. Stand the mixture on ice for 30 min. Vortex it at 0, 10, 20, and 30 min. The synthesis of *N*-[1′-^{14}C]palmitoyl-ethanolamine ([^{14}C]palmitoylethanolamide) is now completed.
8. Add 1 ml of water and 2 ml of diethyl ether into the tube, and vortex the mixture well.

9. Centrifuge the mixture at 1600 × *g* for 10 min at r.t.. Transfer the upper organic layer to another conical 10 ml glass tube with a Pasteur pipette.
10. Add 2 ml of diethyl ether to the lower aqueous layer, and vortex the mixture well. Centrifuge the mixture again at 1600 × *g* for 10 min at r.t. Transfer the resultant upper organic layer into the glass tube containing the upper organic layer collected in **step 9**.
11. Repeat **step 10**.
12. Evaporate the combined organic layer by nitrogen gas stream (*see* **Note 17**).
13. Add 800 μl of ethanol, and vortex the sample (*see* **Note 18**).
14. (Optional) Before the purification by preparative TLC (**steps 15–24**), it is possible to confirm successful synthesis of [^{14}C] palmitoylethanolamide by the following method. Spot 1 μl of the sample on an aluminum TLC sheet (10 cm height, described in Subheading 2.4, **item 11**). Also, spot ~50,000 cpm of [^{14}C] palmitic acid as a control beside the sample. Follow **steps 15–18** except that the development of the TLC sheet and the exposure of an imaging plate to the sheet are 20 min and 30–60 min, respectively. More than 95 % of [^{14}C]palmitic acid is typically converted to [^{14}C]palmitoylethanolamide, which migrates more slowly than [^{14}C]palmitic acid on the TLC sheet.
15. Draw an 18 cm horizontal line at 2 cm apart from the bottom edge on a glass TLC plate by a soft pencil, leaving 1 cm blanks at the both side edges (*see* Fig. 2a). Spot the entire sample along the line with a pipetman and a 200 μl tip under airflow by a hair dryer (*see* **Note 19**). After spotting, leave the plate under the airflow for a few minutes to evaporate the solvent completely.

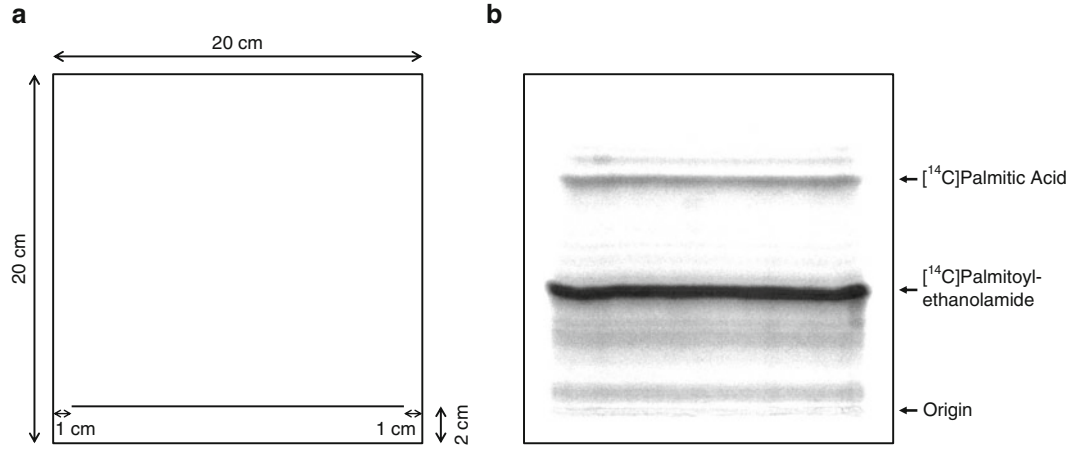

Fig. 2 Marking of TLC plate (a) and an example of thin-layer chromatogram (b) in the purification of the synthesized [^{14}C]palmitoylethanolamide

16. Immerse the lower edge (<2 cm) of the TLC plate in TLC solvent A placed in the TLC tank. Develop the plate at r.t. for 70 min. Take the plate out of the tank, and completely dry it up with airflow (*see* **Note 19**).
17. Wrap the TLC plate with a plastic film, and expose an imaging plate to the wrapped plate in a cassette for about 5 min (*see* **Note 20**).
18. Scan the plate by imaging analyzer FLA-7000 to see the distribution of the radioactivity (*see* Fig. 2b). The radioactive band of [^{14}C]palmitoylethanolamide roughly migrates up to half way of the plate. Print out the image to a paper sheet in its actual size.
19. Remove the plastic film from the TLC plate, and superimpose the plate exactly on the printed paper sheet on an illuminator. Mark the area on the plate that surrounds the [^{14}C]palmitoylethanolamide band with a soft pencil.
20. Scrape the marked area of silica gel from the plate with a chisel above a paraffin paper. Transfer the scraped silica gel to a Buchner funnel.
21. Hold the funnel with a clamp and a stand, and put a conical 10 ml glass tube under the funnel.
22. Elute [^{14}C]palmitoylethanolamide with methanol. First, add 2 ml of methanol to the funnel and crash the silica gel in methanol with a microspatula. Next, add 8 ml of methanol to the funnel. This step can be accelerated by applying a positive pressure with the aid of a rubber bulb with a reservoir.
23. Evaporate methanol by nitrogen gas stream (*see* **Note 17**).
24. Add 2 ml of ethanol, and vortex the sample (*see* **Note 21**).
25. To measure the radioactivity of [^{14}C]palmitoylethanolamide, dilute 5 μl of the obtained sample with 20 μl of ethanol. As in **step 1**, count the radioactivity in 5 μl of the diluted sample in triplicate with a liquid scintillation counter, and calculate the radioactivity per volume (cpm/μl) (*see* **Note 22**).
26. In order to prepare [^{14}C]palmitoylethanolamide with a specific radioactivity of 1000 cpm/nmol, mix 2×10^6 cpm of [^{14}C]palmitoylethanolamide and 50 μl of 40 mM non-radiolabeled palmitoylethanolamide in a conical 10 ml glass tube with a screw cap (*see* **Note 23**).
27. Evaporate the solvent ethanol by nitrogen gas stream (*see* **Note 17**). Add 1 ml of dimethyl sulfoxide, and vortex the solution well. [^{14}C]Palmitoylethanolamide at 10,000 cpm/10 nmol/5 μl used in Subheading 3.4 is now prepared (*see* **Note 24**). Store at −20 °C.

3.4 Enzyme Assay

1. In a 5 ml glass test tube, mix 20 μl of 0.5 M citrate–sodium phosphate buffer (pH 4.5), 5 μl of 60 mM dithiothreitol, 2.5 μl of 4 % Nonidet P-40, 5 μl of 1 % BSA, 5 μl of 3 M NaCl, 5 μl of dimethyl sulfoxide, water, and NAAA preparation (native or

recombinant NAAA prepared as described in Subheading 3.1 or 3.2, respectively) in a final volume of 95 μl (*see* **Note 25**).

2. Add 5 μl of [^{14}C]palmitoylethanolamide (10,000 cpm, 10 nmol, in dimethyl sulfoxide), and vortex the reaction mixture briefly (*see* **Notes 15** and **26**).
3. Incubate the reaction mixture for 30 min at 37 °C in a water bath.
4. Terminate the reaction by adding 320 μl of stop solution and vortexing (*see* **Note 26**).
5. Centrifuge the samples at 2000 × *g* for 5 min at 4 °C.
6. Draw an 18 cm horizontal line at 2 cm apart from the bottom edge on an aluminum TLC sheet by a soft pencil, leaving 1 cm blanks at both side edges. Draw short vertical lines at 1.5 cm interval across the horizontal line to form 12 lanes per sheet (*see* Fig. 3a).
7. Spot 100 μl of the upper ether layer on each lane along the horizontal line. Use a glass micropipette, which is connected to a rubber aspirator tube, under airflow by a hair dryer (*see* **Note 19**). After spotting all the samples, leave the plate under airflow for a few minutes to evaporate the solvent completely.

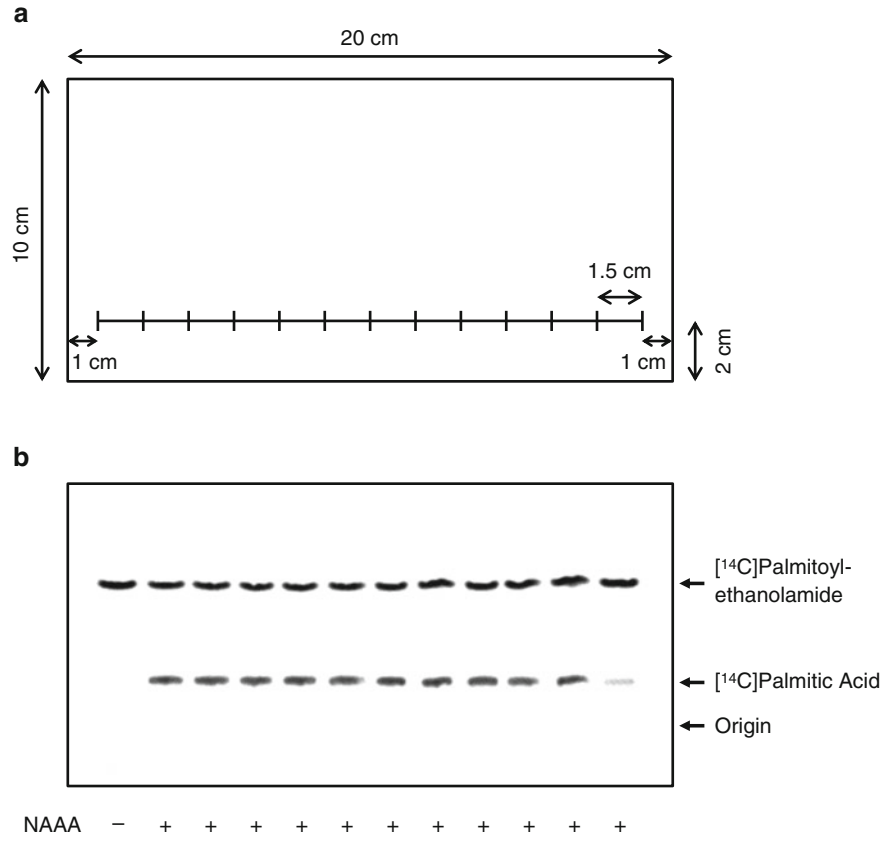

Fig. 3 Marking of TLC plate (a) and an example of thin-layer chromatogram (b) in NAAA assay

8. Immerse the lower edge (<2 cm) of the TLC sheet in TLC solvent B placed in the tank. Develop the plate at 4 °C for 20 min. Take the plate out of the tank, and completely dry it up under airflow (*see* **Note 19**).
9. Wrap the TLC sheet with a plastic film, and expose an imaging plate to the wrapped plate in a cassette for 6–18 h.
10. Scan the plate by imaging analyzer FLA-7000 to see the distribution of the radioactivity (*see* Fig. 3b). Quantify radioactivities of the produced [^{14}C]palmitic acid (a), and the remaining [^{14}C]palmitoylethanolamide (b), and calculate the conversion rate [$c = a/(a+b)$]. Based on the radioactivities of [^{14}C]palmitic acid (a') and [^{14}C]palmitoylethanolamide (b') obtained with an enzyme-free sample, estimate the background value [$c' = a'/(a'+b')$]. Subtract the background value from the conversion rate, and then calculate the enzyme activity [$10 \times (c - c')/30$ (nmol/min)] (*see* **Note 27**).

4 Notes

1. Any strain and age of rats, including retired rats, are eligible. Frozen lungs should not be used because some of NAAA may already be solubilized and may not be recovered in the pellet after centrifugation at 12,000 × *g*.
2. The poly-L-lysine solution is reusable. Store at 4 °C.
3. When recombinant NAAA is prepared as described in Subheading 3.2, cDNA of rat NAAA results in severalfold higher enzyme activity than cDNA of human NAAA. cDNA of mouse NAAA exhibits similar enzyme activity to that of human cDNA.
4. Oxalyl chloride is a corrosive respiratory irritant and lachrymator. It reacts violently with water. Store the bottle of this compound in a sealed 50 ml plastic tube at 4 °C.
5. At least 1–2 h is necessary to saturate the TLC tank with solvent vapors. In order to minimize evaporation of the solvent and change in its composition, put a 1 kg weight on the lid of the tank.
6. There is an obvious lot-to-lot difference in commercially available palmitoylethanolamide affecting the NAAA activity.
7. When an unsaturated *N*-[^{14}C]acylethanolamine is used as a substrate instead of [^{14}C]palmitoylethanolamide, it is recommended to include 5 mM 3(2)-*t*-butyl-4-hydroxyanisole in the stop solution as an antioxidant.
8. The rubber aspirator tube is supplied as an accessory of the glass micropipets.
9. Follow institutional guidelines for the care and use of animals.

10. Lungs of a retired rat weigh around 1.5 g. The capacity of the glass beaker should be 2–3 times larger than the volume of the homogenization buffer added in **step 2**.
11. To prevent temperature rising, take a pause (at least 30 s) between each homogenization or sonication with gentle mixing in the ice-water bath.
12. For effective recovery of the pellet, wash the used centrifuge tube with ice-cold PBS and then collect this PBS to the chamber.
13. The frozen sample can be stored for a long term in both of the freezing steps.
14. The cells may be maintained in the presence of 100 U/ml penicillin and 0.1 mg/ml streptomycin. However, these antibiotics should be removed before starting lipofection in **step 2**.
15. Perform all the steps handling radioactive materials in the controlled areas of radiation work.
16. Since the measured value of radioactivity is typically 50,000–60,000 cpm/vial, the radioactivity of original solution is calculated to be 250,000–300,000 cpm/μl.
17. This step should be done carefully in a safety cabinet. Use a Pasteur pipette connected to a nitrogen gas cylinder via a silicone tube. Since the temperature decreases during evaporation, the glass tube may be placed in a water bath around 25 °C to accelerate evaporation.
18. The sample can be stored at −20 °C at the end of this step.
19. Use normal-temperature airflow from a hair dryer held by a clamp and a stand. Hot airflow should be avoided.
20. In order to know the precise position of the radioactive band, the left and bottom edges of the imaging plate should be overlapped with those of the wrapped TLC plate.
21. White insoluble precipitates, if any, can be removed by low-speed centrifugation.
22. Usually, the yield is more than 80 %.
23. The amount of [^{14}C]palmitoylethanolamide is less than 1 % of non-radiolabeled palmitoylethanolamide and is negligible.
24. Other *N*-[^{14}C]acylethanolamines can also be prepared according to the present method, as long as the corresponding non-radiolabeled *N*-acylethanolamines are available. If not available, before starting the condensation with 2-aminoethanol, mix ^{14}C-labeled free fatty acid with the corresponding non-radiolabeled free fatty acid at the ratio to achieve the desired specific radioactivity and follow this method.
25. The final concentrations of each component are as follows: 100 mM citrate–sodium phosphate buffer (pH 4.5), 3 mM dithiothreitol, 0.1 % Nonidet P-40, 0.05 % BSA, 150 mM

NaCl, 100 μM [^{14}C]palmitoylethanolamide, and 10 % dimethyl sulfoxide. See **Note 27** concerning the amount of NAAA preparation. A master mix solution should be prepared for common components. To screen NAAA inhibitors, dissolve the candidate compound in 5 μl of dimethyl sulfoxide and add the solution to the reaction mixture. However, the final concentration of dimethyl sulfoxide should be always kept at 10 %. A FAAH inhibitor, URB597, should be added at 1–10 μM when coexisting FAAH cannot be ignored in NAAA preparation [3].

26. For multiple samples, each reaction should be started every 15 or 20 s by adding [^{14}C]palmitoylethanolamide and terminated at the same time interval by the stop solution.
27. For the precise measurement of the enzyme activity, the conversion rate (c) should be in the range of 0.01–0.20. Since both of the native and recombinant NAAA, prepared in Subheadings 3.1 and 3.2, respectively, are expected to exhibit the enzyme activities of around 6 nmol/min/mg of protein, the enzyme preparation around 11 μg of protein should achieve the conversion rate around 0.2.

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References

1. Ueda N, Tsuboi K, Uyama T (2013) Metabolism of endocannabinoids and related *N*-acylethanolamines: canonical and alternative pathways. FEBS J 280:1874–1894
2. Tsuboi K, Sun Y-X, Okamoto Y et al (2005) Molecular characterization of *N*-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. J Biol Chem 280: 11082–11092
3. Sun Y-X, Tsuboi K, Zhao L-Y et al (2005) Involvement of *N*-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other *N*-acylethanolamines in macrophages. Biochim Biophys Acta 1736: 211–220
4. Tsuboi K, Zhao L-Y, Okamoto Y et al (2007) Predominant expression of lysosomal *N*-acylethanolamine-hydrolyzing acid amidase in macrophages revealed by immunochemical studies. Biochim Biophys Acta 1771:623–632
5. Bandiera T, Ponzano S, Piomelli D (2014) Advances in the discovery of *N*-acylethanolamine acid amidase inhibitors. Pharmacol Res 86:11–17
6. Ueda N, Yamanaka K, Yamamoto S (2001) Purification and characterization of an acid amidase selective for *N*-palmitoylethanolamine, a putative endogenous anti-inflammatory substance. J Biol Chem 276:35552–35557
7. Petrosino S, Ahmad A, Marcolongo G et al (2015) Diacerein is a potent and selective inhibitor of palmitoylethanolamide inactivation with analgesic activity in a rat model of acute inflammatory pain. Pharmacol Res 91:9–14
8. Solorzano C, Zhu C, Battista N et al (2009) Selective *N*-acylethanolamine-hydrolyzing acid amidase inhibition reveals a key role for endogenous palmitoylethanolamide in inflammation. Proc Natl Acad Sci U S A 106:20966–20971
9. West JM, Zvonok N, Whitten KM et al (2012) Mass spectrometric characterization of human *N*-acylethanolamine-hydrolyzing acid amidase. J Proteome Res 11:972–981

Chapter 16

Assay of DAGLα/β Activity

Tiziana Bisogno

Abstract

The endocannabinoid 2-arachidonoylglycerol (2-AG) exerts its physiological action by binding to and functionally activating type-1 (CB_1) and type-2 (CB_2) cannabinoid receptors. It is thought to be produced through the action of *sn*-1 selective diacylglycerol lipase (DAGL) that catalyzes 2-AG biosynthesis from *sn*-2-arachidonate-containing diacylglycerols. Since 2-AG biosynthetic enzymes have been identified only recently, little information on methodological approaches for measuring DAGL activity is as yet available. Here, a highly sensitive radiometric assay to measure DAGL activity by using 1-oleoyl[1-^{14}C]-2-arachidonoylglycerol as the substrate is reported. All the steps needed to perform lipid extraction, fractionation by thin-layer chromatography (TLC), and quantification of radiolabeled [^{14}C]-oleic acid via scintillation counting are described in detail.

Key words 2-Arachidonoylglycerol, Diacylglycerol lipase, Radiometric assay, Thin-layer chromatography

1 Introduction

DAGL catalyzes the hydrolysis of *sn*-1-acyl-2-arachidonoylglycerol (DAG) to free fatty acid and 2-AG [1]. In tissues, 2-AG is much more abundant than any of the other endocannabinoids (eCBs) identified so far, and acts as a full agonist at CB_1 and CB_2 receptors [2, 3]. Two *sn*-1 DAG lipase isozymes, DAGLα and DAGLβ, have been cloned and enzymatically characterized [1]. DAGLα contains 1042 amino acids and is larger than DAGLβ (672 amino acids). They are mostly located in the plasma membrane, are stimulated by Ca^{2+} and glutathione, appear to possess a catalytic triad typical of serine hydrolases, and do not exhibit strong selectivity for 2-arachidonate-containing DAG [1]. The development of transgenic mice where the DAGL-encoding gene has been ablated confirmed that both DAGLα and DAGLβ regulate 2-AG production in vivo, where the relative contribution made by each enzyme depends on the type of tissue [4, 5]. In particular, DAGLα seems to be the principal regulator of 2-AG in the nervous system and plays an essential role in regulating retrograde synaptic plasticity and

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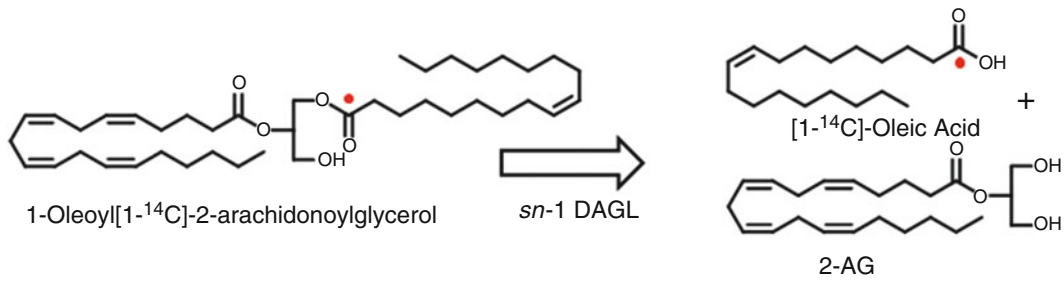

Fig. 1 *sn*-1 DAGL catalyzes [1-^{14}C]-oleic acid and 2-AG formation from 1-oleoyl[1-^{14}C]-2-arachidonoylglycerol

adult neurogenesis [4]. To date, several reports have documented alterations in time and space specificity of production and release of eCBs in relation to pathological conditions [6]. Since 2-AG biosynthetic enzymes have been identified only recently, little information on methodological approaches for measuring DAGL activities is as yet available. Several efforts have been made to fill this gap, culminating in the development of different analytical methodologies that include TLC [1, 7–9], liquid chromatography-mass spectrometry [10], fluorescence resonance energy transfer [11], activity-based protein profiling [12], and fluorescence spectroscopy [13]. Here, the protocol for assaying DAGL activity by means of the highly sensitive, standardized TLC radioassay that uses labeled 1-oleoyl[1-^{14}C]-2-arachidonoylglycerol as a substrate for both DAGLα and DAGLβ is presented (Fig. 1). This procedure has indeed allowed to discover and characterize these two enzymes, and to develop potent and selective inhibitors of their activity [1, 7–9].

2 Materials

Prepare and store all reagents at the suggested temperature, using ultrapure water or specific solvents when indicated. Carefully follow all regulations when disposing waste materials.

2.1 Enzymatic Assay Components

1. Assay buffer: 50 mM Tris–HCl, pH 7.4. Weigh 2.43 g Tris and transfer it to a glass beaker containing 300 ml of water. Mix and adjust pH with HCl (*see* **Note 1**). Make up to 0.4 L with water. Store at 4 °C.
2. Glass homogenizer (Dounce).
3. Centrifuge tubes.
4. 1-Oleoyl[1-^{14}C]-2-arachidonoylglycerol (50–60 mCi/mmol, or 1.85–2.22 GBq/mmol) (American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA): Make a stock solution by taking 0.1 μCi (2 μl) 1-oleoyl[1-^{14}C]-2-arachidonoylglycerol and transferring it to a 0.5 ml tube containing 100 μl of acetonitrile. Use 5 μl of solution to count the radioactivity in a β-counter (*see* **Note 2**). Prepare your working solution by

transferring the required amount of 1-oleoyl[1-^{14}C]-2-arachidonoylglycerol to 2 ml tube containing Tris–HCl buffer up to a concentration of about 250 dpm/μl. Store stock solution at −20 °C and working solution on ice.

5. Stock solution of 10 mM 1-oleoyl-2-arachidonoylglycerol: Weigh 1 mg 1-oleoyl-2-arachidonoylglycerol (*see* **Note 3**), and transfer to a 0.5 ml tube containing 0.156 ml of acetonitrile. Prepare your 125 μM working solution by transferring 12.5 μl of 10 mM stock solution to 2 ml tube containing 1 ml of Tris–HCl. Store stock solution at −20 °C and working solution on ice.
6. Bradford protein assay kit (commercially available).
7. Two milliliter tubes with safe lock (e.g., Eppendorf type).
8. Vortex.
9. Water bath.
10. Chloroform/methanol (2:1, vol/vol) solution: Add 10 ml of chloroform to a 20 ml cylinder, and then add methanol up to 15 ml (*see* **Note 4**). Transfer in a glass conical flask with glass stopper. Store on ice.

2.2 Components for TLC

1. TLC (20 × 20 cm) Silica gel 60 F_{254}.
2. Pencil.
3. Standard solution of 10 mM arachidonic acid (AA): Weigh 2 mg AA and transfer it to a glass vial containing 0.658 ml of methanol (*see* **Note 5**). Store at −20 °C.
4. Chloroform/methanol (9:1, vol/vol) solution (*see* **Note 4**).
5. Chloroform/methanol/ammonium hydroxide (85:15:0.1, vol/vol/vol) solution (*see* **Note 6**).
6. Developing iodine chamber (*see* **Note 7**).
7. Chromatography tanks.
8. Scissors.
9. Tweezers.
10. Ruler.
11. Twenty milliliter liquid scintillation vials with screw cap.
12. Liquid scintillation counter.

3 Methods

3.1 Enzymatic Assay

1. Transfer tissues in a glass homogenizer, add assay buffer (*see* **Note 8**), and then homogenize (*see* **Note 9**).
2. Transfer homogenates to centrifuge tubes and centrifuge at 4 °C sequentially at 800 × *g* for 5 min (to remove insoluble debris) and at 12,000 × *g* for 25 min (*see* **Note 10**).

3. Gently remove the supernatant.
4. Add assay buffer to redissolve the pellet (*see* **Note 11**).
5. Determine protein concentration by means of Bradford protein assay (or similar), following the manufacturer's instructions.
6. Prepare 1 mg/ml protein solution in assay buffer. Store on ice.
7. Mix 0.1 ml of 1-oleoyl[1-^{14}C]-2-arachidonoylglycerol working solution, 0.1 ml of 1-oleoyl-2-arachidonoylglycerol working solution, 0.2 ml of assay buffer, and 0.1 ml of protein solution in a 2 ml tube (*see* **Note 12**). Close the cap, shake, and incubate at 37 °C for 20 min in a preheated water bath (*see* **Note 13**).
8. After the incubation, extract lipids with 2 volumes of chloroform/methanol (2:1) solution. Add 1 ml of chloroform/methanol (2:1) solution to each tube, and shake vigorously with a vortex (*see* **Note 14**).
9. Centrifuge at 10,000 × *g* for 2 min, and transfer the organic phase in a clean 2 ml tube (*see* **Note 15**).
10. Evaporate the organic phase under a stream of gaseous nitrogen or under vacuum (*see* **Note 16**).

3.2 TLC Preparation and Development

1. Chromatography tank with the mobile phase: Use chloroform/methanol/ammonium hydroxide (85:15:0.1) solution as eluting system, and pour it into the chromatography tank, that is the developing chamber. Cover the chamber to saturate the inside with solvent vapor, swirl it gently, and allow it to stand while you prepare TLC plate (*see* **Note 17**).
2. Measure 2 cm from the bottom of the plate (Fig. 2a). Using a pencil, draw two marks on both edges of the plate at 2 cm (*see* **Note 18**). This is the origin: the line on which you will spot the plate. Leave 1 cm from both edges of the plate, draw a 2 cm wide lane (at 2 cm from the bottom of the plate), and under it lightly mark the name "AA standard." Starting from the left edge of the plate, leave 1.5 cm from the spot of AA standard and draw another 2 cm wide lane. Lightly mark the name of the sample you will spot on this plate. Repeat this procedure for as many samples as you have to load (Fig. 2a). The space between different samples prevents them from overlapping during the run (*see* **Note 19**).
3. Spot 15 μl of AA standard solution at the proper location of the TLC plate (*see* **Note 20**).
4. Dissolve each sample in 30 μl of chloroform/methanol (9:1) solution, and then spot samples on the TLC plate (*see* **Note 20**).
5. Place the TLC plate in the developing chamber, cover the chamber, and leave it undisturbed on the bench (*see* **Note 21**). Allow the plate to develop until the solvent is about 1 cm below the top of the plate (Fig. 2b).
6. Remove the plate from the developing chamber and immediately mark the solvent front with a pencil (*see* **Note 22** and Fig. 2b). Next, let the solvent evaporate completely.

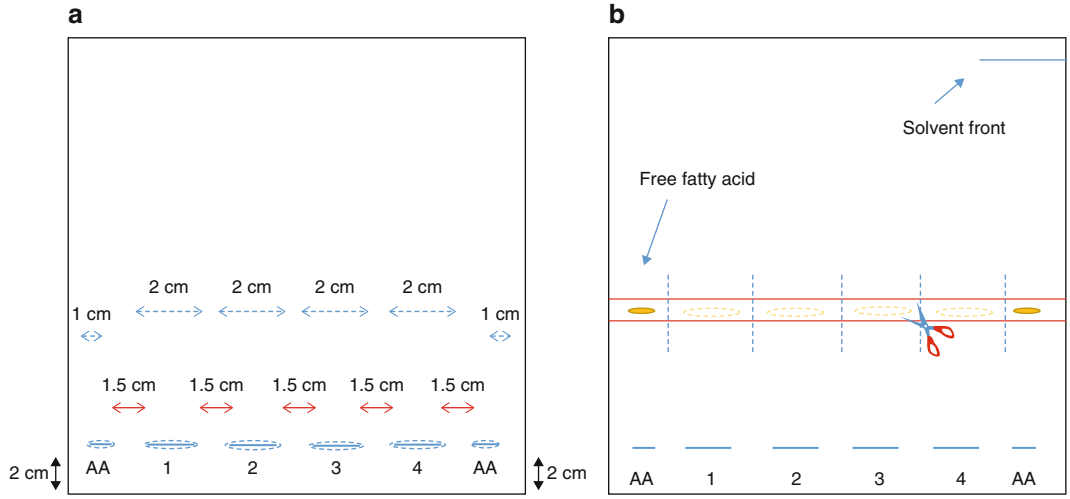

Fig. 2 TLC chromatogram. (a) Preparation and spotting of TLC plate; (b) chromatogram developed with chloroform/methanol/ammonium hydroxide (85:15:0.1, vol/vol/vol), and then visualized with iodine

7. Stain the TLC plate by inserting it in a chamber previously saturated with iodine vapor.
8. Allow TLC to remain in the iodine vapor chamber until the AA standard appears as a dark yellow-brown spot (*see* **Note 23**).
9. Carefully remove the TLC plate from the chamber, and gently circle the spots with a pencil (Fig. 2b).
10. Allow iodine to fully evaporate from the TLC in a chemical hood.
11. Draw two horizontal lines across the whole length of the plate, above and below the circled spot of AA. Draw two vertical lines across the whole length of the plate, both on the right and the left side of each sample (*see* **Note 24**).
12. Use scissors and carefully cut bands (rectangles) corresponding to the position of the AA standard in each sample (*see* **Note 25**).
13. Use tweezers to transfer rectangles into 20 ml scintillation vials.
14. Add 2.5 ml of methanol and shake vigorously.
15. Add 10 ml of scintillation cocktail and count the radioactivity in a β-counter.

4 Notes

1. Having water at the bottom of the beaker helps to dissolve Tris quite easily, because the magnetic stir bar can work immediately. HCl (12 or 6 N) can be used to narrow the gap between the starting pH and the required pH. However, a sudden pH drop below the required pH should be avoided.
2. This procedure must be performed in a radiochemistry laboratory.

3. 1-Oleoyl-2-arachidonoylglycerol is not commercially available, and should be obtained through collaborations, or it should be synthesized as detailed elsewhere [1]. Briefly, 1-oleoyl-2-arachidonoyl-*sn*-glycerol is obtained from the *R*(−)solketal esterified with oleic acid, using *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride/4-dimethylaminopyridin, and deprotecting the acetonide with hydrochloride/methanol. The primary alcoholic group is selectively protected with triisopropylsilyl chloride, whereas the free secondary alcohol is esterified with AA. Finally, the 1-oleoyl-2-arachidonoylglycerol is obtained by selectively removing the sylyl group with tetrabutylammonium fluoride/acetic acid.
4. In order to make the meniscus better visible, place a white card with a black mark behind the cylinder. Align the black mark so that it is just under the meniscus, and get your eye level with the bottom of the meniscus.
5. AA is a colorless oil at room temperature, and weighting 2 mg AA by using an analytical balance might be quite difficult. Yet, AA density is 0.922 g/ml at 25 °C; therefore the best way to prepare a 10 mM solution is to take 2 μl of AA with a micropipette and then transfer it to a glass vial containing 0.6 ml of methanol. Usually AA is delivered in glass ampules that should be spun slowly to allow all oil to reach the bottom.
6. Mix chloroform and methanol in a 100 ml cylinder (*see* **Note 4**), and then add 100 μl of ammonium hydroxide by means of a micropipette.
7. A iodine chamber may be assembled as follows: add to a wide-mouth jar (with cap) a piece of filter paper and few crystals of iodine. The latter have a high vapor pressure, and the chamber will rapidly become saturated with iodine.
8. It is best to add a volume of Tris–HCl that is 20 times that of the tissue sample.
9. Press the pestle on to the sample with a twisting motion. The piston is moved up and down while twisting, to allow exposure of all sample sides to grinding. After the pestle has been turned 360°, it is slowly removed from the tube (a strong vacuum helps to create shearing forces). This procedure must be repeated for at least 20 times on ice.
10. Balance the tubes in the rotor by mass, not by volume. Balancing the masses to the nearest 0.1 g is advised.
11. Avoid to dilute protein solution. Use as little buffer as possible to resuspend the pellet and to obtain a homogeneous protein solution.
12. It is best to add protein solution as the last step. At this time the enzymatic reaction starts.

13. Use only high-quality tubes. Make sure that the cap is closed properly, otherwise you might lose radioactive material and contaminate the surrounding environment.
14. This step allows to denature proteins, to stop the enzymatic reaction and to extract lipids in the organic phase. Therefore, vigorous shaking is advised.
15. Centrifugation of the mixture yields a good separation of the two phases (the lower organic phase and the upper aqueous phase), while proteins remain at the interface between them.
16. If necessary, place parafilm around the tube cap and store at −20 °C until the day of TLC analysis. Keep samples at 20 °C for no longer than 24 h.
17. Whenever you run a TLC, make sure that there is a lid on the TLC chamber (that is the container that holds the TLC plate). Otherwise, the vapor pressure of the TLC solvent will be too low, and too much time will be needed to run TLC. To further aid saturation with solvent vapors, the inside of the TLC chamber can be lined with filter paper.
18. Never use a pen, and be careful not to scratch the surface of the plate. Indeed, you could disturb the thin layer of solid adsorbent, silica.
19. Leave enough space between the samples, so that they do not overlap and do not spread laterally; six samples (including AA standard) can be accommodated in a 20 cm wide plate.
20. Do not allow the spot to become too large. If necessary, you can blow on the spot and repeating this action will keep the wet area on the plate very small.
21. When you place the TLC plate in the chamber, the level of the eluting solution must be below the spots. Otherwise, these will spread unpredictably over the plate, and results will be unreliable. If this happens, you have to prepare a new plate and stat all over again.
22. Prevent the solvent from running over the edge of the plate.
23. AA is a polyunsaturated fatty acid that has a good affinity for iodine, and is easily visualized. Therefore, in our hands it is the best standard to use.
24. Be careful to enclose the samples into rectangles of identical size. Also be careful not to scratch the surface of the plate, because you could disturb the silica layer and lose your samples.
25. Pay attention when using scissors. Search for a pair of scissors that fits your hand. Cut the rectangle by rotating the plate without removing scissors from it. Wear a mask to avoid breathing silica. Also be aware that silica might be easily detached from the plate, and therefore you could lose your sample.

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References

1. Bisogno T, Howell F, Williams G et al (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. J Cell Biol 163:463–468
2. Mechoulam R, Ben-Shabat S, Hanus L et al (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol 50:83–90
3. Sugiura T, Kondo S, Sukagawa A et al (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:89–97
4. Gao Y, Vasilyev DV, Goncalves MB et al (2010) Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. J Neurosci 30:2017–2024
5. Tanimura A, Yamazaki M, Hashimotodani Y et al (2010) The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission. Neuron 65:320–327
6. Di Marzo V (2008) Targeting the endocannabinoid system: to enhance or reduce? Nat Rev Drug Discov 7:438–455
7. Bisogno T, Cascio MG, Saha B et al (2006) Development of the first potent and specific inhibitors of endocannabinoid biosynthesis. Biochim Biophys Acta 176:205–212
8. Bisogno T, Burston JJ, Rai R et al (2009) Synthesis and pharmacological activity of a potent inhibitor of the biosynthesis of the endocannabinoid 2-arachidonoylglycerol. ChemMedChem 4:946–950
9. Bisogno T, Mahadevan A, Coccurello R et al (2013) A novel fluorophosphonate inhibitor of the biosynthesis of the endocannabinoid 2-arachidonoylglycerol with potential anti-obesity effects. Br J Pharmacol 169:784–793
10. Jung KM, Astarita G, Thongkham D et al (2011) Diacylglycerol lipase-alpha and -beta control neurite outgrowth in neuro-2a cells through distinct molecular mechanisms. Mol Pharmacol 80:60–67
11. Johnston M, Bhatt SR, Sikka S et al (2012) Assay and inhibition of diacylglycerol lipase activity. Bioorg Med Chem Lett 22: 4585–4592
12. Hoover HS, Blankman JL, Niessen S et al (2008) Selectivity of inhibitors of endocannabinoid biosynthesis evaluated by activity-based protein profiling. Bioorg Med Chem Lett 18:5838–5841
13. Van der Wel T, Janssen FJ, Baggelaar MP et al (2015) A natural substrate-based fluorescence assay for inhibitor screening on diacylglycerol lipase α. J Lipid Res 56:927–935

Chapter 17

Assay of Monoacylglycerol Lipase Activity

Kwang-Mook Jung and Daniele Piomelli

Abstract

Monoacylglycerol lipase (MGL) is a serine hydrolase involved in the biological deactivation of the endocannabinoid 2-arachidonoyl-*sn*-glycerol (2-AG). 2-AG is one of the main endogenous lipid agonists for cannabinoid receptors in the brain and elsewhere in the body. In the central nervous system (CNS), MGL is localized to presynaptic nerve terminals of both excitatory and inhibitory synapses, where it helps control the regulatory actions of 2-AG on synaptic transmission and plasticity. In this chapter, we describe an in vitro method to assess MGL activity by liquid chromatography/mass spectrometry (LC/MS)-based quantitation of the reaction product. This method may be used to determine the basal or altered MGL activity in various cells or animal tissues after pharmacological, genetic, or biological manipulations. In addition, this assay can be used for MGL inhibitor screening using purified recombinant enzyme or MGL-overexpressing cells.

Key words Monoacylglycerol lipase (MGL), Enzyme assay, 2-Arachidonoyl-*sn*-glycerol (2-AG), Arachidonic acid, Liquid chromatography/mass spectrometry (LC/MS)

1 Introduction

Monoacylglycerol lipase (MGL, also abbreviated MAGL or MGLL; EC 3.1.1.23, acylglycerol lipase), a member of the α/β-hydrolase domain (ABHD) family of serine hydrolases, catalyzes the conversion of long-chain monoglycerides (monoacylglycerols) into free fatty acids and glycerol (Fig. 1). The *MGLL* gene in humans encodes a 33 kDa protein composed of 303 amino acids (NCBI accession number: NP_001003794), which is responsible for the majority of MGL activity found in mammalian tissues [1, 2]. Other mammalian proteins contributing to this activity are ABHD6 and ABHD12 [3, 4].

MGL contains the GXSXG consensus motif common to most serine hydrolases, and harbors a catalytic triad composed of serine, aspartate, and histidine residues (Ser122-Asp239-His269 in human MGL) [1, 2]. Crystal structures (Protein Data Bank accession codes: 3HJU and 3JW8) indicate the presence of a canonical α/β-hydrolase fold characterized by a central β-sheet surrounded

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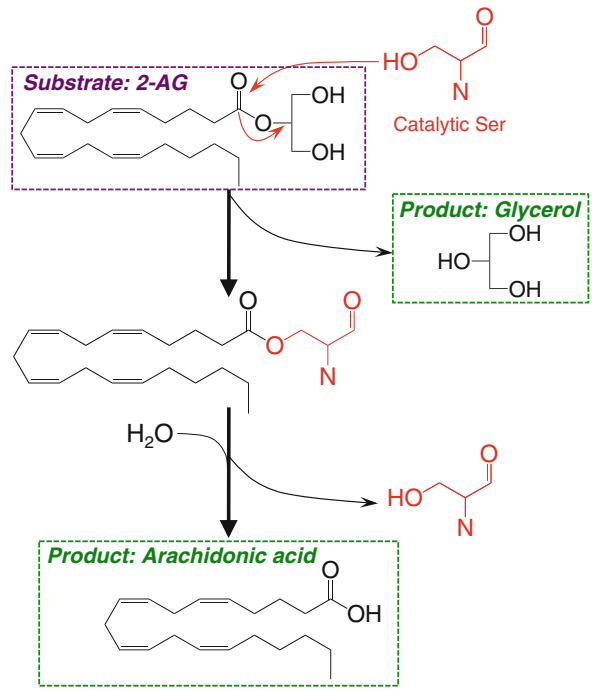

Fig. 1 MGL catalyzes the conversion of monoacylglycerols into free fatty acids and glycerol. In this figure, 2-AG (monoarachidonoylglycerol) is converted to free arachidonic acid and glycerol by MGL. The catalytic serine residue in the MGL active site is shown in *red*

by six α-helices (α1, α2, α3, α6, α7, and α8). In addition, α-helices α4, α5, and α6 form an U-shaped cap (or "lid") domain that is likely to open upon interfacial activation allowing access of substrates to the enzyme's active site [5, 6].

MGL is highly expressed in the central nervous system (CNS) [1, 2, 7], where it is the primary enzyme responsible for the hydrolytic degradation of the endocannabinoid, 2-arachidonoyl-*sn*-glycerol (2-AG), into free arachidonic acid and glycerol [8–11] (Fig. 1). Approximately 85 % of the 2-AG-hydrolyzing activity found in the rodent brain is attributable to this protein [1, 3, 12, 13]. MGL is also present in peripheral tissues where, in addition to degrading 2-AG, it completes the hydrolysis of triacylglycerols initiated by hormone-sensitive lipase and triacylglycerol lipase [14].

Earlier methods to measure MGL activity employed radioactive substrates such as 2-[^{3}H]arachidonoylglycerol and thin-layer chromatographic separation, to assess radioactivity in breakdown products [1]. Advances in liquid chromatography/mass spectrometry (LC/MS) technology and greater availability of LC/MS instruments have contributed to the development of innovative methods for lipid analyses with greatly improved sensitivity, fidelity,

and accuracy [15, 16]. Here, we describe an LC/MS-based MGL assay that is routinely used in our laboratory [11, 13, 17]. The protocol includes details on enzyme preparation, lipid extraction, LC/MS analysis, and data processing.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. LC-grade solvents must be used for LC/MS analyses.

2.1 Reagents

2.1.1 Lipids

2-Oleoylglycerol (2-monoolein, 2-OG) (Sigma-Aldrich, St. Louis, MO, USA), heptadecanoic acid, and oleic acid (Nu-Chek Prep, MN, USA).

2.1.2 Buffers and Growth Medium

1. MGL homogenization buffer: 50 mM Tris–HCl, pH 8.0. Prepare 10× MGL homogenization buffer (0.5 M Tris–HCl, pH 8.0) and dilute to 1× when needed. For the 10× solution, weigh 60.57 g Tris and transfer to a glass graduated flask containing approximately 900 ml of water. Mix and adjust pH to 8.0 by adding HCl drop by drop. Add water to adjust the volume to 1 l. Store at 4 °C.
2. MGL reaction buffer: 50 mM Tris–HCl, pH 8.0, 0.05 % fatty acid-free bovine serum albumin (BSA). Weigh 25 mg of fatty acid-free BSA and dissolve them in 50 ml of 1× MGL Homogenization buffer. Store at 4 °C for 1–2 weeks.
3. 10× substrate solution: 0.1 mM 2-OG. Prepare just before use (*see* **Note 1**).
4. Stop solution: 100 % methanol containing internal standard (5 nmol heptadecanoic acid per sample). Prepare just before use (*see* **Note 2**).
5. Complete growth medium for HeLa cells: Dulbecco's modified Eagle medium plus 10 % fetal bovine serum and penicillin/streptomycin.

2.1.3 Solvents and Chemicals

1. Mobile phase A: Methanol, 0.25 % acetic acid, 5 mM ammonium acetate.
2. Mobile phase B: Water, 0.25 % acetic acid, 5 mM ammonium acetate.

2.2 Equipment

1. Tissue homogenizer.
2. Vortex mixer.
3. Water bath (37 °C).
4. Low-speed refrigerated centrifuge.
5. Spectrophotometer.

6. LC/MS system equivalent to or higher than Agilent 1200-LC system with autosampler linked to Ion Trap XCT or single-quadrupole 1946D MS detectors, and interfaced with ESI or APCI (Agilent Technologies, Wilmington, DE).
7. High-purity N_2 gas for sample drying.
8. Chemical fume hood (*see* **Note 3**).

2.3 Supplies and Apparatuses

1. Glass tubes (13 × 100 mm) or glass vials (8 ml) with Teflon liner cap.
2. Glass vials (1.5 ml) for LC/MS autosampler and conical inserts.
3. Glass pipettes and pipette aid.
4. (Micro)centrifuge tubes, micropipettes, and pipette tips.
5. Vial and tube racks.
6. 5′ Glass Pasteur pipettes. Plug with sterile hydrophilic cotton.
7. LC column: Reverse-phase Zorbax XDB Eclipse C18 column (50 × 4.6 mm i.d., 1.8 μm, Agilent Technologies) or equivalent.

3 Methods

3.1 Enzyme Preparation

Comparative MGL assays can determine the basal or altered MGL activity in cells or in animal tissues under various conditions. Also, in vitro MGL activity assays can be performed to screen MGL inhibitors using MGL-overexpressing cells or purified recombinant enzyme (*see* **Note 4**). The following is a general protocol for enzyme preparation, which can be adapted by individual laboratories.

3.1.1 Cell Homogenate

1. Culture cells in appropriate tissue culture dishes, and treat them with test drug(s) if required.
2. On the day of harvesting cells, prepare an ice bucket, micro-centrifuge tubes, glass tubes, and/or glass vials. Label tubes and vials appropriately.
3. Aspirate/discard the cultured media and wash cell cultures twice with a sufficient volume of ice-cold phosphate-buffered saline (PBS). Remove PBS completely.
4. Add ice-cold MGL homogenization buffer (1 ml per a 100 mm culture dish). Scrape and collect cells in (micro)centrifuge tubes.
5. Homogenize the cells using a tissue homogenizer or sonicator on ice. Settings may vary depending on the system (*see* **Note 5**). Be careful to prevent samples from heating up during homogenization.
6. Centrifuge samples for 10 min at 1000 × *g* at 4 °C. Carefully collect the supernatant into clean tubes.

7. Determine protein concentration of the supernatant using the bicinchoninic acid (BCA) assay or Bradford protein assay, with BSA as a standard (*see* **Note 6**).

3.1.2 Tissue Homogenate

1. Collect the tissues of interest. If the tissues are going to be processed right away, keep them in cold MGL homogenization buffer on ice. Otherwise, snap-freeze them after sacrifice using liquid N_2 or powdered dry ice.
2. Place the (frozen) tissues into 10 tissue volumes of MGL homogenization buffer in an appropriate tube.
3. Homogenize the tissues using a tissue homogenizer or sonicator on ice. Be careful to prevent samples from heating up during homogenization. Settings may vary depending on the homogenization system and the amount and nature of tissue (*see* **Note 5**).
4. Centrifuge the samples for 10 min at $1000 \times g$ at 4 °C. Carefully collect the supernatant into clean tubes.
5. Determine protein concentration of the supernatant by using the BCA assay or Bradford protein assay with BSA as a standard (*see* **Note 6**).

3.1.3 Homogenate of MGL-Overexpressing HeLa Cells

1. The day before transfection, detach HeLa cells from culture dishes by trypsin treatment and count them. Plate cells into 100 mm dishes using complete growth medium. The optimal number of cells to be plated varies depending on the transfection method.
2. Transfect the cells with plasmid DNA encoding MGL under a strong mammalian promoter. Our laboratory uses vectors encoding rat MGL under the human EF-1α promoter [17].
3. Incubate the cells at 37 °C in a CO_2 incubator for 24–72 h after transfection.
4. Prepare cell homogenate according to Subheading 3.1.1.
5. Confirm MGL expression by Western blotting or other methods (*see* **Note 7**).

3.2 Setting Up the Enzyme Reaction

The final reaction consists of 50 mM Tris–HCl, pH 8.0, 0.05 % fatty acid-free BSA, an appropriate amount of enzyme, and 10 μM 2-OG, in a total reaction volume of 0.5 ml. If required, MGL can be pre-incubated with test drugs for 10 min, alongside an appropriate vehicle. MGL activity is unaltered by dimethyl sulfoxide (DMSO) at concentrations ≤1 %.

1. Design the experiment. Determine the volume of enzyme source (χ μl) that will be used for the assay, referring to the protein quantitation results (*see* **Note 8**). Run all reactions in triplicate. Include a blank triplicate assayed without any enzyme for blank subtraction.

2. Label glass tubes (13 × 100 mm) or glass vials (8 ml). Place them on ice.
3. Add (450 − χ) μl of MGL reaction buffer and χ μl of enzyme source.
4. If testing a drug, add it to the mixture, vortex gently, and incubate in a 37 °C water bath for 10 min.
5. Quickly add 50 μl of 10× substrate, vortex, and incubate at 37 °C for 30 min.

3.3 Lipid Extraction

1. Quench the reaction by adding 1 ml of Stop solution to each sample.
2. Add 2 ml of chloroform.
3. Vortex for about 60 s.
4. Add 0.5 ml of water.
5. Vortex for about 60 s.
6. Centrifuge the samples at 4 °C, 2000 × *g* for 15 min. After centrifugation, the mixture should be separated into two phases with a whitish protein disk at the interface. The lower phase is mainly chloroform and contains most of the lipids; the upper phase is methanol and water containing more polar metabolites.
7. Prepare another set of 8 ml glass vials with the same labeling.
8. Collect the lower (organic) phase using a glass Pasteur pipette attached to a pipette aid (do not pipette by mouth!). Carefully transfer the organic phase to a new 8 ml glass vial (*see* **Note 9**). Discard the protein disk and the upper (aqueous) phase (*see* **Note 10**).
9. Evaporate the solvent to dryness in the vials with a gentle N_2 stream (*see* **Note 11**).
10. Resuspend the dried lipids in 0.1 ml of chloroform:methanol (1:3, vol/vol) mixture.
11. Transfer them into 1.5 ml glass LC vials with 0.2 ml conical inserts and proceed to LC/MS analysis.

3.4 LC/MS Analysis

Fatty acids are identified based on their retention times and MS properties.

1. Set up the column and the LC/MS parameters. We use a reverse-phase Zorbax XDB Eclipse C18 column (50 × 4.6 mm i.d., 1.8 μm, Agilent Technologies). Detection and analysis are performed using Agilent Chemistation and Bruker Daltonics software.
2. Lipids are eluted with a linear gradient from 90 to 100 % of A in B for 2.5 min at a flow rate of 1.5 ml/min with the column

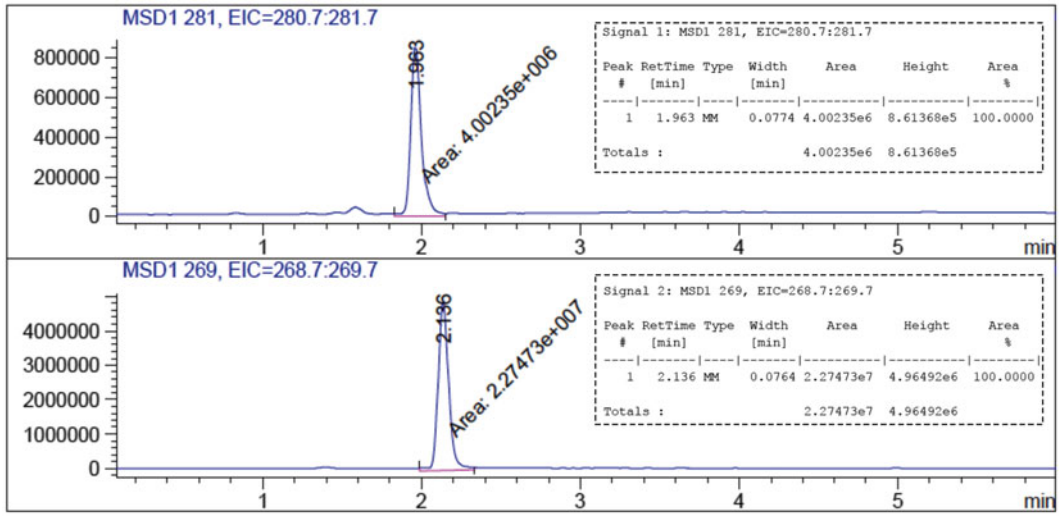

Fig. 2 Representative LC/MS chromatograms of oleic acid and heptadecanoic acid. Signals from LC/MS are extracted for oleic acid ($m/z = 281$) and heptadecanoic acid ($m/z = 269$). *Insets* show the peak integration summary report

temperature at 40 °C. ESI is in the negative mode, capillary voltage is set at 4 kV, and the fragmentor voltage is 100 V. N_2 is used as a drying gas at a flow rate of 13 l/min and a temperature of 350 °C. Nebulizer pressure is set at 60 psi. Fatty acids are measured by monitoring the mass-to-charge ratio (m/z) of deprotonated molecular ions $[M–H]^-$ in selected ion monitoring mode.

3. Extract chromatograms for oleic acid and heptadecanoic acid from LC/MS runs: For oleic acid, $m/z = 281$, and for heptadecanoic acid, $m/z = 269$. Figure 2 shows a representative LC/MS chromatogram, where the integrated peak area can be obtained using the LC/MS software.

3.5 Calculation of MGL Activity

The fatty acid product of MGL activity in this assay, oleic acid ($18{:}1\Delta^9$ FA), is quantified using the internal standardization method. Briefly, the method consists in adding known amounts of a structurally related internal standard to the sample under analysis. Unlike traditional analytical methods that rely on signal intensity, this method employs signal ratios.

In the current method, a fixed amount of standard heptadecanoic acid (17:0 FA) is added to the sample immediately following the incubation. Extracted chromatograms for oleic acid and heptadecanoic acid from LC/MS analyses are used to obtain the integrated peak area and the ratio of oleic acid to heptadecanoic acid for each sample. Then, the amount of oleic acid is determined based on a standard curve that is generated from mixtures of oleic acid and heptadecanoic acid with known ratios. This protocol is

very similar to the isotope dilution method that is commonly used to determine the quantity of endocannabinoids such as 2-AG and anandamide [15].

3.5.1 Standard Curve

1. Prepare 1 mM heptadecanoic acid and 1 mM oleic acid in a chloroform:methanol (1:3, vol/vol) mixture. Mix equal parts of 1 mM heptadecanoic acid and 1 mM oleic acid, to make the standard for 5 nmol oleic acid.
2. Dilute 1 mM oleic acid twofold with a chloroform:methanol (1:3, vol/vol) mixture to make 0.5 mM oleic acid solution. Mix equal parts of 1 mM heptadecanoic acid and 0.5 mM oleic acid, to make standard for 2.5 nmol oleic acid.
3. Dilute 0.5 mM oleic acid twofold with chloroform:methanol (1:3, vol/vol) mixture to make 0.25 mM oleic acid solution. Mix equal parts of 1 mM heptadecanoic acid and 0.25 mM oleic acid, to make standard for 1.25 nmol oleic acid.
4. Repeat twofold serial dilution of oleic acid and mix with 1 mM heptadecanoic acid to obtain (in nmol) 0.625, 0.3125, 0.15625, 0.078125, 0.0390625, and 0.01953125 standards.
5. Run each standard in duplicate by LC/MS. Generate a standard curve using the average peak area of the duplicates of each standard. The *X*-axis is the quantity (nmol) of oleic acid and the *Y*-axis is the ratio of oleic acid to heptadecanoic acid (Fig. 3) (*see* **Note 12**).

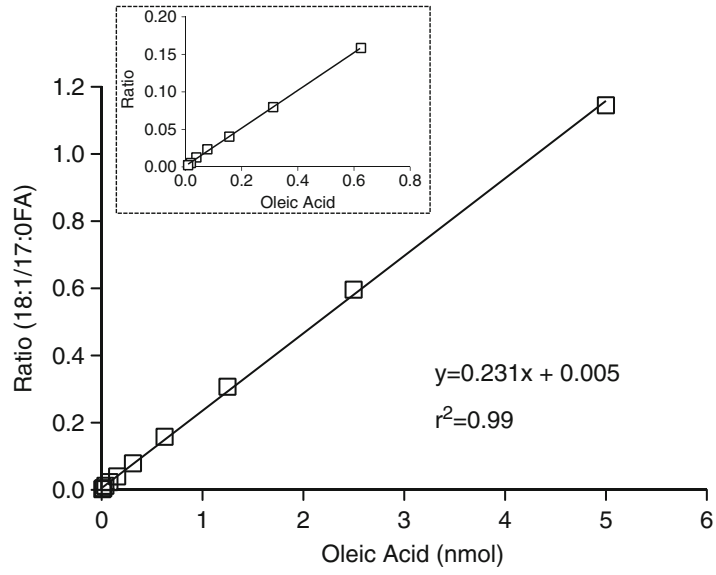

Fig. 3 A representative standard curve. An example standard curve for oleic acid quantitation using 5 pmol heptadecanoic acid as an internal standard is shown here. The result from a linear regression analysis of the data is also displayed. *Inset*: Magnification of the same graph in the range of 0–0.625 nmol oleic acid. 18:1 FA, oleic acid; 17:0 FA, heptadecanoic acid

3.5.2 Calculations

1. Using the peak area of samples obtained in Subheading 3.4, **step 3**, determine the ratio of oleic acid to heptadecanoic acid for each sample. Then, calculate the quantity of oleic acid by entering this ratio into the standard curve.
2. Calculate the quantity of oleic acid from the blank samples. Subtract the average of the blank samples from each sample run.
3. Normally, enzyme specific activity can be displayed as the amount of product formed per unit time and protein. In the current assay, MGL-specific activity is calculated as follows:

$$\text{specific activity (nmol/[min mg of protein])} = \text{oleic acid (nmol)} \times 1000/\mu\text{g protein added to reaction} \times 30.$$

4 Notes

1. First, prepare 10 mM 2-OG stock solution in DMSO and divide it into small aliquots (10–100 µl). Store at –20 °C. Avoid repeated freeze-thaw. Just before use, dilute the aliquot 100 times with MGL reaction buffer (e.g., add 10 µl of 10 mM 2-OG to 990 µl MGL reaction buffer) and vortex to make 10× substrate solution.
2. First, prepare 10 mM heptadecanoic acid stock solution in chloroform in a glass vial with Teflon liner cap. Protect from light and store at –20 °C. Just before use, warm the 10 mM heptadecanoic acid stock solution at room temperature for about 5 min, and vortex. Prepare the Stop solution by adding the appropriate amount of 10 mM heptadecanoic acid stock solution to methanol, according to the number of samples to be analyzed (e.g., for ten samples, add 5 µl of 10 mM heptadecanoic acid stock solution in 10 ml of cold methanol). Keep on ice until use.
3. All procedures involving the use of chloroform should be handled in a chemical fume hood.
4. Our laboratory uses the pET15b vector system (Novagen, La Jolla, CA, USA) that produces rat MGL protein with an N-terminal 6x histidine tag. MGL is over-expressed in Rosetta 2(DE3)pLysS *E. coli* cells (Novagen), by using isopropyl-β-D-thiogalactopyranoside (IPTG) induction, and is purified from the 1 % Triton X-100-soluble fraction using a TALON column (Clontech, Mountain View, CA). For additional details, refer to our previous publication [13].
5. We use a Branson probe-type sonicator (Digital Sonifier 250, Branson Ultrasonics, Danbury, CT, USA) for cell homogenization, and a Polytron homogenizer (PT 6100, Kinematica AG, Luzern, Switzerland) for tissue homogenization. For example, we use 1-s pulse on-1-s pulse off, 10-s total running

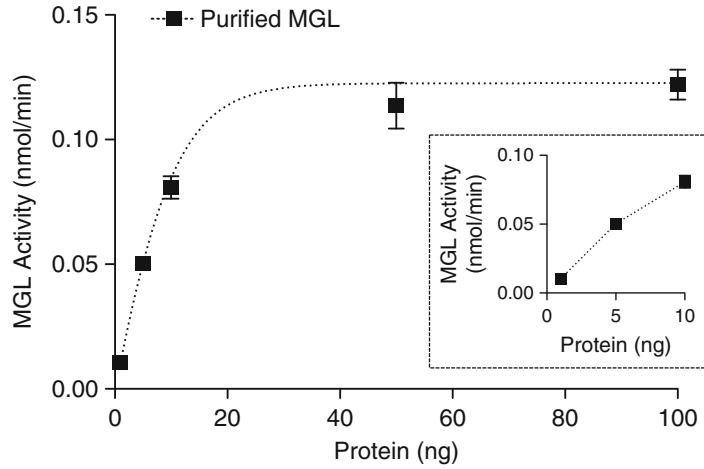

Fig. 4 Dose-response curve for the MGL assay. MGL activity assay was performed as described in this chapter, using the indicated amounts of purified recombinant MGL. *Inset* shows the linear range of the dose-response relationship (0–10 ng purified MGL protein)

time for the Branson probe-type sonicator. Using the Polytron homogenizer, we homogenize tissues for 30 s to 1 min. All procedures should be done on ice and the samples should be kept cold at all times.

6. We determine protein concentration using a BCA assay kit (Life Technologies), and a spectrophotometer, following the manufacturer's manual.
7. We confirm MGL overexpression by Western blot using either anti-MGL antibody [1] or antibody for the C-terminal tag sequence of recombinant MGL [11]. Alternatively, quantitative PCR or MGL activity assay can be performed [11].
8. Determining the amount of protein being used for the assay is an important factor for success. As shown in Fig. 4, the increment of MGL enzyme in the assay shall eventually result in depletion of substrate, which causes the concentration of product to reach a plateau and saturate the assay system. Therefore, a comparative study must use protein amounts within the linear range of the dose-response relationship (1–10 ng for the purified MGL, inset of Fig. 4). Although the concentration of the 2-OG substrate vastly exceeds the concentration of enzyme under normal conditions, it is recommended to run an enzyme dose-response curve whenever a new type of protein source (cells, tissues, etc.) is used. In our laboratory, we use 0.2–5.0 μg protein for HeLa MGL homogenate, 10–50 μg for cell or brain homogenate, or 1–10 ng for purified recombinant MGL.

9. When the bottom organic phase is removed, it is important not to contaminate it with the upper aqueous phase. We suggest inserting the tip of the glass Pasteur pipette through the upper phase while giving a very gentle positive pressure (which will result in a gentle bubbling). When the tip has reached the bottom layer, carefully withdraw the organic phase from the very bottom of the glass tube. To prevent contamination, it is better not to try recovering the last drops from the bottom phase, instead leaving 5–10 % of the phase in the vial.
10. At this step, the procedure can be stopped. Keep the organic phase at –20 °C.
11. (*Optional*) *After* **step 9**, the sample can be further purified by open-bed silica column purification if better separation is required for LC/MS analysis. Follow the **steps Opt 1** to **Opt 9** below.

    Opt 1. Reconstitute the dried lipid pellet from **step 9** in 2 ml of chloroform.

    Opt 2. Prepare a suspension of Silica Gel G (60-Å 230–400 Mesh ASTM; Whatman, Clifton, NJ) in chloroform (1:1, vol/vol).

    Opt 3. Add 1 ml of the Silica Gel G suspension to the glass columns.

    Opt 4. Wash the columns with 2 ml of chloroform.

    Opt 5. Load the samples onto the columns. Wait until all liquid drops down by gravity.

    Opt 6. Elute the lipids with 2 ml of chloroform/methanol (9:1, vol/vol), and collect the eluate in another set of 8 ml glass vials with the same labeling.

    Opt 7. Evaporate the eluate to dryness under a gentle N_2 stream.

    Opt 8. Resuspend the dried lipids in 0.1 ml of chloroform:methanol (1:3, vol/vol) mixture.

    Opt 9. Transfer them into 1.5 ml glass LC vials with 0.2 ml conical inserts, and proceed to LC/MS analysis.
12. The standard curve may be used to obtain oleic acid levels only when 5 nmol heptadecanoic acid is used as an internal standard. It is recommended to renew the standard curve periodically.

Acknowledgements

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References

1. Dinh TP, Carpenter D, Leslie FM et al (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A 99:10819–10824
2. Karlsson M, Contreras JA, Hellman U et al (1997) cDNA cloning, tissue distribution, and identification of the catalytic triad of monoglyceride lipase. Evolutionary relationship to esterases, lysophospholipases, and haloperoxidases. J Biol Chem 272:27218–27223
3. Blankman JL, Simon GM, Cravatt BF (2007) A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem Biol 14:1347–1356
4. Marrs WR, Blankman JL, Horne EA et al (2010) The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. Nat Neurosci 13:951–957
5. Labar G, Bauvois C, Borel F et al (2010) Crystal structure of the human monoacylglycerol lipase, a key actor in endocannabinoid signaling. Chembiochem 11:218–227
6. Bertrand T, Augé F, Houtmann J et al (2010) Structural basis for human monoglyceride lipase inhibition. J Mol Biol 396:663–673
7. Gulyas AI, Cravatt BF, Bracey MH et al (2004) Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. Eur J Neurosci 20:441–458
8. Piomelli D (2003) The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 4:873–884
9. Chanda PK, Gao Y, Mark L et al (2010) Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system. Mol Pharmacol 78:996–1003
10. Schlosburg JE, Blankman JL, Long JZ et al (2010) Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. Nat Neurosci 13:1113–1119
11. Jung KM, Clapper JR, Fu J et al (2012) 2-Arachidonoylglycerol signaling in forebrain regulates systemic energy metabolism. Cell Metab 15:299–310
12. Dinh TP, Kathuria S, Piomelli D (2004) RNA interference suggests a primary role for monoacylglycerol lipase in the degradation of the endocannabinoid 2-arachidonoylglycerol. Mol Pharmacol 66:1260–1264
13. King AR, Duranti A, Tontini A et al (2007) URB602 inhibits monoacylglycerol lipase and selectively blocks 2-arachidonoylglycerol degradation in intact brain slices. Chem Biol 14:1357–1365
14. Fredrikson G, Tornqvist H, Belfrage P (1986) Hormone-sensitive lipase and monoacylglycerol lipase are both required for complete degradation of adipocyte triacylglycerol. Biochim Biophys Acta 876:288–293
15. Astarita G, Piomelli D (2009) Lipidomic analysis of endocannabinoid metabolism in biological samples. J Chromatogr B Analyt Technol Biomed Life Sci 877:2755–2767
16. Piomelli D, Astarita G, Rapaka R (2007) A neuroscientist's guide to lipidomics. Nat Rev Neurosci 8:743–754
17. King AR, Dotsey EY, Lodola A et al (2009) Discovery of potent and reversible monoacylglycerol lipase inhibitors. Chem Biol 16: 1045–1052

Chapter 18

A Sensitive and Versatile Fluorescent Activity Assay for ABHD6

Juha R. Savinainen, Dina Navia-Paldanius, and Jarmo T. Laitinen

Abstract

The α/β-hydrolase domain-containing 6 (ABHD6) enzyme is a newly found serine hydrolase whose substrate profile resembles that of monoacylglycerol lipase (MAGL), the major 2-arachidonoyl glycerol (2-AG) hydrolase in the brain. Here, we describe a sensitive fluorescent assay of ABHD6 activity in a 96-well-plate format that allows parallel testing of inhibitor activities of up to 40 compounds in a single assay. The method utilizes lysates of HEK293 cells transiently overexpressing human ABHD6 as the enzymatic source, and kinetically monitors glycerol liberated in the hydrolysis of 1(3)-AG, the preferred arachidonoyl glycerol isomer. Glycerol output is coupled to an enzymatic cascade generating the fluorescent end-product resorufin. The approach has major benefits compared to laborious traditional mass spectrometric methods and liquid scintillation-based assays, or approaches using unnatural substrates.

Key words 2-AG hydrolase, Endocannabinoid, Fluorescence, Glycerol, Inhibitor, Lipase, Monoacylglycerol, Natural substrate, Screening

1 Introduction

The endocannabinoid 2-arachidonoyl glycerol (2-AG) is a unique bioactive lipid that, based on recent evidence, has three major hydrolyzing enzymes in the body: monoacylglycerol lipase (MAGL), α/β-hydrolase domain-containing 6 (ABHD6), and 12 (ABHD12) [1]. The newly characterized ABHD6 has been associated with 2-AG metabolism both in the central nervous system [2] and in peripheral tissues [3]. It is estimated that at the bulk brain level, ~4 % of 2-AG hydrolysis is due to ABHD6 [4]. The MAG substrate profile of human ABHD6 (hABHD6) closely resembles that of hMAGL with the exception that hABHD6 prefers the MAG 1(3)-isomers over 2-isomers, as shown in our previous in vitro study [5]. Retrospectively, development of sensitive and rapid methods to monitor endocannabinoid hydrolysis has appeared challenging, probably mostly because of instability and lipophilic nature of 2-AG and other endocannabinoids. Mass spectrometric approaches in this field appear

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DOI 10.1007/978-1-4939-3539-0_18,

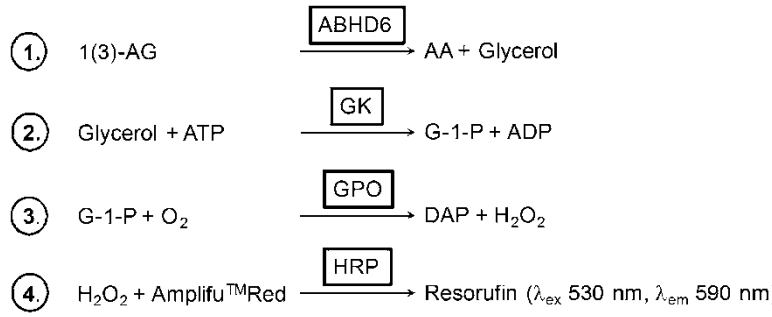

Fig. 1 Principle of the coupled-enzyme system to detect ABHD6 activity. ABHD6-catalyzed hydrolysis of 1(3)-AG generates equimolar amounts of arachidonic acid (AA) and glycerol. In the coupled-enzyme system, glycerol is converted to glycerol-1-phosphate (G-1-P) in the presence of ATP, in a reaction catalyzed by glycerol kinase (GK). Glycerol 3-phosphate oxidase (GPO)-catalyzed oxidation of G-1-P generates H_2O_2 which in the presence of horseradish peroxidase (HRP) converts Amplifu™ Red into the fluorescent product resorufin whose fluorescence (λ_{ex} 530 nm; λ_{em} 590 nm) is kinetically monitored

rather laborious. On the other hand, relying on radioactive-based liquid scintillation analysis has many disadvantages. Here, we describe in detail a sensitive fluorescence-based method for assessment of hABHD6 activity and its inhibition in 96-well-plate format, based on our recent publication [5]. The method relies on detection of glycerol liberated from the natural MAG substrate 1(3)-AG as a result of ABHD6 hydrolytic activity. Glycerol is then converted by a coupled-enzyme system to generate the fluorescent end-product resorufin. The principle of the coupled-enzyme system to detect glycerol formed by ABHD6 activity is detailed in Fig. 1.

The method is highly sensitive, allowing detection of low picomolar quantities of the enzymatic product (water-soluble glycerol), and requires only a small amount of lysate (0.3 μg protein/well) prepared from HEK293 cells transiently overexpressing hABHD6. The kinetic assay format has been validated for the three 2-AG hydrolases (hMAGL, hABHD6, and hABHD12) [5], and has been recently modified further to allow diacylglycerol lipase (DAGL) activity measurements [6].

2 Materials

Prepare all solutions using Millipore-purified, deionized high-quality water (HQW). Store buffer stocks at room temperature (RT) and other reagents at −80 °C (*see* **Note 1**).

2.1 Basic Incubation Cocktail

1. 500 mM Tris–HCl, pH 7.4: Dissolve 60.55 g Tris in 800 ml of HQW with constant stirring for at least 2 h. Adjust pH to 7.40 with strong HCl, adjust volume to 1 l with HQW. Store at RT in a dark bottle.

2. 100 mM EDTA: Dissolve 3.7224 g Na_2EDTA in 100 ml of HQW. Store at 4 °C in a dark bottle.
3. 500 mM $MgCl_2$: Dissolve 20.33 g $MgCl_2 \cdot 6H_2O$ in 200 ml of HQW. Store at 4 °C in a dark bottle.
4. 4 M NaCl: Dissolve 23.376 g NaCl in 100 ml of HQW. Store at 4 °C in a dark bottle.
5. Basic incubation cocktail (BIC): Prepare BIC from the fresh stocks by pipetting 50 ml of 500 mM Tris–HCl (pH 7.4) in a 250 ml volumetric flask. Add 5 ml of 100 mM EDTA, 5 ml of 500 mM $MgCl_2$, and 12.5 ml of 4 M NaCl, and adjust the volume to 250 ml with HQW. Final concentrations of the buffer components are 100 mM Tris–HCl (pH 7.4), 2 mM EDTA, 10 mM $MgCl_2$, and 200 mM NaCl. Store at RT in a dark bottle.

2.2 Components of the Coupled-Enzyme System

For the preparations of coupling enzyme stocks (GPO, GK, HRP, see details below) in HQW, calculate the amount of HQW needed for the stock preparation, which depends on the specific activity of each enzyme batch. Mix well and divide in 25 µl single-use aliquots for storage at −80 °C. Thaw one aliquot of each just prior to the experiment and keep on ice until use.

1. 200 U/ml glycerol 3-phosphate oxidase (GPO) in HQW: GPO from *Pediococcus* sp. (Sigma), 100 U, 40–80 U/mg solid, pH 8.1.
2. 200 U/ml glycerokinase (GK) in HQW: GK from *Cellulomonas* sp. (Sigma), 1000 U, 25–75 U/mg protein.
3. 240–250 U/ml horseradish peroxidase (HRP) in HQW: HRP (Sigma), 5000 U, 45 mg solid.

2.3 Other Reagents

1. 10 mM Amplifu Red™ in DMSO: Dissolve the vial content of Amplifu Red™ (Fluka) in DMSO (e.g., 5 mg in 1944 µl) and divide in 25 µl single-use aliquots to be stored at −80 °C. Thaw one aliquot just prior to the experiment and protect from light (*see* **Note 2**).
2. 10 mM ATP in HQW: This needs to be prepared fresh for each experiment. Weigh ATP and dissolve in HQW just prior to use, e.g., 2.536 mg/0.5 ml of HQW. Keep on ice until use.
3. 5 % BSA (w/v) in HQW: Carefully dissolve BSA (essentially fatty acid free) with constant stirring, e.g., 10 g in 200 ml of HQW. Divide in 1 ml aliquots for storage at −20 °C (*see* **Note 3**).
4. 10 mM 1(3)-AG in ethanol: 1(3)-AG is commonly supplied in acetonitrile. Transfer 5 mg 1(3)-AG into a preweighed Eppendorf tube, evaporate the solvent under a stream of nitrogen, weight the residue, and dissolve in ethanol (1321 µl/5 mg). Divide in 100 µl aliquots for storage at −80 °C.

5. DMSO for control samples and for dilution of ABHD6 inhibitors.
6. Phosphate-buffered saline (PBS) for the preparation and dilution of cellular lysates.
7. 10 mM Tetrahydrolipstatin (THL) in DMSO: Weigh, e.g., 2.55 mg THL and dissolve it in 514 μl of DMSO. Divide in 10 μl aliquots for storage at −80 °C. THL serves as a reference inhibitor and is included at 10^{-5} and 10^{-7} M concentrations in each experiment to monitor assay performance (*see* **Note 4**). Prepare 1 mM and 10 μM intermediate dilutions in DMSO; these will give 10^{-5} and 10^{-7} M final concentrations during the preincubation with the enzyme (1:100 ratio).

2.4 Glycerol Standards and Glycerol Quality Control

1. Glycerol (liquid with a density of 1.25 g/ml): 1 μl corresponds to 13.57 μmol glycerol (13.57 M stock). This needs to be diluted several times, in order to reach the pmol concentration range.
2. Std-A: Dilute glycerol stock 1:100 with HQW as follows: Weigh 125 mg of glycerol into a 10 ml volumetric flask, and dilute to 10 ml ⇒ 1 μl corresponding to 135.7 nmol glycerol. This can be divided into 0.5 ml aliquots for storage at −20 °C.
3. Std-B: Dilute Std-A 1:100 with HQW (10 μl + 990 μl) ⇒ 1 μl corresponding to 1.357 nmol glycerol.
4. Glycerol quality control (GQC): Prepare a larger volume (50 ml) by pipetting 25 ml of BIC + 5 ml of 5 % (w/v) BSA + 50 μl Std-B + HQW. Divide in 900 μl aliquots for storage at −20 °C. In the assay, pipette 99 μl of GQC together with DMSO or inhibitors (1 μl). This equals to 134 pmol glycerol per well (*see* **Note 5**).

2.5 Reagents for Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM).
2. Fetal bovine serum (FBS).
3. Antibiotics (penicillin and streptomycin).

2.6 Equipment

1. Black 96-well plates suitable for fluorescence reading.
2. Automatic (or manual) multichannel pipettes for rapid pipetting of enzyme dilutions and the glycerol assay mix (containing substrate and the coupling enzymes). Normal set of pipettes is needed for preparation of inhibitor dilutions and other assay components.
3. Plastic pipetting reservoirs for multichannel pipetting substrate mix and enzyme dilutions (*see* **Note 1**).
4. A fluorescence reader capable of kinetic measurements (λ_{ex} 530 nm; λ_{em} 590 nm) (*see* **Note 6**).
5. Equipment for cell culture.

3 Methods

We have validated the assay by using lysates of HEK293 cells transiently overexpressing hABHD6 [5]. HEK293 cells have negligible endogenous ABHD6 activity and thus serve as excellent host cells for transient overexpression of the enzyme. The fluorescent assay is highly sensitive requiring merely 0.3 μg of lysate protein/well [5]. Under these conditions, 1(3)-AG hydrolysis by the cellular background is low and the signal-to-noise is therefore optimal [5]. We recommend including also mock-transfected HEK293 cells as negative control, in order to facilitate verification of successful ABHD6 expression (*see* **Note 7**).

3.1 Enzyme Preparation

1. Transfection: Culture HEK293 cells as monolayers in DMEM containing 10 % FBS under antibiotics (penicillin/streptomycin) at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air. Use plasmids containing ABHD6 for introducing cDNAs to cells by a standard transient transfection procedure [5]. Culture mock cells (i.e., cells transfected with an empty vector) in parallel to facilitate verification of successful enzyme expression and activity.
2. Preparation of cell lysates: Wash cells twice with ice-cold PBS. Scrape off and pellet cells at 250 × *g* for 10 min at 4 °C. Freeze-thaw cell pellets three times, resuspend in ice-cold PBS, briefly sonicate, and aliquot for storage at −80 °C. Determine protein concentration by using BSA as a standard. For the assay, dilute an aliquot to a protein concentration of 3 mg/ml using PBS. Total volume required for a full 96-well plate is 10 μl (*see* **Note 8**).
3. Analysis of ABHD6 expression: We recommend verifying successful expression of ABHD6 before routine use in the glycerol assay, preferentially by using activity-based protein profiling (ABPP), as this method discloses a catalytically active enzyme with proper size [5]. If this methodology is not available, Western blotting can be used to detect immunoreactive protein [5].

3.2 Activity Assay

Carry out all procedures at RT unless otherwise specified. Here, we detail our routine assay platform for 96-well plates allowing determination of hABHD6 hydrolytic activity together with activity screening of potential hABHD6 inhibitors. With this setup, up to 40 distinct inhibitors (or different concentrations of selected inhibitors) can be tested in duplicates in a single full-plate assay. The procedure can be completed within 3–4 h.

Overview of the method. Final volume per well is 200 μl. The solvent (DMSO) or the inhibitors are first pipetted in a volume of 1 μl into appropriate wells of the 96-well plate (Fig. 2). After this, 99 μl of blank buffer (without enzyme), hABHD6-HEK lysate (0.3 μg protein/well), or GQC (134 pmol glycerol/well) will be added to the indicated wells using a multichannel pipette, and the plate is

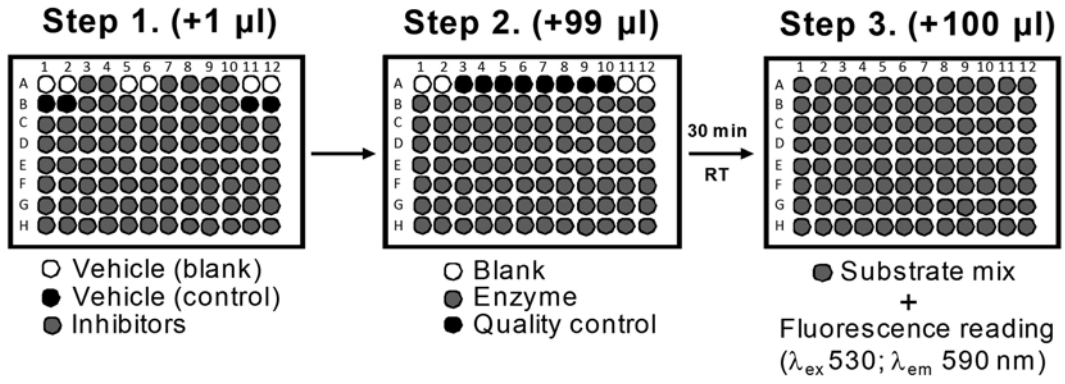

Fig. 2 Platform allowing simultaneous monitoring of ABHD6 hydrolytic activity and its inhibition in a 96-well-plate format. Final volume is 200 µl per well. DMSO/inhibitors (1 µl) are added first into the appropriate wells, and then blank buffer, glycerol quality control (GQC), or the diluted enzyme preparation are added in 99 µl of assay buffer. Following 30-min preincubation of enzyme with inhibitors, 100 µl of glycerol assay mix containing the substrate and the coupling enzymes is added by using a multichannel pipette. The plate is vortexed and the fluorescence (λ_{ex} 530 nm; λ_{em} 590 nm) is kinetically monitored at 10-min intervals for 90 min at RT

vortexed and incubated for 30 min at RT. Following this, glycerol assay mix (100 µl/well) containing the substrate and the coupled-enzyme system is added to all wells using a multichannel pipette, the plate is vortexed, and fluorescence is kinetically monitored at 10-min intervals for 90 min at RT.

1. Pipetting DMSO/inhibitors into the wells: Prepare 10 mM inhibitor stocks in DMSO (*see* **Note 9**). The stocks are further diluted in DMSO, so that the solutions to be pipetted into the wells (1 µl) are 100-fold concentrated over the desired final concentrations during the preincubation. For wells without inhibitor, use 1 µl of DMSO (*see* **Note 10**). In each experiment, include THL at the two final concentrations (10^{-7} and 10^{-5} M) as the reference inhibitor, in order to monitor assay performance (*see* **Note 4**).
2. Preincubation of enzyme with DMSO/inhibitors: Prepare the blank buffer and the hABHD6-HEK lysate working dilution into test tubes according to Table 1. Transfer the solutions into plastic reservoirs, and by using a multichannel pipette add 99 µl into indicated wells (Fig. 2). Finally, pipette 99 µl of GQC into indicated wells (Fig. 2) (*see* **Note 11**). At this stage, all wells should have a total volume of 100 µl. Vortex the plate and incubate at RT for 30 min with the plate covered.
3. Kinetic assay with the substrate: Just prior to use (i.e., during the final 5–10 min of the preincubation step), prepare 11 ml of glycerol assay mix containing the substrate and the coupled-enzyme system (Table 2). Amplifu™ Red should be added last in order to minimize decomposition (*see* **Note 2**). Mix the cocktail well by vortexing several times, and avoid bubbles (*see* **Note 3**), as well as unnecessary delays. Transfer into a plastic reservoir, and by using a multichannel pipette add 100 µl into

Table 1
Preparation of blank and hABHD6-HEK dilutions

Component (for full 96-well plate test)	Blank	hABHD6-HEK lysate (0.3 µg/well final)	Final concentration in the mix
BIC	500 µl	5000 µl	50 mM Tris–HCl, pH 7.40, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl
HQW	400 µl	3990 µl	
hABHD6-HEK lysate (3 mg protein/ml)	–	10 µl	3 µg/ml
5 % (w/v) BSA	100 µl	1000 µl	0.5 % (w/v)
Total volume	1000 µl	10,000 µl	

Table 2
Preparation of the glycerol assay mix

Component	Glycerol assay mix	Final concentration in the mix
HQW	4007.5 µl	
BIC	5500 µl	50 mM Tris–HCl, pH 7.40, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl
BSA 5 % (w/v)	1100 µl	0.5 % (w/v)
HRP (200 U/ml)	22 µl	0.4 U/ml[a]
GPO (200 U/ml)	22 µl	0.4 U/ml[a]
GK (200 U/ml)	22 µl	0.4 U/ml[a]
ATP (10 mM)	277 µl	0.25 mM[a]
Amplifu™ Red (10 mM in DMSO)	22 µl	20 µM[a]
1(3)-AG (10 mM in ethanol)	27.5 µl	25 µM 1(3)-AG; 1 % ethanol (v/v)[a]
Final volume	11 ml	

[a]Final concentrations of these components in the wells during the kinetic assay will be 1/2× of the values indicated here

all wells (*see* **Note 11**), vortex the plate (*see* **Note 12**), and in order to obtain initial fluorescence reading at time point 0 min, start the kinetic measurement without delay. Record fluorescence unit (FU) readings at 10-min intervals for 90 min at RT (*see* **Note 13**).

3.3 Guidelines for Calculations

We use well-based changes in fluorescence ($FU_{90min} - FU_{0min}$) as the basis to calculate all results, as explained below (*see* **Note 14**).

1. Calculate the **net fluorescence (NetFU)** ($FU_{90min} - FU_{0min}$) for individual wells by subtracting the mean value ($FU_{90min} - FU_{0min}$) of the blank wells from the ($FU_{90min} - FU_{0min}$) values of all other wells.

2. Calculate **ABHD6 activity** in the DMSO-ABHD6 wells (representing total activity in the absence of any inhibitor), based on the mean NetFU of these wells obtained above and that of the GQC wells (*see* **Note 5**):

   $$\text{ABHD6 activity (pmol/well)} = (\text{NetFU}_{\text{ABHD6}} \times 134)/\text{NetFU}_{\text{GQC}}.$$

3. Calculate **substrate consumption**, based on ABHD6 activity and the amount of substrate. Final 1(3)-AG concentration in wells is initially 12.5 μM; thus 2500 pmol substrate is initially available:

   $$\%\ \text{substrate consumption} = (\text{ABHD6 activity}/2500\ \text{pmol}) \times 100.$$

   Ideally, substrate consumption during the 90-min incubation should be <20 %.

4. For wells with inhibitors, calculate **percentage of remaining activity** (*see* **Note 9**):

   $$\%\ \text{remaining activity} = (\text{NetFU}_{\text{inhibitor}}/\text{NetFU}_{\text{ABHD6-DMSO}}) \times 100.$$

4 Notes

1. Any detergent residues (containing glycerol) from the washing-up steps of laboratory glassware and plastic reservoirs are potential contaminators of the assay. Therefore, careful washing of containers and plastic pipetting reservoirs with HQW is recommended before use.
2. Amplifu™ Red is light sensitive, and thus protect the reagent from light and cover the plate after substrate mix addition. Check also the color of the substrate mix after preparation: if it is reddish, the reagent may be giving too high values and rise the background.
3. Fatty acid-free BSA is included in this assay to minimize nonspecific binding of hydrophobic compounds to the plastic. Be aware that strong vortexing of BSA solutions may cause bubbles that disturb fluorescence reading. Also, in the black wells used in these experiments small bubbles are difficult to see.
4. Using this methodology, the IC_{50} value for THL towards hABHD6 is 47.9 nM [5]; the two concentrations are expected to produce ~60 % (10^{-7} M) and 100 % (10^{-5} M) inhibition.
5. The inclusion of GQC is important for the following reasons: (1) Fixed amount of glycerol added to the wells (134 pmol/well in the case of GQC) allows to monitor from assay to assay that the coupled-enzyme system properly coverts glycerol to resorufin; that is, the net fluorescence of the GQC wells should be comparable between different assays. If fluorescence dramatically drops, component(s) of the coupled-enzyme system are not working properly and fresh stock(s) may need to be prepared. (2) Using GQC as a standard, it is possible to calcu-

late the amount of glycerol formed (pmol/well) for each individual well. The assay is highly linear in the range of 0–500 pmol glycerol/well [5]. Inclusion of GQC makes it possible to determine the amount of glycerol formed (pmol/well) for each well also at various time points of the kinetic assay [5]. (3) In inhibitor discovery, it is important to rule out that the coupled-enzyme system is not the target of the inhibitor. Wells with GQC ± inhibitor giving the same net fluorescence indicate no interference with the coupled-enzyme system.

6. When optimizing condition to detect fluorescent signal from fluorescence reader, test suitable gain values to prevent overflow values during the kinetic analysis. We use Tecan Infinite M200 plate reader with a gain setting of 60.
7. An identical protocol using lysates of HEK293 cells with transient overexpression of hMAGL or hABHD12 can be used to assess hMAGL or hABHD12 activity, respectively [5]. Furthermore, the assay format can, in principle, be adapted for hABHD6 produced in other host cell lines and different biological samples (tissue and cell membranes, cytosolic and nuclear samples), but elaborate validation is needed in each case to demonstrate that the assay specifically reports ABHD6 activity. This is because the relative expression of ABHD6 may be low in cell lines or tissues with endogenous expression of ABHD6, and multiple enzymes possessing MAG hydrolytic activity could contribute to the pool of glycerol formed.
8. Although ABHD6 is predicted to be an integral membrane protein [4], we found no particular enrichment of ABHD6 activity in HEK293 cell membrane preparations when compared to lysates [5]. As only a fraction of cellular material is needed to produce the same amount of protein from lysates as compared to membranes, this economy aspect also favors the use of lysates in these assays. Lysates may sometimes contain material that is difficult to pipette after freezing. This DNA-containing material can be removed by sucking it into the pipette tip but after this step the protein content needs to be determined again.
9. This point is relevant in the case that inhibitors are to be tested in the assay. If not, total number of wells as well as volumes of enzyme preparation (Table 1) and assay mix (Table 2) can be scaled down to fit the size of the experiment. On the other hand, for initial validation purposes it may be useful to construct dose-response curves and to determine IC_{50} values for inhibitors (e.g., MAFP, IDFP, HDSF, THL, RHC-80267, WWL70) that have been previously evaluated in this assay [5]. At the very least, we advice to include THL at the two concentrations (*see* **Note 4**). Noteworthy, it may be important to periodically test that for samples receiving the same treatment, fluorescence readings are uniform throughout the plate regardless of the position.

Especially when pipetting cell lysates, the wells receiving the lysate mix last may contain slightly more enzymatic activity due to tendency of the lysates to sediment in the reservoirs. The assay is highly sensitive and therefore robustly reports inaccuracies in pipetting, especially those concerning the enzyme preparation. We recommend to test multiple DMSO-hABHD6-HEK samples distributed randomly throughout the plate, and to confirm uniform fluorescence readings for these samples to rule out the possibility that any gradient could exist.

10. When dispending individual inhibitors, pipette (by touching the bottom of the well) 1 μl of compound in the middle of the well. The drop is readily visible to the naked eye, so this serves as a useful control step to check that all wells have received DMSO/inhibitor. DMSO is not readily evaporated, so this step is not rate limiting. However, use parafilm to protect the wells.
11. We recommend to use inverse pipetting when adding the enzyme dilution and glycerol assay mix.
12. When vortexing the plate after addition of the glycerol assay mix, use medium-speed settings to avoid splashing.
13. Prepare in advance the fluorometer for the assay before starting the pipetting steps. This ensures that initial fluorescence readings (approximating time point 0 min) can be obtained without unnecessary delays immediately after the addition of the glycerol assay mix.
14. We recommend to prepare an Excel sheet to facilitate calculations of the results.

Acknowledgement

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References

1. Savinainen JR, Saario SM, Laitinen JT (2012) The serine hydrolases MAGL, ABHD6 and ABHD12 as guardians of 2-arachidonoylglycerol signalling through cannabinoid receptors. Acta Physiol (Oxf) 204:267–276
2. Naydenov AV, Horne EA, Cheah CS et al (2014) ABHD6 blockade exerts antiepileptic activity in PTZ-induced seizures and in spontaneous seizures in R6/2 mice. Neuron 83: 361–371
3. Thomas G, Betters JL, Lord CC et al (2013) The serine hydrolase ABHD6 is a critical regulator of the metabolic syndrome. Cell Rep 5:508–520
4. Blankman JL, Simon GM, Cravatt BF (2007) A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem Biol 14:1347–1356
5. Navia-Paldanius D, Savinainen JR, Laitinen JT (2012) Biochemical and pharmacological characterization of human α/β-hydrolase domain containing 6 (ABHD6) and 12 (ABHD12). J Lipid Res 53:2413–2424
6. Van der Wel T, Janssen FJ, Baggelaar MP et al (2015) A natural substrate-based fluorescence assay for inhibitor screening on diacylglycerol lipase α. J Lipid Res 56:927–935

Chapter 19

A Sensitive and Versatile Fluorescent Activity Assay for ABHD12

Juha R. Savinainen, Dina Navia-Paldanius, and Jarmo T. Laitinen

Abstract

Despite great progress in identifying and deorphanizing members of the human metabolic serine hydrolase (mSH) family, the fundamental role of numerous enzymes in this large protein class has remained unclear. One recently found mSH is α/β-hydrolase domain containing 12 (ABHD12) enzyme, whose natural substrate in vivo appears to be the lysophospholipid lysophosphatidylserine (LPS). In vitro, ABHD12 together with monoacylglycerol lipase (MAGL) and ABHD6 hydrolyzes also monoacylglycerols (MAGs) such as the primary endocannabinoid 2-arachidonoyl glycerol (2-AG). Traditional approaches for determining 2-AG hydrolase activity are rather laborious, and often utilize unnatural substrates. Here, we describe a sensitive fluorescent assay of ABHD12 activity in a 96-well-plate format that allows simultaneous testing of inhibitor activities of up to 40 compounds in a single assay. The method utilizes lysates of HEK293 cells transiently overexpressing human ABHD12 as the enzymatic source, and kinetically monitors glycerol liberated in the hydrolysis of 1(3)-AG, the preferred MAG substrate of this enzyme. Glycerol output is coupled to an enzymatic cascade generating the fluorescent end-product resorufin. This methodology has helped to identify the first class of inhibitors showing selectivity for ABHD12 over the other mSHs.

Key words 2-AG hydrolase, Endocannabinoid, Fluorescence, Glycerol, Inhibitor, Lipase, Monoacylglycerol, Natural substrate, Screening

1 Introduction

Physiological significance of MAGL in the metabolism of the endocannabinoid 2-AG has been firmly established, whereas the in vivo role of the recently found ABHD12 as a 2-AG hydrolase is still elusive [1]. Recent research supports the idea that ABHD12 regulates 2-AG metabolism in immune cells, whereas in the brain it appears to metabolize LPS, a non-cannabinoid lysophospholipid [2, 3]. Dysfunctional ABHD12 has been linked to PHARC, a rare neurodegenerative disease with symptoms of *p*olyneuropathy, *h*earing loss, *a*taxia, *r*etinitis pigmentosa, and early-onset *c*ataract [2, 4]. In vitro, the MAG substrate preference of hABHD12 is distinct from that of hMAGL and hABHD6, the two other 2-AG hydrolases [5]. Like

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hABHD6, hABHD12 shows preference for the MAG 1(3)-isomers, and from a panel of 12 tested MAG species its best substrate was found to be 1(3)-AG [5]. During the past 20 years, assay development in the field of 2-AG hydrolases has been relatively challenging, not least because of instability and lipophilic nature of endocannabinoids. Mass spectrometric approaches to detect 2-AG hydrolysis are quite laborious and thus they do not offer a shortcut for inhibitor discovery. Furthermore, radioactive substrates in liquid scintillation-based assays bring their own apparent inconveniences. Some efforts to develop alternative approaches, more suitable for high-throughput screening (HTS), have been made but to the best of our knowledge none takes advantage of natural substrates. To gain better insight into the in vitro activities of the 2-AG hydrolases MAGL, ABHD6, and ABHD12, we have developed and optimized a highly sensitive fluorescent 96-well-plate assay that allows kinetic monitoring of glycerol liberated from the natural MAG substrates by the hydrolytic activity of these enzymes [5]. Here, we describe this methodology in a platform allowing simultaneous assessment of hABHD12 activity and its inhibition. Glycerol is converted by a coupled-enzyme system to generate the fluorescent end-product resorufin. The principle of the coupled-enzyme system to detect glycerol formed by ABHD12 activity is detailed in Fig. 1.

The method is highly sensitive, allowing detection of low picomolar quantities of the enzymatic product (water-soluble glycerol), and requires only a small amount of lysate (0.3 μg protein/well) prepared from HEK293 cells transiently overexpressing hABHD12 [5]. The kinetic assay format has been validated for the three 2-AG hydrolases (hMAGL, hABHD6, and hABHD12) [5], and has been

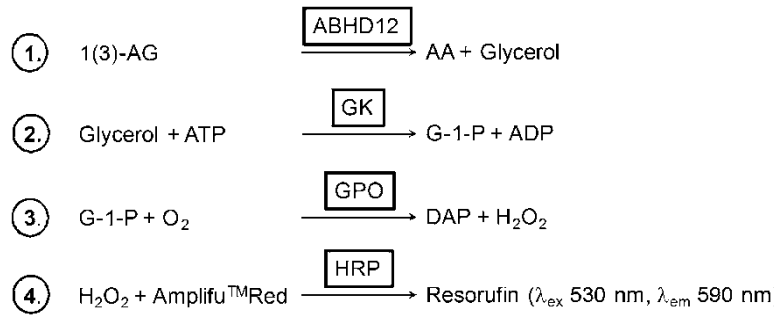

Fig. 1 Principle of the coupled-enzyme system to detect ABHD12 activity. ABHD12-catalyzed hydrolysis of 1(3)-AG generates equimolar amounts of arachidonic acid (AA) and glycerol. In the coupled-enzyme system, glycerol is converted to glycerol-1-phosphate (G-1-P) in the presence of ATP in a reaction catalyzed by glycerol kinase (GK). Glycerol 3-phosphate oxidase (GPO)-catalyzed oxidation of G-1-P generates H_2O_2 that in the presence of horseradish peroxidase (HRP) converts Amplifu™ Red into the fluorescent product resorufin, whose fluorescence (λ_{ex} 530 nm; λ_{em} 590 nm) is kinetically monitored

recently modified further to allow diacylglycerol lipase (DAGL) activity measurements [6]. It has also played an important role in the discovery of the first class of reversible triterpene-based inhibitors with selectivity for ABHD12 over the other mSHs [7].

2 Materials

Prepare all solutions using Millipore-purified, deionized high-quality water (HQW). Store buffer stocks at RT and other reagents at –80 °C (*see* **Note 1**).

2.1 Basic Incubation Cocktail

1. 500 mM Tris–HCl, pH 7.4: Dissolve 60.55 g Tris in 800 ml of HQW with constant stirring for at least 2 h. Adjust pH to 7.40 with strong HCl, and adjust volume to 1 l with HQW. Store at RT in a dark bottle.
2. 100 mM EDTA: Dissolve 3.7224 g Na_2EDTA in 100 ml of HQW. Store at 4 °C in a dark bottle.
3. 500 mM $MgCl_2$: Dissolve 20.33 g $MgCl_2 \cdot 6H_2O$ in 200 ml of HQW. Store at 4 °C in a dark bottle.
4. 4 M NaCl: Dissolve 23.376 g NaCl in 100 ml of HQW. Store at 4 °C in a dark bottle.
5. Basic incubation cocktail (BIC): Prepare BIC from the fresh stocks by pipetting 50 ml of 500 mM Tris–HCl (pH 7.4) in a 250 ml volumetric flask. Add 5 ml of 100 mM EDTA, 5 ml of 500 mM $MgCl_2$, and 12.5 ml of 4 M NaCl, adjusting the volume to 250 ml with HQW. Final concentrations of the buffer components are 100 mM Tris–HCl (pH 7.4), 2 mM EDTA, 10 mM $MgCl_2$, and 200 mM NaCl. Store at RT in a dark bottle.

2.2 Components of the Coupled-Enzyme System

For the preparations of coupling enzyme stocks (GPO, GK, HRP, see details below) in HQW, calculate the amount of HQW needed for the stock preparation which depends on the specific activity of each enzyme batch. Mix well and divide in 25 µl single-use aliquots for storage at –80 °C. Thaw one aliquot of each just prior to the experiment and keep on ice until use.

1. 200 U/ml Glycerol 3-phosphate oxidase (GPO) in HQW: GPO from *Pediococcus* sp. (Sigma), 100 U, 40–80 U/mg solid, pH 8.1.
2. 200 U/ml Glycerokinase (GK) in HQW: GK from *Cellulomonas* sp. (Sigma), 1000 U, 25–75 U/mg protein.
3. 240–250 U/ml Horseradish peroxidase (HRP) in HQW: HRP (Sigma), 5000 U, 45 mg solid.

2.3 Other Reagents

1. 10 mM Amplifu Red™ in DMSO: Dissolve the vial content of Amplifu Red™ (Fluka) in DMSO (e.g., 5 mg in 1944 μl), and divide into 25 μl single-use aliquots to be stored at −80 °C. Thaw one aliquot just prior to the experiment and protect from light (*see* **Note 2**).
2. 10 mM ATP in HQW: This needs to be prepared fresh for each experiment. Weigh ATP and dissolve it in HQW just prior to use, e.g., 2.536 mg/0.5 ml of HQW. Keep on ice until use.
3. 5 % BSA (w/v) in HQW: Dissolve BSA (essentially fatty acid free) carefully with constant stirring, e.g., 10 g in 200 ml. Divide into 1 ml aliquots for storage at −20 °C (*see* **Note 3**).
4. 10 mM 1(3)-AG in ethanol: 1(3)-AG is commonly supplied in acetonitrile. Transfer 5 mg 1(3)-AG into a preweighed Eppendorf tube, evaporate the solvent under a stream of nitrogen, weight the residue, and dissolve in ethanol (1321 μl/5 mg). Divide into 100 μl aliquots for storage at −80 °C.
5. DMSO for control samples and for dilution of ABHD12 inhibitors.
6. Phosphate-buffered saline (PBS) for the preparation and dilution of cellular lysates.
7. 10 mM Tetrahydrolipstatin (THL) in DMSO: Weigh THL and dissolve it in DMSO (e.g., 2.55 mg in 514 μl). Divide into 10 μl aliquots for storage at −80 °C. THL serves as a reference inhibitor and is included at 10^{-5} and 10^{-7} M concentrations in each experiment to monitor assay performance (*see* **Note 4**). Prepare 1 mM and 10 μM intermediate dilutions in DMSO; these will give 10^{-5} and 10^{-7} M final concentrations during the preincubation with the enzyme (1:100 ratio).

2.4 Glycerol Standards and Glycerol Quality Control

1. Glycerol (liquid with a density of 1.25 g/ml): 1 μl corresponds to 13.57 μmol glycerol (13.57 M stock). This needs to be diluted several times, in order to reach the pmol concentration range.
2. Std-A: Dilute glycerol stock 1:100 with HQW as follows: Weigh 125 mg of glycerol into a 10 ml volumetric flask and dilute to 10 ml ⇒ 1 μl corresponding to 135.7 nmol glycerol. This can be divided into 0.5 ml aliquots for storage at −20 °C.
3. Std-B: Dilute Std-A 1:100 with HQW (10 μl + 990 μl) ⇒ 1 μl corresponding to 1.357 nmol glycerol.
4. Glycerol quality control (GQC): Prepare a larger volume (50 ml) by pipetting 25 ml BIC + 5 ml 5 % (w/v) BSA + 50 μl Std-B + HQW. Divide into 900 μl aliquots for storage at −20 °C. In the assay, pipette 99 μl of GQC together with 1 μl of DMSO or inhibitors. This equals to 134 pmol glycerol per well (*see* **Note 5**).

2.5 Reagents for Cell Culture

1. Dulbecco's modified eagle's medium (DMEM).
2. Fetal bovine serum (FBS).
3. Antibiotics (penicillin and streptomycin).

2.6 Equipment

1. Black 96-well plates suitable for fluorescence reading.
2. Automatic (or manual) multichannel pipettes for rapid pipetting of enzyme dilutions and the glycerol assay mix (containing substrate and the coupling enzymes): Normal set of pipettes is needed for preparation of inhibitor dilutions and other assay components.
3. Plastic pipetting reservoirs for multichannel pipetting substrate mix and enzyme dilutions (*see* **Note 1**).
4. A fluorescence reader capable of kinetic measurements (λ_{ex} 530 nm; λ_{em} 590 nm) (*see* **Note 6**).
5. Equipment for cell culture.

3 Methods

We have validated the assay by using lysates of HEK293 cells transiently overexpressing hABHD12 [5]. HEK293 cells have negligible endogenous ABHD12 activity and thus serve as excellent host cells for transient overexpression of the enzyme. The fluorescent assay is highly sensitive requiring merely 0.3 μg of lysate protein/well [5]. Under these conditions, 1(3)-AG hydrolysis by the cellular background is low and the signal-to-noise is therefore optimal [5]. We recommend including also mock-transfected HEK293 cells as negative control, in order to facilitate verification of successful ABHD12 expression (*see* **Note 7**).

3.1 Enzyme Preparation

1. Transfection: Culture HEK293 cells as monolayers in DMEM containing 10 % FBS under antibiotics (penicillin/streptomycin) at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air. Use plasmids containing ABHD12 for introducing cDNAs to cells by a standard transient transfection procedure [5]. Culture mock cells (i.e., cells transfected with an empty vector) in parallel to facilitate verification of successful enzyme expression and activity.
2. Preparation of cellular lysates: Wash cells twice with ice-cold PBS. Scrape off and pellet cells at 250×*g* for 10 min at 4 °C. Freeze-thaw cell pellet three times, resuspend in ice-cold PBS, briefly sonicate, and aliquot for storage at −80 °C. Determine protein concentration by using BSA as a standard. For the assay, dilute an aliquot to a protein concentration of 3 mg/ml by using PBS. Total volume required for a full 96-well plate is 10 μl (*see* **Note 8**).

3. Analysis of ABHD12 expression: We recommend verifying successful expression of ABHD12 before routine use in the glycerol assay, preferentially by using activity-based protein profiling (ABPP), as this method discloses a catalytically active enzyme with proper size [5]. If this methodology is not available, Western blotting can be used to detect immunoreactive protein [5].

3.2 Activity Assay

Carry out all procedures at RT unless otherwise specified. Here, we detail our routine assay platform for 96-well plates allowing determination of hABHD12 hydrolytic activity together with activity screening of potential hABHD12 inhibitors. With this setup, up to 40 distinct inhibitors (or different concentrations of selected inhibitors) can be tested in duplicates in a single full-plate assay. The procedure can be completed within 3–4 h.

Overview of the method. Final volume is 200 µl per well. The solvent (DMSO) or the inhibitors are first pipetted in a volume of 1 µl into appropriate wells of the 96-well plate (Fig. 2). After this, 99 µl of blank buffer (without enzyme), hABHD12-HEK lysate (0.3 µg protein/well), or GQC (134 pmol glycerol/well) will be added to the indicated wells by using a multichannel pipette, and the plate is vortexed and incubated for 30 min at RT. Then, glycerol assay mix (100 µl/well) containing the substrate and the coupled-enzyme system is added to all wells with a multichannel pipette, the plate is vortexed, and fluorescence is kinetically monitored at 10-min intervals for 90 min at RT.

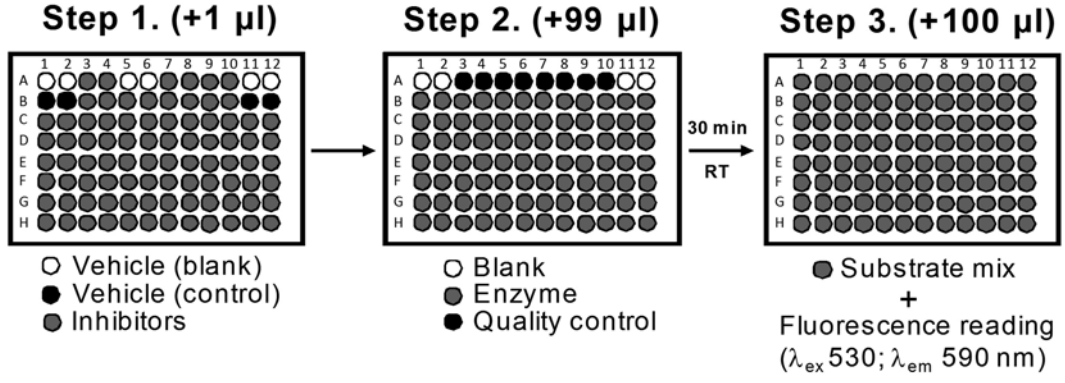

Fig. 2 Platform allowing simultaneous monitoring of ABHD12 hydrolytic activity and its inhibition in a 96-well-plate format. Final volume is 200 µl per well. DMSO/inhibitors (1 µl) are added first into the appropriate wells, and then blank buffer, glycerol quality control (GQC), or the diluted enzyme preparation are added in 99 µl of assay buffer. Following 30-min preincubation of enzyme with inhibitors, 100 µl of glycerol assay mix containing the substrate and the coupling enzymes is added by using a multichannel pipette. The plate is vortexed and fluorescence (λ_{ex} 530 nm; λ_{em} 590 nm) is kinetically monitored at 10-min intervals for 90 min at RT

1. Pipetting DMSO/inhibitors into the wells: Prepare 10 mM inhibitor stocks in DMSO (*see* **Note 9**). The stocks are further diluted in DMSO, so that the solutions to be pipetted into the wells (1 µl) are 100-fold concentrated over the desired final concentrations during the preincubation. For wells without inhibitor, use 1 µl of DMSO (*see* **Note 10**). In each experiment, include THL at the two final concentrations (10^{-7} and 10^{-5} M) as the reference inhibitor to monitor assay performance (*see* **Note 4**).
2. Preincubation of enzyme with DMSO/inhibitors: Prepare the blank buffer and the hABHD12-HEK lysate working dilution into test tubes according to Table 1. Transfer the solutions into plastic reservoirs, and using a multichannel pipette add 99 µl into indicated wells (Fig. 2). Finally, pipette 99 µl of GQC into indicated wells (Fig. 2) (*see* **Note 11**). At this stage, all wells should have a total volume of 100 µl. Vortex the plate and incubate for 30 min at RT with the plate covered.
3. Kinetic assay with the substrate: Just prior to use (i.e., during the final 5–10 min of the preincubation step), prepare 11 ml of glycerol assay mix containing the substrate and the coupled-enzyme system (Table 2). Amplifu™ Red should be added last in order to minimize decomposition (*see* **Note 2**). Mix the cocktail well by vortexing several times, and avoid bubbles (*see* **Note 3**), as well as unnecessary delays. Transfer into a plastic reservoir, and using a multichannel pipette add 100 µl into all wells (*see* **Note 11**), vortex the plate (*see* **Note 12**), and in order to obtain initial fluorescence reading at time point 0 min, start the kinetic measurement without delay. Record fluorescence unit (FU) readings at 10-min intervals for 90 min at RT (*see* **Note 13**).

Table 1
Preparation of blank and hABHD12-HEK dilutions

Component (for full 96-well plate test)	Blank	hABHD12-HEK lysate (0.3 µg/well final)	Final concentration in the mix
BIC	500 µl	5000 µl	50 mM Tris–HCl, pH 7.40, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl
HQW	400 µl	3990 µl	
hABHD12-HEK lysate (3 mg protein/ml)	–	10 µl	3 µg/ml
5 % (w/v) BSA	100 µl	1000 µl	0.5 % (w/v)
Total volume	1000 µl	10,000 µl	

Table 2
Preparation of the glycerol assay mix

Component	Glycerol assay mix	Final concentration in the mix
HQW	4007.5 µl	
BIC	5500 µl	50 mM Tris–HCl, pH 7.40, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl
BSA 5 % (w/v)	1100 µl	0.5 % (w/v)
HRP (200 U/ml)	22 µl	0.4 U/ml[a]
GPO (200 U/ml)	22 µl	0.4 U/ml[a]
GK (200 U/ml)	22 µl	0.4 U/ml[a]
ATP (10 mM)	277 µl	0.25 mM[a]
Amplifu™ Red (10 mM in DMSO)	22 µl	20 µM[a]
1(3)-AG (10 mM in ethanol)	27.5 µl	25 µM 1(3)-AG; 1 % ethanol (v/v)[a]
Final volume	11 ml	

[a]Final concentrations of these components in the wells during the kinetic assay will be 1/2× of the values indicated here

3.3 Guidelines for Calculations

We use well-based changes in fluorescence ($FU_{90min} - FU_{0min}$) as the basis to calculate all results, as explained below (*see* **Note 14**).

1. Calculate the **net fluorescence (NetFU)** ($FU_{90min} - FU_{0min}$) for individual wells by subtracting the mean value ($FU_{90min} - FU_{0min}$) of the blank wells from the ($FU_{90min} - FU_{0min}$) values of all other wells.
2. Calculate **ABHD12 activity** in the DMSO-ABHD12 wells (representing total activity in the absence of any inhibitor), based on the mean NetFU of these wells obtained above and that of the GQC wells (*see* **Note 5**):

$$\text{ABHD12 activity (pmol/well)} = (\text{NetFU}_{\text{ABHD12}} \times 134)/\text{NetFU}_{\text{GQC}}.$$

3. Calculate **substrate consumption**, based on ABHD12 activity and the amount of substrate. Final 1(3)-AG concentration in wells is initially 12.5 µM; thus 2500 pmol substrate is initially available:

$$\%\ \text{substrate consumption} = (\text{ABHD12 activity}/2500\ \text{pmol}) \times 100.$$

   Ideally, substrate consumption during the 90-min incubation should be <20 %.

4. For wells with inhibitors, calculate **percentage of remaining activity** (*see* **Note 9**):

$$\%\ \text{remaining activity} = (\text{NetFU}_{\text{inhibitor}}/\text{NetFU}_{\text{ABHD12-DMSO}}) \times 100.$$

4 Notes

1. Any detergent residues (containing glycerol) from the washing-up steps of laboratory glassware and plastic reservoirs are potential contaminators of the assay. Therefore, careful washing of containers and plastic pipetting reservoirs with HQW is recommended before use.
2. Amplifu™ Red is light sensitive, and thus protect the reagent from light and cover the plate after substrate mix addition. Check also the color of the substrate mix after preparation: if it is reddish, the reagent may be giving too high values and rise the background.
3. Fatty acid-free BSA is included in this assay to minimize nonspecific binding of hydrophobic compounds to the plastic. Be aware that strong vortexing of BSA solutions may cause bubbles that disturb fluorescence reading. Also, in the black wells used in these experiments small bubbles are difficult to see.
4. Using this methodology, the IC_{50} value for THL towards hABHD12 is 191 nM [5]; the two concentrations are expected to produce ~35 % (10^{-7} M) and 100 % (10^{-5} M) inhibition.
5. The inclusion of GQC is important for the following reasons: (1) Fixed amount of glycerol added to the wells (134 pmol/well in the case of GQC) allows to monitor from assay to assay that the coupled-enzyme system properly coverts glycerol to resorufin; that is, the net fluorescence of the GQC wells should be comparable between different assays. If fluorescence dramatically drops, component(s) of the coupled-enzyme system are not working properly and fresh stock(s) may need to be prepared. (2) Using GQC as a standard, it is possible to calculate the amount of glycerol formed (pmol/well) for each individual well. The assay is highly linear in the range of 0–500 pmol glycerol/well [5]. Inclusion of GQC makes it possible to determine the amount of glycerol formed (pmol/well) for each well also at various time points of the kinetic assay [5]. (3) In inhibitor discovery, it is important to rule out that the coupled-enzyme system is not the target of the inhibitor. Wells with GQC ± inhibitor giving the same net fluorescence indicate no interference with the coupled-enzyme system.
6. When optimizing conditions to detect fluorescent signal from fluorescence reader, test suitable gain values to prevent overflow values during the kinetic analysis. We use Tecan Infinite M200 plate reader with a gain setting of 60.
7. An identical protocol using lysates of HEK293 cells with transient overexpression of hMAGL or hABHD6 can be used to assess hMAGL or hABHD6 activity, respectively [5]. Furthermore, the assay format can, in principle, be adapted for

hABHD12 produced in other host cell lines or in different biological samples (tissue and cell membranes, cytosolic and nuclear samples), but elaborate validation is needed in each case to demonstrate that the assay specifically reports ABHD12 activity. This is because the relative expression of ABHD12 may be low in cell lines or tissues with endogenous expression of ABHD12, and multiple enzymes possessing MAG hydrolytic activity could contribute to the pool of glycerol formed.

8. Although ABHD12 is predicted to be an integral membrane protein [4], we found no particular enrichment of ABHD12 activity in HEK293 cell membrane preparations when compared to lysates [5]. As only a fraction of cellular material is needed to produce the same amount of protein from lysates as compared to membranes, this economy aspect also favors the use of lysates in these assays. Lysates may sometimes contain material that is difficult to pipette after freezing. This DNA-containing material can be removed by sucking it into the pipette tip, but after this step the protein content needs to be determined again.
9. This point is relevant in the case that inhibitors are to be tested in the assay. If not, total number of wells as well as volumes of enzyme preparation (Table 1) and assay mix (Table 2) can be scaled down to fit the size of the experiment. On the other hand, for initial validation purposes it may be useful to construct dose-response curves and to determine IC_{50} values for inhibitors (e.g., MAFP, IDFP, HDSF, THL, maslinic acid) that have been previously evaluated in this assay [5]. At the very least, we advice to include THL at the two concentrations (*see* **Note 4**). Noteworthy, it may be important to periodically test that for samples receiving the same treatment, fluorescence readings are uniform throughout the plate regardless of the position. Especially when pipetting cell lysates, the wells receiving the lysate mix last may contain slightly more enzymatic activity due to tendency of the lysates to sediment in the reservoirs. The assay is highly sensitive and therefore robustly reports inaccuracies in pipetting, especially those concerning the enzyme preparation. We recommend to test multiple DMSO-hABHD12-HEK samples distributed randomly throughout the plate, and to confirm uniform fluorescence readings for these samples to rule out the possibility that any gradient could exist.
10. When dispending individual inhibitors, pipette (by touching the bottom of the well) 1 μl of compound in the middle of the well. The drop is readily visible to the naked eye, so this serves as a useful control step to check that all wells have received DMSO/inhibitor. DMSO is not readily evaporated, so this step is not rate limiting. However, use parafilm to protect the wells.

11. We recommend to use inverse pipetting when adding the enzyme dilution and glycerol assay mix.
12. When vortexing the plate after addition of the glycerol assay mix, use medium-speed settings to avoid splashing.
13. Prepare in advance the fluorometer for the assay before starting the pipetting steps. This ensures that initial fluorescence readings (approximating time point 0 min) can be obtained without unnecessary delays immediately after the addition of the glycerol assay mix.
14. We recommend to prepare an Excel sheet to facilitate calculations of the results.

Acknowledgement

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References

1. Savinainen JR, Saario SM, Laitinen JT (2012) The serine hydrolases MAGL, ABHD6 and ABHD12 as guardians of 2-arachidonoylglycerol signalling through cannabinoid receptors. Acta Physiol (Oxf) 204:267–276
2. Blankman JL, Long JZ, Trauger SA et al (2013) ABHD12 controls brain lysophosphatidylserine pathways that are deregulated in a murine model of the neurodegenerative disease PHARC. Proc Natl Acad Sci U S A 110:1500–1505
3. Kamat SS, Camara K, Parsons WH et al (2015) Immunomodulatory lysophosphatidylserines are regulated by ABHD16A and ABHD12 interplay. Nat Chem Biol 11:164–171
4. Fiskerstrand T, H'mida-Ben Brahim D, Johansson S et al (2010) Mutations in ABHD12 cause the neurodegenerative disease PHARC: an inborn error of endocannabinoid metabolism. Am J Hum Genet 87:410–417
5. Navia-Paldanius D, Savinainen JR, Laitinen JT (2012) Biochemical and pharmacological characterization of human α/β-hydrolase domain containing 6 (ABHD6) and 12 (ABHD12). J Lipid Res 53:2413–2424
6. Van der Wel T, Janssen FJ, Baggelaar MP et al (2015) A natural substrate-based fluorescence assay for inhibitor screening on diacylglycerol lipase α. J Lipid Res 56:927–935
7. Parkkari T, Haavikko R, Laitinen T et al (2014) Discovery of triterpenoids as reversible inhibitors of α/β-hydrolase domain 12 (ABHD12). PLoS One 9, e98286

Chapter 20

Assay of Endocannabinoid Uptake

Mark Rau, Simon Nicolussi, Andrea Chicca, and Jürg Gertsch

Abstract

Endocannabinoids at physiological concentrations are crossing cellular membranes by facilitated diffusion, a process that can be studied by measuring transport kinetics. Here, we describe a radiosubstrate-based assay using arachidonoyl[1-^{3}H]ethanolamine or arachidonoyl[1,2,3-^{3}H]glycerol to measure the cellular endocannabinoid uptake in a three-phase assay with human U937 cells.

Key words Endocannabinoid uptake, Anandamide, 2-Arachidonoylglycerol

1 Introduction

Endocannabinoids (ECs) are arachidonic acid-derived lipids that interact either with extracellular targets, such as type 1 and type 2 cannabinoid receptors (CB_1R and CB_2R, respectively), or with intracellular targets like ion channels, peroxisome proliferator-activated receptors (PPARs), intracellularly located CB_1 receptors (e.g., in mitochondria), cytoplasmic binding proteins (e.g., albumin, heat-shock protein-70, FABP5), and metabolic enzymes. While the EC family includes at least four lipid mediators, the most abundant and best characterized molecules are 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (anandamide, AEA). The biological effects of AEA and 2-AG are regulated by their cellular biosynthesis, extracellular release, reuptake, trafficking, and enzymatic cleavage. In contrast to the relatively clear knowledge about the EC biosynthetic and metabolic pathways, their cellular uptake remains poorly understood. Different models of EC uptake have been proposed and cell type-dependent mechanisms of EC cellular transport exist that have been recently reviewed in detail [1]. However, the measurement of AEA and 2-AG uptake is hampered by numerous confounding factors [2–5]. One of the principal issues in elucidating EC membrane transport is the tight interplay between the movement of ECs across plasma membranes and their rapid and almost complete cellular cleavage by the fatty acid amide hydrolase (FAAH) and the monoacylglycerol

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lipase (MAGL) or alpha/beta hydrolase 6 (ABHD6), respectively. At physiological concentrations, ECs are transported across the plasma membrane by facilitated diffusion, as best characterized by differential Michaelis-Menten kinetics in different cell types , independent of enzyme concentration [1, 6]. The uptake of AEA is driven by its breakdown primarily by FAAH, which belongs to the family of serine hydrolases and is expressed at the endoplasmatic reticulum, Golgi apparatus, and mitochondria. Although the cellular uptake of 2-AG has been poorly investigated, several studies suggest that AEA and 2-AG may share the same mechanism of membrane uptake despite their distinct intracellular fates [1, 7]. In addition, all ECs seem to compete for the same uptake process [7]. Importantly, the cellular uptake of AEA and 2-AG can be selectively and potently inhibited by small molecules that might be inhibitors of a putative endocannabinoid membrane transporter (EMT), and therefore constitute an independent class of modulators of the ECS [1, 20]. In order to identify and characterize novel EMT inhibitors, reproducible and sensitive assays are indispensable. Here, we employ the tritiated radiosubstrates [^{3}H]AEA and [^{3}H]2-AG, which allow robust measurements of EC cellular uptake by using differential extraction and radiometric-based assays. Radiometric assays show advantages over fluorescent and colorimetric assays, as chemically modified substrates might interfere with the membrane lipid environment leading to amplification or quenching of the signal. Moreover, the addition of a fluorophore to the EC core structure leads to modifications of its physicochemical properties, thus potentially altering cellular uptake kinetics. The quantitative measurements of EC intracellular levels by GC-MS/MS and LC-MS/MS are more costly and time consuming, and they do not seem optimal to screen for EMT inhibitors. Therefore, radiometric assays are most frequently used to assess EC uptake inhibition in combination with assays that measure inhibition of EC hydrolysis.

2 Materials

Prepare buffers using deionized H_2O and analytical grade reagents. Follow waste disposal regulations when disposing cells and radioactive waste material. For all radioactive substances, the half-life of radioactive isotopes must be taken into account. Store stock solutions at −20 °C, and perform regular analytical analyses to ensure product stability.

2.1 Components and Reagents

1. 2-Arachidonoylglycerol (2-AG).
2. Anandamide (AEA).
3. Arachidonoyl[1-^{3}H]ethanolamine (AEA).
4. Arachidonoyl[1,2,3-^{3}H]glycerol (2-AG) (*see* **Note 1**).
5. Bovine serum albumin (BSA), essentially fatty acid free (*see* **Note 2**).
6. Refrigerated centrifuge for 24 samples (e.g., Eppendorf centrifuge 5415 R).

7. Centrifuge, suited for Falcon tubes (e.g., Eppendorf centrifuge 5804 R).
8. Chloroform ($CHCl_3$).
9. Dimethyl sulfoxide (DMSO).
10. Ethanol (EtOH), ≥99 % pure.
11. Incubator for cell culture, at 37 °C and 5 % CO_2.
12. Liquid scintillation counter (e.g., Tri-Carb 2100TR).
13. Methanol (MeOH).
14. Micropipettes (10, 100, 200, and 1000 μl).
15. Mini-scintillation vials (6 ml).
16. Inverted microscope.
17. Neubauer "improved" cell counting chamber.
18. 0.01 M Phosphate-buffered saline (PBS), pH 7.4.
19. Reference inhibitors OMDM-2 and UCM707, and guineensine (available from J. Gertsch, University of Bern).
20. SafeSeal microtubes (2 ml, PP).
21. Screw-cap microtubes (1.5 ml, PP) (*see* **Note 3**).
22. Silanization fluid (AquaSil™), to be used according to instructions (Thermo Fisher Scientific).
23. ThermoMixer (1.5 ml).
24. Ultima Gold™ scintillation cocktail.
25. Vortex.
26. Water bath or thermoblock at 37 °C.

2.2 Cell Culture Material

- 150 cm^2 Tissue culture flasks.
- Amphotericin B.
- Cell line: Human monocytic lymphoma cells U937.
- Cell culture hood, biological safety cabinet (SterileGARD, Class 2A/B3).
- Falcon tubes (15/50 ml, PP).
- Fetal bovine serum (FBS) (*see* **Note 4**).
- Penicillin/streptomycin.
- RPMI-1640 medium with L-glutamine.

2.3 Buffers and Stock Solutions

1. [^{3}H]AEA-mix: 1 nM Arachidonoyl[1-^{3}H]ethanolamine, 99 nM AEA (prepare 100× stock solution in EtOH; 5 μl of [^{3}H]AEA mix will be used per sample):
   - 3 μl (167 nM) of arachidonoyl[1-^{3}H]ethanolamine in EtOH per sample.
   - 1.72 μl (28.8 μM) of AEA in EtOH per sample.
   - 0.28 μl of EtOH per sample.

2. [^{3}H]2-AG mix: 1 nM Arachidonoyl[1,2,3-^{3}H]glycerol (*see* **Note 1** and ref. 7), 999 nM 2-AG (prepare 100× stock solution in EtOH).
   - 2.5 µl (200 nM) of arachidonoyl[1,2,3-^{3}H]glycerol in EtOH per sample.
   - 2.5 µl (199.8 µM) of 2-AG in EtOH per sample.
3. 1 % (w/v) BSA in PBS, pH 7.4, at 4 °C.
   - Add 2 g BSA into 200 ml of PBS.

3 Methods

3.1 Cell Culture Maintenance

Grow human U937 cells in suspension at a density of approximately 1×10^6/ml in 150 cm^2 tissue culture flasks An endotoxin-free enviornment is essential. Use RPMI-1640 medium supplemented with 10 % FBS, 1 µg/ml amphotericin B, and 1 % (100 µg/ml) penicillin/streptomycin. Incubate cells at 37 °C with 5 % CO_2 for optimal growth (*see* **Note 5**).

3.2 Three-Phase AEA Uptake Assay

The workflow of this assay is illustrated in Fig. 1 (for details *see* **Note 6**).

1. Prepare inhibitor dilutions and sufficient [^{3}H]AEA mix. Heat PBS and RPMI-1640 in the water bath to 37 °C.
2. Count the cells using the "Neubauer improved" chamber (*see* **Note 7**), take the necessary number of U937 cells, and centrifuge them at 180 × *g* for 5 min at room temperature; wash with 10 ml of PBS (37 °C), centrifuge again at 180 × *g* for 5 min at room temperature, and resuspend in pre-warmed RPMI-1640 medium (without any additive) or PBS, to a density of 2×10^6 cells/ml.
3. Use 490 µl of cell suspension (~10^6 cells) per sample in silanized flip-cap microtubes.
4. Add 5 µl of OMDM-2 or UCM707 inhibitors (and of DMSO vehicle in positive controls); use 100× stock solutions (e.g., 1 mM solution to reach a final concentration of 10 µM), and vortex the tubes gently for 2 s (300 rpm in the ThermoMixer). Preincubate the cells for 30 min at 37 °C and 5 % CO_2. Run each sample in triplicate.
5. Add 100 nM [^{3}H]AEA mix (5 µl of 100× stock) to the cells. Vortex tubes carefully to distribute AEA homogenously. Incubate for 5 min at 37 °C.
6. Stop the uptake reaction by putting the tubes on ice, and rapidly centrifuge the samples at 800 × *g* for 5 min at 4 °C. Meanwhile, prefill 1.5 ml screw-cap microtubes with 2 volumes of an ice-cold $CHCl_3$:CH_3OH (1:1) mixture (*see* **Note 3**).

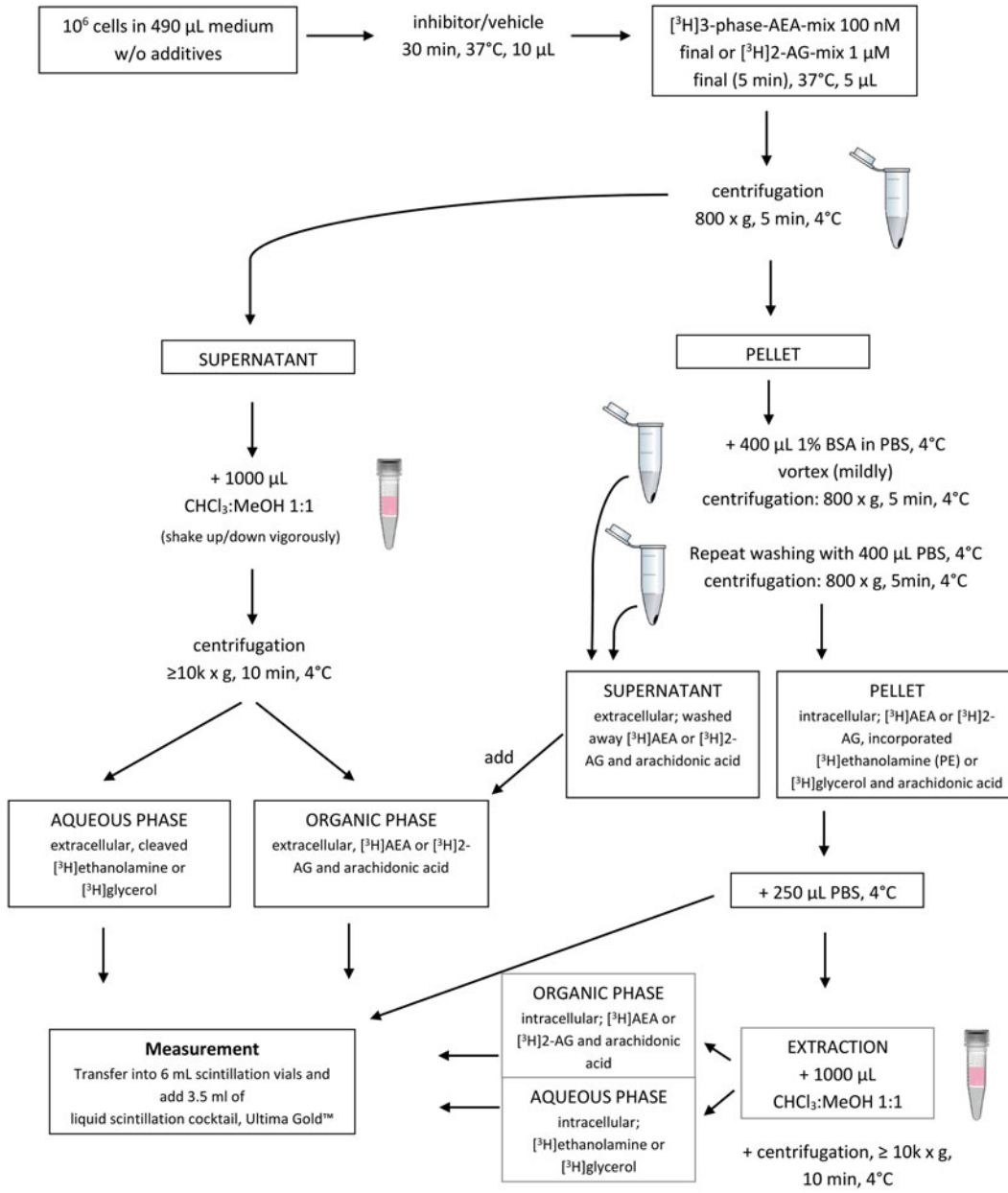

Fig. 1 Workflow of the three-phase AEA/2-AG uptake assay, according to the protocol

7. Transfer sample supernatants to the prefilled screw-cap microtubes including $CHCl_3$:CH_3OH, and put cell pellets on ice (pellet processing continues in **step 10**, also *see* **Note 8**).
8. Vortex or shake sample supernatants vigorously for extraction.
9. Centrifuge for 10 min at ≥10,000 × *g* at 4 °C, to separate aqueous and organic phases. Transfer the aqueous (upper) phase and organic (lower) phase separately into scintillation tubes for radioactive measurement (*see* **Note 9**).

10. Wash cell pellets from **step 6** with 400 µl of an ice-cold 1 % BSA in PBS, and shake carefully. Centrifuge at 800 × *g*, for 5 min and 4 °C. Add the supernatant to the scintillation tube containing the organic phase (from **step 9**), leave 5–10 µl of liquid to make sure that cell pellet remains intact, and cells are not removed with the supernatant.
11. Wash the pellets again with 400 µl of ice-cold PBS to remove any residual amounts of washing solution, and shake carefully (*see* **Note 10**).
12. Centrifuge for 5 min at 800 × *g* and 4 °C, and then transfer the supernatants to the scintillation tube containing the supernatant organic phase (from **step 9**).
13. Resuspend cell pellets in 250 µl of PBS, add 2 volumes (0.5 ml) of ice-cold $CHCl_3$:CH_3OH (1:1), and vortex vigorously.
14. Sonicate cell solutions for 5 min on ice, and then centrifuge for 10 min at ≥10,000 × *g* at 4 °C. Transfer the upper (aqueous) phase to the scintillation tube containing the aqueous phase derived from the extracted supernatant (*see* **step 9**) while collecting the cellular organic phase in a separate scintillation tube.
15. Perform liquid scintillation counting after addition of 3.5 ml of Ultima Gold™ scintillation liquid to all scintillation vials, followed by 1-h shaking. Also measure total radioactive signal of the substrate by adding 5 µl of [^{3}H]AEA mix directly to new scintillation vials.

Examples of the reference inhibitors OMDM-2 and UCM707 are shown in Fig. 2. This assay allows to characterize differential EC cellular uptake inhibition in U937 cells [7], to investigate the profiles of FAAH and MAGL inhibitors [7]], suitable and to characterize new selective AEA uptake inhibitors like guineensine [20], or highly potent inhibitors of FAAH [4].

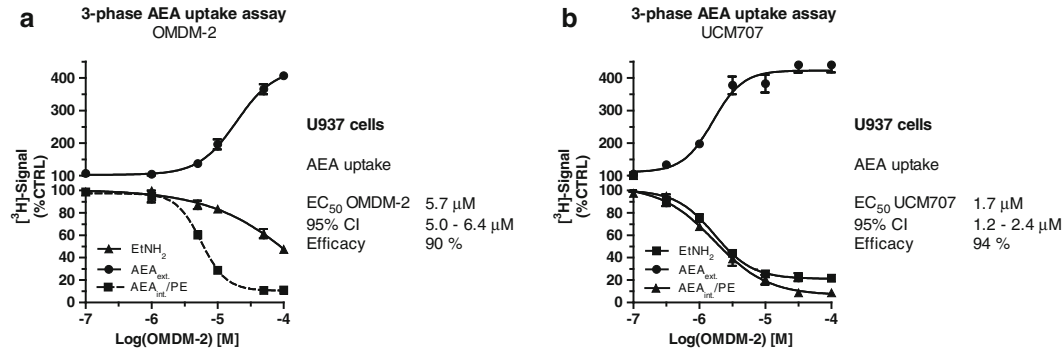

Fig. 2 Concentration-dependent effects of the reference inhibitors (**a**) OMDM-2 and (**b**) UCM707, determined in the three-phase AEA uptake assay. $AEA_{int./PE}$ describes the intracellular free AEA and the AEA-derived ethanolamine which was incorporated into phosphatidylethanolamine (PE). $EtNH_2$ is the free ethanolamine cleaved but not incorporated, and AEA_{ext} (extracellular) expresses the amount of free AEA which did not penetrate into the cell. 1×10^6 U937 cells were used per sample. Experiments were performed in triplicate with $n = 3$ ($N = 9$); means ± SEM are reported

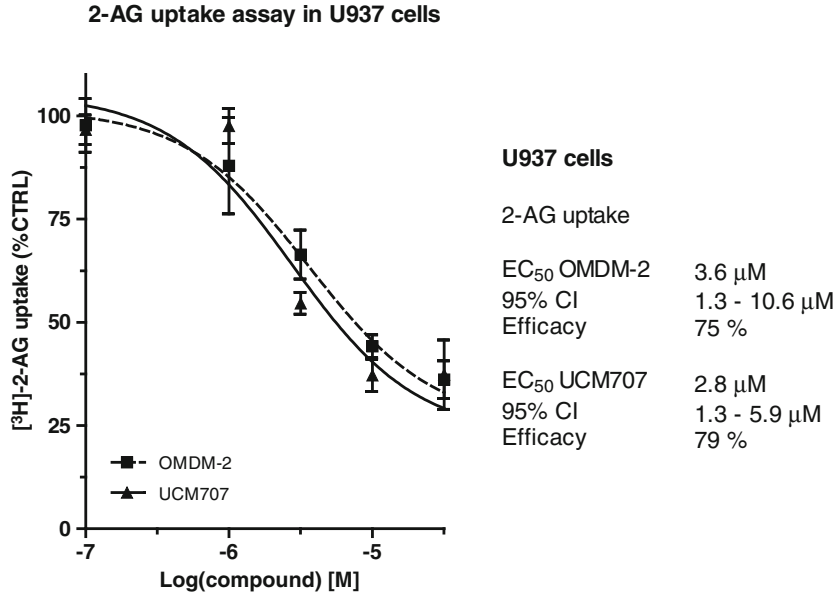

Fig. 3 Concentration-dependent effects of the reference inhibitors OMDM-2 and UCM707 in the three-phase 2-AG uptake assay. 1×10^6 U937 cells were used per sample. The cellular fraction is shown. For further details and graphs *see* [7]. Experiments were performed in triplicate with $n = 3$ ($N = 9$); means ± SEM are reported

3.3 Three-Phase 2-AG Uptake Assay

Perform as described in the three-phase assay for AEA uptake but apply the following adaptions:

Use 5 µl of 1 µM [^{3}H]2-AG mix per sample instead of 100 nM [^{3}H]AEA mix. Incubate [^{3}H]2-AG mix for 2–5 min.

Examples of reference inhibitors OMDM-2 and UCM707 are shown in Fig. 3.

4 Notes

1. 2-AG uptake is more difficult to measure than AEA uptake. The radioactive label in arachidonoyl[1-^{3}H]ethanolamine is in the ethanolamine moiety, which is quickly cleaved and then incorporated into phospholipids [7–9]. Therefore, the radioactive signal associated to the organic phase of the cell pellets is derived from free [^{3}H]AEA (minimal contribution) and [^{3}H]phosphatidylethanolamine (higher abundance). From the experimental point of view, this leads to higher radioactivity counts, which result in a robust signal-to-noise ratio. In case of arachidonoyl[1,2,3-^{3}H]glycerol uptake, the cleaved [1,2,3-^{3}H]glycerol freely and rapidly moves out of the cell *via* aquaporins, leading to a "loss" of intracellular radioactive signal [10]. The remaining signal is therefore only dependent on intracellular arachidonoyl[1,2,3-^{3}H]glycerol [7]. Furthermore, while the most relevant AEA hydrolytic enzyme (FAAH) is located predominantly in intracellular mem-

branes such as ER, Golgi apparatus, and mitochondria [8, 11, 12], 2-AG is degraded by at least three different enzymes, which are all located at the plasma membrane [12, 13]. Therefore, once taken up by the cell 2-AG is quickly cleaved, mainly by the membrane-associated enzyme MAGL (~85 % of the total 2-AG hydrolysis) and by the membrane-bound enzymes ABHD6 and ABHD12 (~15 %). Interestingly, the predicted structure of the latter two hydrolases suggests that they face the catalytic site towards the intracellular lumen and the extracellular milieu, respectively [13]. Direct cleavage of 2-AG at the outer membrane by ABHD12 might represent an important confounding factor when measuring 2-AG uptake, especially in specific cell lines lacking MAGL but not ABHDs (e.g., some macrophages and microglial cells) [14]. Nonetheless, it must be taken into account that inhibitors of arachidonic acid incorporation into phospholipids might reduce 2-AG uptake [15]. Moreover, short incubation times (≤5 min) are crucial to measure 2-AG uptake, since the arachidonoyl[1,2,3-^{3}H]glycerol is cleaved quickly after being taken up by the cells.

2. Serum albumin (fatty acid free) is an endogenous carrier of endocannabinoids [16, 17] and has been shown to affect endocannabinoid uptake [4]. Indeed, BSA (1 % in PBS) is the optimal washing solution to remove endocannabinoids associated to the outer leaflet of the plasma membrane.
3. Screw-cap microtubes are well suited for Folch's extraction, because they do not leak the MeOH/$CHCl_3$ mixture upon vigorous vortexing.
4. FBS is a nutritional supplement used for many cell cultures. Different batches were shown to contain variable amounts of endocannabinoids [18]. FBS low in endocannabinoid content should be selected. Uptake experiments should always be performed without FBS, in order to avoid biases.
5. Cell culture maintenance is one of the most crucial factors for the success of the EC uptake assay. When cells are only once malnourished, overgrown, activated by cell debris/endotoxins, or at unsuitable pH levels (i.e., yellow medium), they will acquire different phenotypes. U937 cells should be well-round shaped (Fig. 4a) and devoid of any dendritic structures which would indicate differentiation (Fig. 4b). Under suboptimal conditions, incorporation of ethanolamine into phospholipids (PE) is decreased (Fig. 4d), compared to healthy cells (Fig. 4c). Furthermore, endotoxin activated cells show reduced AEA uptake efficacy and FAAH enzymatic activity (Fig. 5a, b). Although the cells might recover and proliferate steadily, they will not be suitable for EC uptake any longer, and should be replaced. The RPMI-1640 medium contains the pH indicator phenol red, and turns from purple (pH > 8.2) to yellow (pH < 6.8) depending on the cell metabolic activity. Usually,

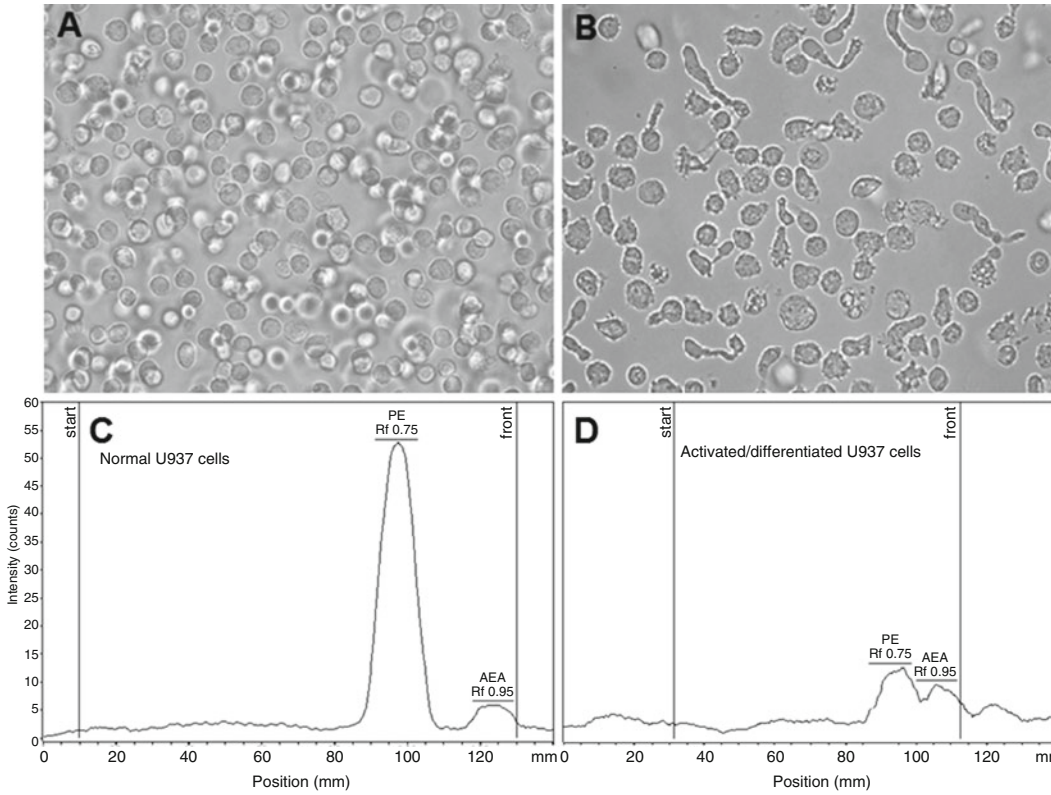

Fig. 4 (**a**) Normal U937 cells at 400× magnification. Cells are well rounded in suspension. (**b**) Activated and partially differentiated U937 cells at 400× magnification. Cells show deformations, dendritic structures, and partial adherence. (**c**) Incorporation of [^{3}H]ethanolamine into phosphatidylethanolamine (PE) and AEA on a TLC. (Assay was performed as described in [7].) (**d**) Activated/differentiated cells show a heavily reduced incorporation of [^{3}H]ethanolamine into phosphatidylethanolamine in comparison to normal U937 cells

when cells reach the density of about 1×10^6/ml, the medium turns into a yellowish color, implying a high metabolic activity. These cells should be used for EC uptake on the same day. When U937 cells are maintained under optimal culturing conditions, the generation time is roughly 24 h at cell densities $>0.8 \times 10^6$/ml. However, our experience is that under these exponential growth conditions the EC uptake assay works best.

6. The three-phase assay is an advanced EC uptake assay providing at once multiple readouts, which lead to a characteristic profile of an EC uptake inhibitor (Fig. 2a, b; also *see* refs. 7, 20). The assay reveals the intracellular and extracellular distribution pattern of AEA or 2-AG and their metabolites in the cells. For instance, the extracellular aqueous phase contains cleaved glycerol from 2-AG or ethanolamine from AEA, revealing the activity of EC-degrading enzymes; the extracellular organic phase contains intact 2-AG or AEA, which was not

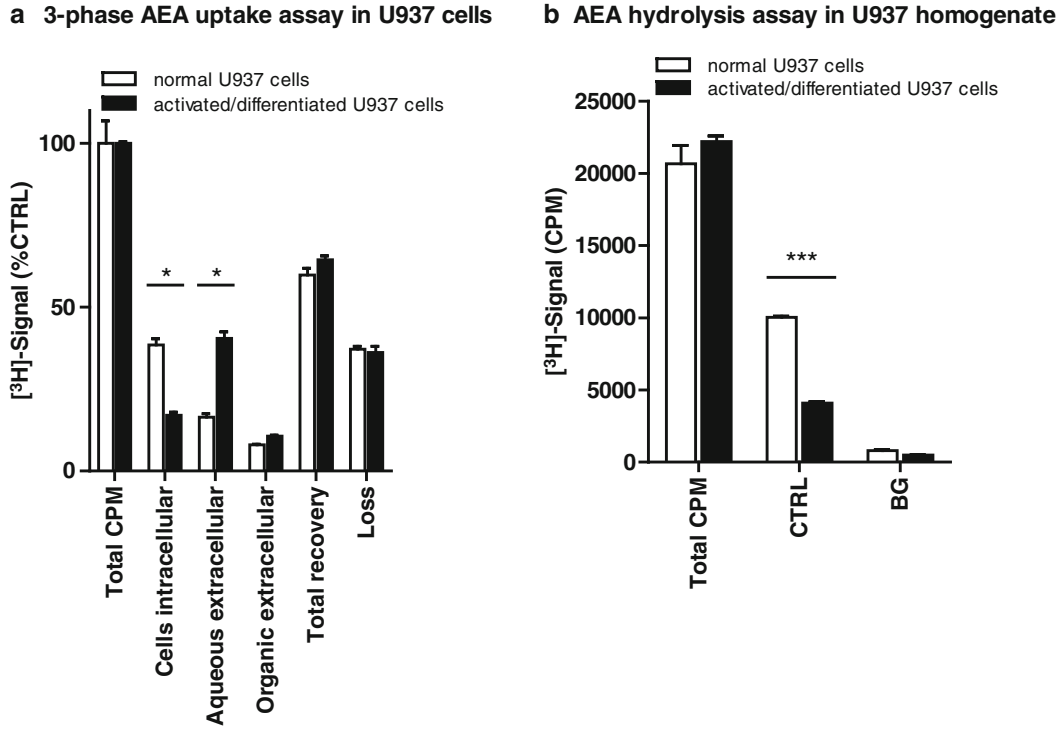

Fig. 5 (**a**) Three-phase AEA uptake assay of normal U937 cells versus "activated" U937 cells. The retention of radioactivity inside the activated/differentiated cells was significantly decreased and the [^{3}H]ethanolamine was found to be increased in the aqueous extracellular phase instead of being trapped in the cell by incorporation into PE. Overall, total signal of [^{3}H]3-phase-AEA mix, recovery, and remaining extracellular intact [^{3}H]AEA remained the same. Experiments were performed in triplicate with $n = 3$ ($N = 9$); means ± SEM and 95% CI are reported. (**b**) AEA hydrolysis assay in U937 cell homogenates, performed as previously described [4, 19]. The activated/differentiated cells showed significantly lower FAAH activity than normal U937 cells. Total [^{3}H]AEA added and background (BG) signals were equal. Experiments were performed in triplicate, $n = 3$ ($N = 9$); means ± SEM are reported

taken up by the cells. The three-phase assay enables collection of a complete set of information regarding the distribution and degradation of AEA or 2-AG as a concentration-dependent function of an EC uptake inhibitor. The assay is also suitable to characterize different cell types for AEA and 2-AG uptake, and to compare the pharmacological properties of new EMT inhibitors. AEA uptake in particular is heavily driven by the activity of FAAH that generates an inward concentration gradient. Thus, an inhibitor of FAAH will always inhibit EC uptake as well [2, 4]. However, each process can be inhibited independently, thus leading to $EtNH_2$ curve shifts that reveal an apparent selectivity for AEA cellular uptake (e.g., with OMDM-2 (Fig. 2a) or guineensine [20], but see also [4, 7] in comparison to FAAH inhibitors). After identification of a new AEA uptake inhibitor, further potential targets such as intracellular shuttles

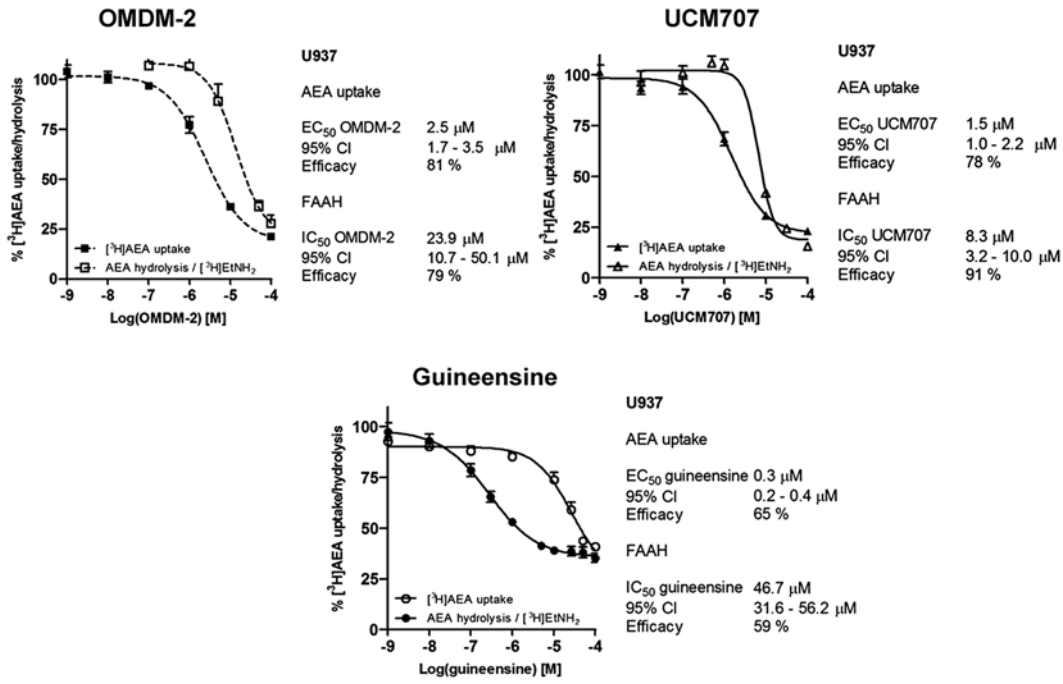

Fig. 6 Inhibition of [^{3}H]AEA uptake and [^{3}H]AEA hydrolysis by the reference inhibitors OMDM-2, UCM707, and guineensine in separate assays, as previously described [20]. 0.5×10^6 U937 cells or cell homogenates, respectively, were used per sample. Curves are shown without normalization. For AEA uptake, EC_{50} values (i.e., relative IC_{50} values) are shown because complete inhibition (i.e., efficacy of 100 %) cannot be reached due to non-inhibitable passive diffusion of the substrate, while absolute IC_{50} values are shown for FAAH activity. Reference FAAH inhibitors using the same assay protocol are described in [4]. Experiments were performed in triplicate with $n = 3$ ($N = 9$); means ± SEM and 95% CI are reported

[16, 21] and most importantly FAAH should be addressed as well (Fig. 6). To confirm an observed apparent selectivity (i.e., $EtNH_2$ right shift) of a new inhibitor of AEA uptake over FAAH inhibition, two independent assays can be performed as previously reported [4, 19, 20], as shown in Fig. 6.

7. We use the Neubauer improved cell counting chamber to quickly determine cell density, and to check at the same time their viability by trypan blue.
8. Consider that in EC uptake assays, the steps with the cell pellet are the most crucial and sensitive (the supernatant is transferred into $CHCl_3$:MeOH (1:1) and does not react any further). It is therefore advised to continue with **step 10** straight after **step 7** and do **steps 8** and **9** at the end.
9. Folch's extraction leads to a three-phasic separation. A bottom phase with $CHCl_3$ and lipophilic substances such as the arachidonic acid, phospholipids, 2-AG, or AEA; a precipitated phase of proteins in the middle; and an upper aqueous phase with MeOH, H_2O, and either free glycerol or ethanolamine, depending on the assay. After centrifugation at maximal speed

($\geq$10,000 $\times g$) the entire upper phase can be collected. Alternatively, a constant volume (e.g., 800 µl) per sample can be collected to reduce signal deviations.

10. In the presence of EMT inhibitors, most of [^{3}H]AEA and [^{3}H]2-AG which does not penetrate into the cells will stick to the membrane phospholipid bilayer, due to their high hydrophobic nature. Washing cell pellets with 1 % BSA solution ensures almost complete removal of this "extracellular fraction" of [^{3}H]AEA and [^{3}H]2-AG, which sticks on the external leaflet of the membrane. In our experience, this fraction usually contains a high radioactive signal; therefore further washing of cell pellets with PBS after the washing step with 1 % BSA is done to remove the residual extracellular signal from the cell pellet. Avoiding this additional washing step with PBS alone can lead to a high variability of the radioactive signal associated to the cell pellets.

References

1. Nicolussi S, Gertsch J (2015) Endocannabinoid transport revisited. Vitam Horm 98:441–485
2. Fowler CJ, Tiger G, Ligresti A et al (2004) Selective inhibition of anandamide cellular uptake versus enzymatic hydrolysis-a difficult issue to handle. Eur J Pharmacol 492:1–11
3. Oddi S, Fezza F, Catanzaro G et al (2010) Pitfalls and solutions in assaying anandamide transport in cells. J Lipid Res 51:2435–2444
4. Nicolussi S, Chicca A, Rau M et al (2014) Correlating FAAH and anandamide cellular uptake inhibition using N-alkylcarbamate inhibitors: from ultrapotent to hyperpotent. Biochem Pharmacol 92:669–689
5. Fowler CJ (2013) Transport of endocannabinoids across the plasma membrane and within the cell. FEBS J 280:1895–1904
6. Felder CC, Dickason-Chesterfield AK, Moore SA (2006) Cannabinoids biology: the search for new therapeutic targets. Mol Interv 6:149–161
7. Chicca A, Marazzi J, Nicolussi S et al (2012) Evidence for bidirectional endocannabinoid transport across cell membranes. J Biol Chem 287:34660–34682
8. Ahn K, McKinney MK, Cravatt BF (2008) Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. Chem Rev 108:1687–1707
9. Gibellini F, Smith TK (2010) The Kennedy pathway—de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. IUBMB Life 62:414–428
10. Madeira A, Moura TF, Soveral G (2015) Aquaglyceroporins: implications in adipose biology and obesity. Cell Mol Life Sci 72:759–771
11. Cravatt BF, Giang DK, Mayfield SP et al (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384:83–87
12. Gulyas AI, Cravatt BF, Bracey MH et al (2004) Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. Eur J Neurosci 20:441–458
13. Blankman JL, Simon GM, Cravatt BF (2007) A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem Biol 14:1347–1356
14. Muccioli GG, Xu C, Odah E et al (2007) Identification of a novel endocannabinoid-hydrolyzing enzyme expressed by microglial cells. J Neurosci 27:2883–2889
15. Beltramo M, Piomelli D (2000) Carrier-mediated transport and enzymatic hydrolysis of the endogenous cannabinoid 2-arachidonylglycerol. Neuroreport 11:1231–1235
16. Oddi S, Fezza F, Pasquariello N et al (2009) Molecular identification of albumin and Hsp70 as cytosolic anandamide-binding proteins. Chem Biol 16:624–632
17. Bojesen IN, Hansen HS (2003) Binding of anandamide to bovine serum albumin. J Lipid Res 44:1790–1794

18. Marazzi J, Kleyer J, Paredes JM et al (2011) Endocannabinoid content in fetal bovine sera—unexpected effects on mononuclear cells and osteoclastogenesis. J Immunol Methods 373:219–228
19. Hajdu Z, Nicolussi S, Rau M et al (2014) Identification of endocannabinoid system-modulating N-alkylamides from Heliopsis helianthoides var. scabra and Lepidium meyenii. J Nat Prod 77:1663–1669
20. Nicolussi S, Viveros-Paredes JM, Gachet MS et al (2014) Guineensine is a novel inhibitor of endocannabinoid uptake showing cannabimimetic behavioral effects in BALB/c mice. Pharmacol Res 80:52–65
21. Kaczocha M, Glaser ST, Deutsch DG (2009) Identification of intracellular carriers for the endocannabinoid anandamide. Proc Natl Acad Sci U S A 106:6375–6380

Chapter 21

Assay of Endocannabinoid Oxidation by Cyclooxygenase-2

Shalley N. Kudalkar, Philip J. Kingsley, and Lawrence J. Marnett

Abstract

The endocannabinoids, 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide (AEA), are endogenous ligands for the cannabinoid receptors (CB_1 and CB_2) and are implicated in a wide array of physiological processes. These neutral arachidonic acid (AA) derivatives have been identified as efficient substrates for the second isoform of the cyclooxygenase enzyme (COX-2). A diverse family of prostaglandin glycerol esters (PG-Gs) and prostaglandin ethanolamides (PG-EAs) is generated by the action of COX-2 (and downstream prostaglandin synthases) on 2-AG and AEA. As the biological importance of the endocannabinoid system becomes more apparent, there is a tremendous need for robust, sensitive, and efficient analytical methodology for the endocannabinoids and their metabolites. In this chapter, we describe methodology suitable for carrying out oxygenation of endocannabinoids by COX-2, and analysis of products of endocannabinoid oxygenation by COX-2 and of endocannabinoids themselves from in vitro and cell assays.

Key words Cyclooxygenase-2, Endocannabinoids, PG-Gs, PG-EAs, In vitro assay, Cell assay, LC-MS/MS

1 Introduction

The endocannabinoids 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide (AEA) are neutral arachidonic acid (AA) derivatives that exert analgesic and anti-inflammatory effects via the activation of cannabinoid receptors, CB_1 and CB_2 [1, 2]. Much like arachidonic acid (AA), 2-AG and AEA are oxygenated by the second isoform of the cyclooxygenase enzyme, COX-2, that produces prostaglandin H_2-glycerol ester (PGH_2-G) and prostaglandin H_2-ethanolamide (PGH_2-EA), respectively [3, 4]. Each PGH_2 derivative undergoes further metabolism via prostaglandin synthases to a range of PG-glycerol esters (PG-Gs) and PG-ethanolamides (PG-EAs) that exhibit biological activities, such as activation of calcium mobilization in tumor cells and macrophages, modulation of inhibitory synaptic transmission, induction of neurotoxicity by enhancement of excitatory glutamatergic synaptic transmission, and induction of hyperalgesia and anti-inflammatory responses [5–10] (Fig. 1). Additionally, when the macrophage cell line (RAW264.7) is treated

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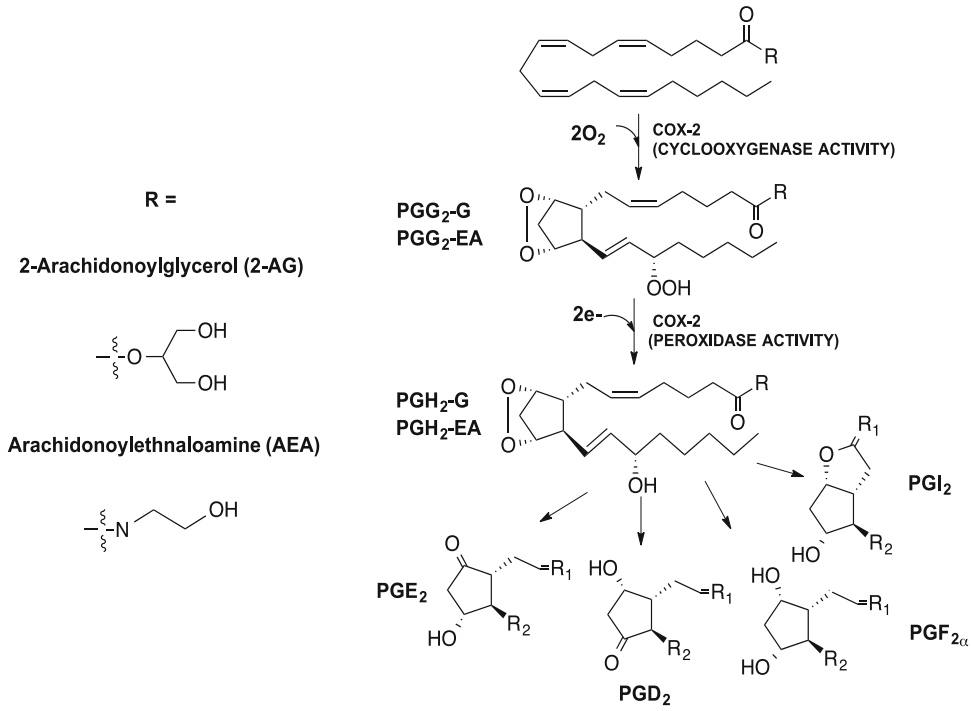

Fig. 1 Structures of endocannabinoids, 2-arachidonoylglycerol (2-AG), and arachidonylethanolamide (AEA), and their conversion by COX-2 and various PG synthases to prostaglandin glyceryl esters (PG-Gs) and prostaglandin ethanolamides (PG-EAs), respectively

with lipopolysaccharide and ionomycin, PG-Gs are produced which stimulate Ca^{2+} mobilization in the RAW264.7 cells [7, 11], suggesting that PG-Gs may exert independent biological activities.

Given the physiological importance of the endocannabinoids and the potential biological relevance of their COX-2-derived oxygenated products, dependable methods for investigating these interactions are indispensable. In this chapter, we provide stepwise instructions for (1) establishing reactions of 2-AG and/or AEA with COX-2 both in vitro and in the RAW264.7 macrophage cell line, and (2) LC-MS/MS methodology that enables quantitative analysis of the endocannabinoids and their oxygenated metabolites.

We have utilized two liquid chromatography-mass spectrometry (LC-MS/MS) methods: one for the analysis of COX-2 substrates (2-AG and AEA) and another one for the analysis of COX-2 oxygenation products (PG-Gs and PG-EAs). A silver cation (Ag^+) coordination, liquid-chromatography, electrospray-ionization, and tandem mass spectrometry (LC-ESI-MS-MS) method for analyzing 2-AG and AEA has been developed by our lab [12]. In this method, the silver cation coordinates with the four double bonds of the arachidonate backbone of 2-AG and AEA, which is rich in π electrons. This coordination of silver to 2-AG and AEA forms an $[M+Ag]^+$ complex that is amenable to electrospray ionization and tandem mass spectrometric techniques. Figure 2 shows the chromatograms of 2-AG, AEA,

and their respective internal standards coordinated with silver. In Fig. 2a, two peaks are seen corresponding to 1-AG and 2-AG, which are two isomers of arachidonoylglycerol. While 2-AG is the biologically relevant isomer of arachidonoylglycerol, 2-AG readily undergoes acyl migration under biological settings to form 1-AG [13].

Our laboratory has also published a method for the simultaneous analysis of several PG-Gs and PG-EAs [14]. A method describ-

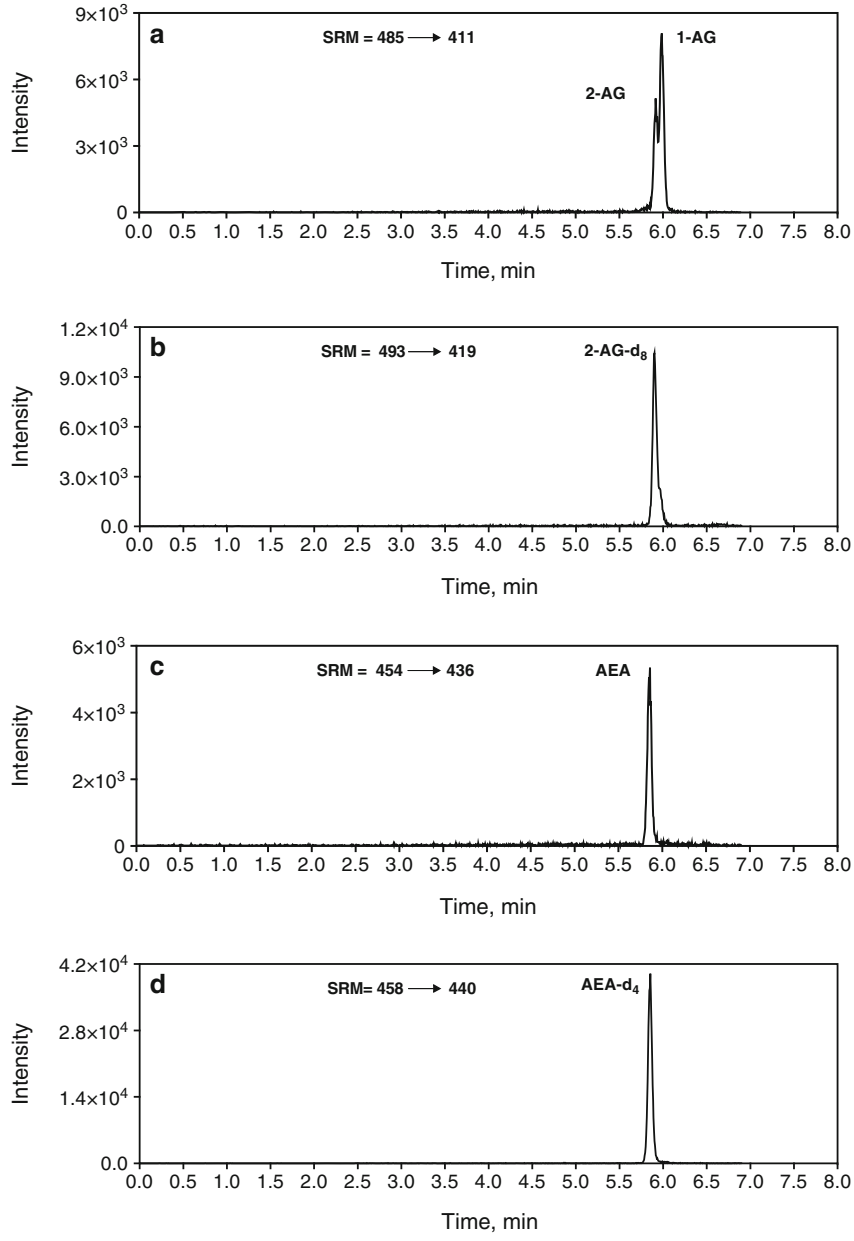

Fig. 2 LC-MS/MS chromatograms of endocannabinoids and their deuterated internal standards. 2- and 1-AG (a), 2-AG-d_8 (b), AEA (c), and AEA-d_4 (d)

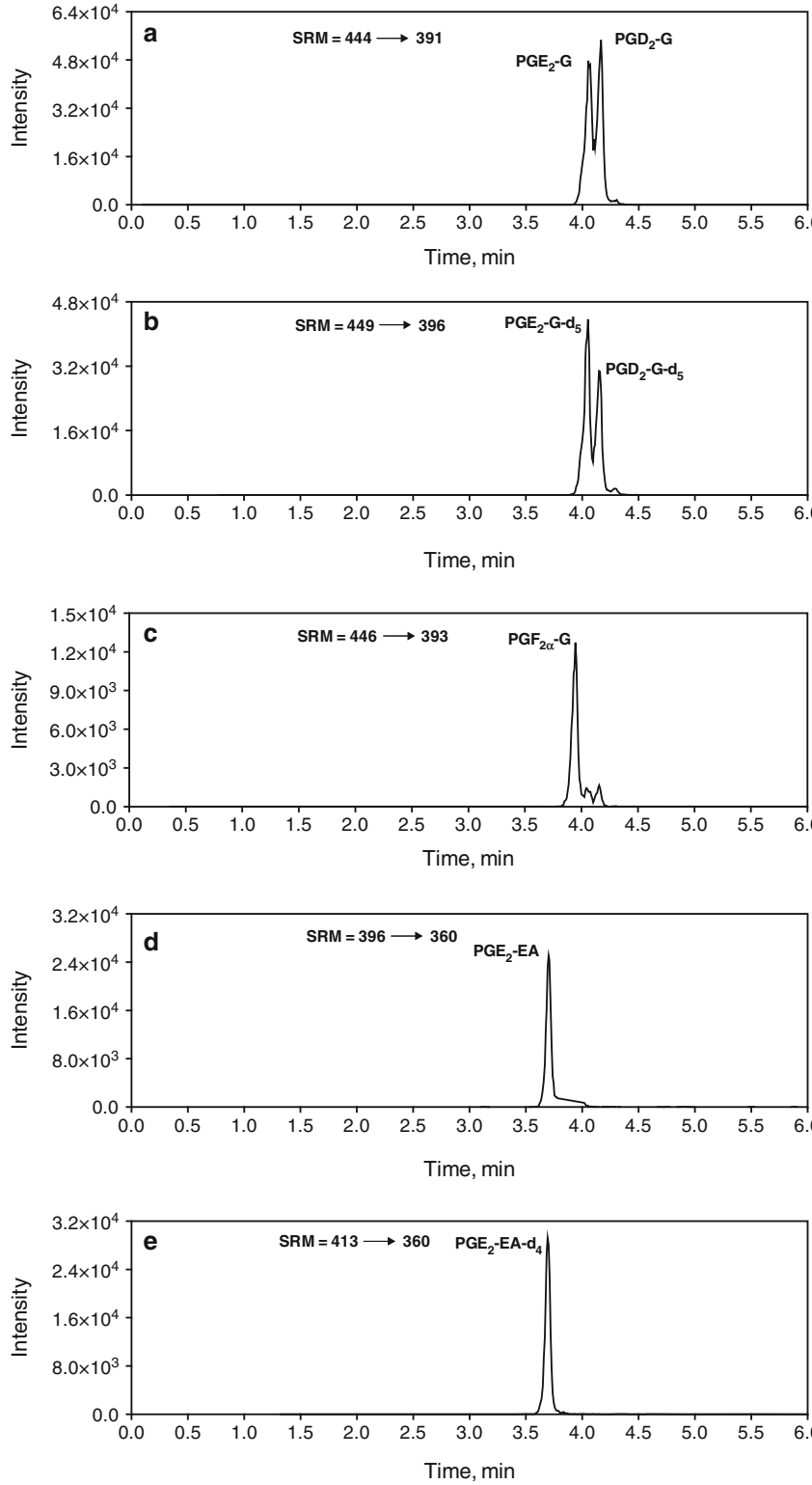

Fig. 3 LC-MS/MS chromatograms of selected PG-Gs and PG-EAs. PGE_2-G and PGD_2-G (a), PGE_2-G-d_5 and PGD_2-G-d_5 (b), $PGF_{2\alpha}$-G (c), PGE_2-EA (d), and PGE_2-EA-d_4 (e)

ing the analysis of $PGF_{2\alpha}$-EA also exists in the literature [15]. The methodology developed in our laboratory [14] involves complexing the neutral PG-Gs and PG-EAs with either ammonium (NH_4^+) or a proton (H^+). The resultant $[M+NH_4]^+$ or $[M+H]^+$ complexes yield multiple intense fragments upon collisionally induced dissociation (CID), several of which may be employed in selected reaction monitoring (SRM). Chromatograms of different species of PG-Gs and PG-EAs along with the respective internal standards are shown in Fig. 3.

2 Materials

2.1 General

1. 2-AG, AEA, deuterated 2-AG, and deuterated AEA: Additionally, the penta-deuterated analogue of PGE_2-G and tetra-deuterated PGE_2-EA were synthesized as described previously [3] (*see* **Note 1**).
2. 96-Well plate or auto-sampler vials.

2.2 In Vitro Assays

1. 5 mM Hematin stock solution in dimethyl sulfoxide (DMSO). Store at room temperature.
2. Reaction buffer: 100 mM Tris–HCl (pH 8.0) with 500 μM phenol. Store at room temperature.
3. Purified COX-2 enzyme [16].
4. Substrates or inhibitors of interest.
5. Quench solution: Internal standards dissolved in ethyl acetate with 0.5 % acetic acid. Place on ice before use in assay. The concentration of the internal standards in the quench solution should be such that the amount of internal standard delivered to each sample upon quenching is within tenfold of the amount of the analyte that the internal standard will be used to quantitate.

2.3 Cell Assay

1. RAW264.7 macrophages.
2. Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum.
3. 100 ng/ml Working solution of Kdo_2-lipid A (KLA) in Ca^{2+}/Mg^{2+}-free sterile Dulbecco's phosphate-buffered saline (DPBS) buffer. Make the working solution of KLA in serum-free medium.
4. 2 mM Ionomycin stock solution in DMSO: Our lab uses 2–5 μM of ionomycin as the final concentration.
5. Inhibitors of interest (*see* **Note 2**).
6. Extraction solution: It is the same as the "quench solution" in Subheading 2.2, **item 5**.

2.4 LC-MS/MS

1. Silver complexation mobile-phase components:
   A—150 μM silver acetate in HPLC-grade water plus 0.1 % formic acid.
   B—150 μM silver acetate in HPLC-grade methanol plus 0.1 % formic acid.
2. Ammonium complexation mobile-phase components:
   A—5 mM ammonium acetate in HPLC-grade water, pH adjusted to 3.2–3.4 with formic acid.
   B—6 % component A in HPLC-grade acetonitrile with 0.1 % formic acid.
3. HPLC column: C18, 5 × 0.2 cm, either 3 or 5 μm particle size.
4. LC-MS system: An HPLC system with a binary pump and autosampler in-line with a triple-quadrupole mass spectrometer and appropriate data acquisition software (*see* **Note 3**).
5. Standard mixture of analytes in methanol at a concentration of 2 μM—store at −20 °C.
6. Internal standard recovery solution (*see* **Note 4**).

3 Methods

3.1 In Vitro Assay

1. Prepare a 40× substrate stock solution whose concentration is 40 times the concentration of substrate in the reaction vessel. Prepare in DMSO and make enough to provide 5 μl for each 200 μl reaction.
2. Prepare a 40× inhibitor stock solution whose concentration is 40 times the concentration of inhibitor in the reaction vessel. Prepare in DMSO and make enough to provide 5 μl for each 200 μl reaction.
3. Prepare a COX-2 solution in 100 mM Tris-HCl buffer (pH 8.0) containing 500 μM phenol (the usual concentration of COX-2 is 50–100 nM). Add 3 equivalents of hematin solution to this enzyme solution 10–15 min prior to the experiment. Keep this enzyme solution on ice.
4. Prepare the quench solution and keep on ice.
5. For inhibition assays, aliquot 190 μl of the COX-2 solution into a 1.5 ml microfuge tube and incubate in heat block set at 37 °C for 3 min. For assays with weak-reversible inhibitors, add 5 μl of 40× inhibitor stock solution to the enzyme solution, and incubate at 37 °C for an additional 3 min. For assays with slow-tight binding inhibitors, add 5 μl of 40× inhibitor stock solution to 190 μl of the enzyme solution, and incubate at 37 °C for an additional 15 min. After the incubation period, add 5 μl of stock substrate solution and wait for 30 s (*see* **Note 5**).
6. For assays with only COX-2 and substrate, aliquot 195 μl of the COX-2 solution into a 1.5 ml microfuge tube, and incubate

in a heat block set at 37 °C for 3 min. Then, add 5 μl of 40× substrate stock solution and wait for 30 s (*see* **Note 5**).

7. After the 30-s reaction period, quench the reaction by adding 200 μl of the quench solution. Vortex vigorously and keep on ice.
8. Collect the top layer from the quenched reaction and add it to a small glass tube. Dry the solution under a stream of N_2.
9. Resuspend the dried solution with 200 μl of 1:1 methanol:water (HPLC-grade water) solution and vortex vigorously.
10. Aliquot ~200 μl of this solution to either auto-sampler vials or a 96-well plate.
11. Load the vials or plates into the autosampler of the LC-MS system; prepare a queue with an appropriate method and start the program as described in Subheading 3.3.

3.2 Cell Assay

1. Plate 3×10^6 cells in 8 ml of DMEM.
2. After 24 h, co-treat with KLA and inhibitor solution for 6 h, to get COX-2 activation (*see* **Note 6**).
3. Collect the media and transfer it to a 15 ml Falcon tube.
4. Extract PG-G or PG-EA species from the media by adding the extraction solution (twice the media volume) to the Falcon tube. Vortex vigorously and keep on ice.
5. Transfer the top layer to a clean vessel and dry it down under N_2.
6. Resuspend the dried solution with 200 μl of 1:1 methanol:water solution and vortex vigorously.
7. Aliquot ~200 μl of this solution to either auto-sampler vials or a 96-well plate.
8. Load the vials or plates into the autosampler of the LC-MS system, prepare a queue with an appropriate method, and start the program as described in Subheading 3.3.

3.3 LC-MS Analysis

This section describes two LC-MS/MS methods: one for the analysis of COX-2 substrates (2-AG and AEA) and another one for the analysis of COX-2 oxygenation products (PG-Gs and PG-EAs). Both methods employ reverse-phase chromatography and mass spectrometric detection, where the mass spectrometer is equipped with an electrospray source, operated in positive ion mode and configured for selected reaction monitoring (SRM). Obviously, not all analytes described here need to be included in one's assay, the choice of analytes depending on the experimental parameters and the interest of the investigator. These methods are based on literature data for endocannabinoids [12] and prostanoids [14].

1. Prime the LC system with appropriate mobile phase (*see* Subheading 2.4, **item 1** or **2**) and establish mobile phase flow through the chosen column at the initial conditions.

Table 1
SRM transitions for endocannabinoids *via* silver complexation analysis

Compound	M.W.	Q1 (*m*/*z*)	Q3 (*m*/*z*)	Collision energy
2-AG	378.6	485	411	33
2-AG-d_8	386.6	493	419	33
AEA	347.5	454	436	33
AEA-d_4	351.5	458	440	33

Table 2
SRM transitions for oxygenated products of COX-2

Compound	M.W.	Q1 (*m*/*z*)	Q3 (*m*/*z*)	Collision energy
PGE_2-G and PGD_2-G	426.6	444	391	19
$PGF_{2\alpha}$-G	428.6	446	393	19
PGE_2-G-d_5	431.6	449	396	19
PGE_2-EA	395.6	396	360	13
PGE_2-EA-d_4	399.6	400	364	13

Table 3
Gradient for silver complexation LC-MS/MS

Time	%B
Initial	70
0.5	70
4.5	100
5.5	100
6.0	70
7.5	70

2. Create (or modify an existing) instrument method containing the desired SRM transitions and chromatographic gradient profile, as specified in Tables 1, 2, 3, and 4 (*see* **Note 7**).
3. Place samples in sample tray and create a run sequence. It is best to bracket all unknowns with standards, and then randomize the order in which the unknowns are analyzed.
4. Inject the standard solution, and verify that all analytes are observed.
5. Inject the internal standard recovery solution, and verify that the retention times are very similar to **step 4**, that no analyte peak is observed in the appropriate SRM transition, and that all internal standards are observed (*see* **Note 4**).

Table 4
Gradient for oxygenated product LC-MS/MS

Time	%B
Initial	30
0.5	30
2.0	80
3.70	80
3.74	30
3.75	30

6. Inject a blank and ensure that no peaks appear in any transition.
7. Start the sequence.
8. After the samples have been successfully injected, prepare a processing method and process the resultant raw files.
9. Export data to Excel or other spreadsheet, and calculate analyte amounts (*see* **Note 8**).

   The representative chromatograms of COX-2 substrates, 2-AG and AEA, and their oxygenated products are shown in Figs. 2 and 3. For SRM analysis of 2-AG and 2-AG-d_8, the transitions of *m/z* 485–411 and of *m/z* 493–419 are used (Fig. 2a, b). For AEA, the *m/z* 454–436 transition is employed for SRM analysis, while the *m/z* 462–444 transition is used for detection of AEA-d_8 (Fig. 2b, c). For PG-Gs and PG-G-d_5 PGs, *m/z* 444–391 and *m/z* 449–396 transitions are used for SRM analysis, respectively (Fig. 3a, b). A transition of *m/z* 446–393 is used for PG-$F_{2\alpha}$-G (Fig. 3c). For SRM analysis of PG-EA and PG-EA-d_4, *m/z* 396–360 and *m/z* 400–364 transitions are used (Fig. 3d, e).

4 Notes

1. Many vendors sell deuterated internal standards. It is in the researchers' best interest to establish the isotopic purity of purchased internal standards before use with unknown samples. Our lab has found that some isotopically labeled compounds have a range of stable isotope incorporation, and that some isotopically labeled compounds will give a signal in the SRM channel for the native compound of interest.
2. Stock solutions of inhibitors are made in DMSO.
3. Our lab has employed both a Thermo Quantum triple-quadrupole (with Xcalibur software) and a SCIEX 3200 QTrap (with Analyst software) instruments. Any reasonably modern triple-quadrupole or ion trap mass spectrometer should give reasonable results for the methods discussed here.

4. The recovery internal standard is a standard where the amount of internal standard in each sample is dissolved in the reconstitution volume used for each sample. This sample is important because it (1) establishes whether the instrument is working acceptably, and (2) gives the experimenter the recovery level of his/her analytes, which is a useful parameter when assessing the experimental results.
5. It is important to limit the percent DMSO to ≤ 5 % to prevent protein precipitation.
6. Slow-tight binding inhibitors are added along with KLA for 6 h. In contrast, weak-reversible inhibitors plus 5 μM ionomycin are added after 6 h of treatment with KLA. The addition of ionomycin releases 2-AG. Cells are incubated for an additional 45 min before extracting PGs.
7. Flow rate of 0.3–0.4 ml/min is recommended for both LC-MS-MS methods.
8. Stable isotope dilution is used for quantification of analytes in the assays described above. With stable isotope dilution, the amount of analyte in each sample equals the response ratio (analyte peak area divided by the internal standard peak area) of the analyte multiplied by the amount of internal standard added to the sample.

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References

1. Devane WA, Hanus L, Breuer A et al (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258:1946–1949
2. Stella N, Schweitzer P, Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. Nature 388:773–778
3. Kozak KR, Rowlinson SW, Marnett LJ (2000) Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. J Biol Chem 275:33744–33749
4. Yu M, Ives D, Ramesha CS (1997) Synthesis of prostaglandin E_2 ethanolamide from anandamide by cyclooxygenase-2. J Biol Chem 272:21181–21186
5. Sang N, Zhang J, Chen C (2006) PGE_2 glycerol ester, a COX-2 oxidative metabolite of 2-arachidonoyl glycerol, modulates inhibitory synaptic transmission in mouse hippocampal neurons. J Physiol (Lond) 572:735–745
6. Sang N, Zhang J, Chen C (2007) COX-2 oxidative metabolite of endocannabinoid 2-AG enhances excitatory glutamatergic synaptic transmission and induces neurotoxicity. J Neurochem 102:1966–1977
7. Nirodi CS, Crews BC, Kozak KR et al (2004) The glyceryl ester of prostaglandin E_2 mobilizes calcium and activates signal transduction in RAW264.7 cells. Proc Natl Acad Sci U S A 101:1840–1845
8. Hu SS-J, Bradshaw HB, Chen JSC et al (2008) Prostaglandin E-2 glycerol ester, an endogenous COX-2 metabolite of 2-arachidonoylglycerol,

induces hyperalgesia and modulates nf kappa b activity. Br J Pharmacol 153:1538–1549

9. Valdeolivas S, Pazos MR, Bisogno T et al (2013) The inhibition of 2-arachidonoylglycerol (2-AG) biosynthesis, rather than enhancing striatal damage, protects striatal neurons from malonate-induced death: A potential role of cyclooxygenase-2-dependent metabolism of 2-AG. Cell Death Dis 4:e862
10. Alhouayek M, Masquelier J, Cani PD et al (2013) Implication of the anti-inflammatory bioactive lipid prostaglandin d_2-glycerol ester in the control of macrophage activation and inflammation by abhd6. Proc Natl Acad Sci U S A 110:17558–17563
11. Rouzer CA, Marnett LJ (2005) Glyceryl prostaglandin synthesis by resident peritoneal macrophages in response to a zymosan stimulus. J Biol Chem 280:26690–26700
12. Kingsley PJ, Marnett LJ (2003) Analysis of endocannabinoids by Ag^+ coordination tandem mass spectrometry. Anal Biochem 314:8–15
13. Rouzer CA, Ghebreselasie K, Marnett LJ (2002) Chemical stability of 2-arachidonylglycerol under biological conditions. Chem Phys Lipids 119:69–82
14. Kingsley PJ, Rouzer CA, Saleh S et al (2005) Simultaneous analysis of prostaglandin glyceryl esters and prostaglandins by electrospray tandem mass spectrometry. Anal Biochem 343:203–211
15. Weber A, Ni JS, Ling KHJ et al (2004) Formation of prostamides from anandamide in FAAH knockout mice analyzed by HPLC with tandem mass spectrometry. J Lipid Res 45:757–763
16. Marnett LJ, Rowlinson SW, Goodwin DC et al (1999) Arachidonic acid oxygenation by COX-1 and COX-2 - mechanisms of catalysis and inhibition. J Biol Chem 274:22903–22906

Chapter 22

Oxygenation of Anandamide by Lipoxygenases

Guus van Zadelhoff and Mario van der Stelt

Abstract

The endocannabinoids anandamide and 2-arachidonoylglycerol are not only metabolized by serine hydrolases, such as fatty acid amide hydrolase, monoacylglycerol lipase, and α,β-hydrolases 6 and 12, but they also serve as substrates for cyclooxygenases and lipoxygenases. These enzymes oxygenate the 1*Z*,4*Z*-pentadiene system of the arachidonic acid backbone of endocannabinoids, thereby giving rise to an entirely new array of bioactive lipids. Hereby, a protocol is provided for the enzymatic synthesis, purification, and characterization of various oxygenated metabolites of anandamide generated by lipoxygenases, which enables the biological study and detection of these metabolites.

Key words Lipoxygenase, Endocannabinoid, Anandamide, 2-Arachidonoylglycerol, Oxygenated metabolite

1 Introduction

The endocannabinoids anandamide (AEA, (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(2-hydroxyethyl)icosa-5,8,11,14-tetraenamide) and 2-arachidonoylglycerol (2-AG, 1,3-dihydroxypropan-2-yl (5*Z*,8*Z*,11*Z*,14*Z*)-icosa-5,8,11,14-tetraenoate) are signaling lipids that act on type 1 and type 2 (CB_1 and CB_2) cannabinoid receptors [1, 2]. They modulate a wide array of physiological functions. For example, in the brain they act as retrograde messengers that inhibit neurotransmitter release, whereas at the periphery they influence immune cell migration [3, 4]. The biological effect of endocannabinoids at CB_1 and CB_2 receptors depends on their life-span in the extracellular space. Both endocannabinoids serve as substrates for metabolic serine hydrolases that hydrolyze the amide bond of AEA or the ester bond of 2-AG, thereby releasing arachidonic acid and ethanolamine or glycerol, respectively. Fatty acid amide hydrolase is the main enzyme responsible for anandamide hydrolysis, whereas 2-AG is converted by monoacylglycerol lipase, α,β-hydrolase 6 and 12 [5]. Hydrolysis of the amide or ester bond results in termination of CB_1 and CB_2 receptor signaling [6].

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Both AEA and 2-AG contain an arachidonic acid backbone, which may serve as a substrate for enzymes such as cyclooxygenases and lipoxygenases [7–11]. Indeed, both enzyme classes convert endocannabinoids into oxygenated metabolites, such as prostamides, prostaglandin-glyceryl esters, and hydro(pero)xy-derivatives. These oxygenated products do not necessarily serve to terminate endocannabinoid signaling, but may exert biological actions of their own and/or modulate the pharmacology of endocannabinoids by interfering with fatty acid amide hydrolase activity. The action of cyclooxygenases on endocannabinoids has been studied in detail and specific receptors have been found for the prostamides, whereas the characterization of the biological presence and action of the lipoxygenase products is lacking behind [12].

There are three types of lipoxygenases (5-, 12-, and 15-lipoxygenases) that insert O_2 at C5, C12, or C15 of arachidonic acid, respectively. The same enzymes oxygenate in a regio- and stereospecific manner also AEA and 2-AG, adding O_2 at different positions of their arachidonic acid backbone (Fig. 1). The resulting hydroperoxide products are rapidly and nonenzymatically reduced in the cell to their corresponding hydroxyl derivatives.

12(*S*)-Hydroxy-AEA has been shown to bind to CB_1 receptor with comparable affinity as AEA, whereas the other metabolites showed reduced binding affinity [13]. Of note, all lipoxygenase products were found to be inhibitors of fatty acid amide hydrolase in the low to submicromolar range. In various physiological processes, action of lipoxygenase metabolites of endocannabinoids has been suggested, based on intervention studies with lipoxygenase inhibitors [14]. To interrogate the physiological relevance and function of lipoxygenase metabolites of endocannabinoids in more detail, it is important to have sufficient quantities of the oxygenated products. Here, we provide a protocol for the enzymatic synthesis, purification, and analysis of 5(*S*)-hydroxy-AEA, 11(*S*)-hydroxy-AEA, 12(*S*)-hydroxy-AEA, 15(*S*)-hydroxy-AEA, 5(*S*),15(*S*)-dihydroxy-AEA, and 8(*S*),15(*S*)-dihydroxy-AEA (*see* **Note 1**) [13–15].

2 Materials

2.1 Buffers and Solutions

1. 0.1 M Sodium borate buffer (pH 9.0): Weigh 6.18 g boric acid and transfer to a 1 l conical flask. Add 900 ml of water and dissolve boric acid under magnetic stirring. Adjust the pH to 9.0 with 1 M NaOH, and bring the volume to 1 l.
2. 0.1 M Sodium phosphate buffer (pH 7.0): Weigh 15.60 g sodium phosphate monobasic dihydrate and transfer to a 1 l conical flask. Add 900 ml of water and dissolve sodium phosphate under magnetic stirring. Adjust the pH to 7.0 with 1 M NaOH and bring the volume to 1 l.

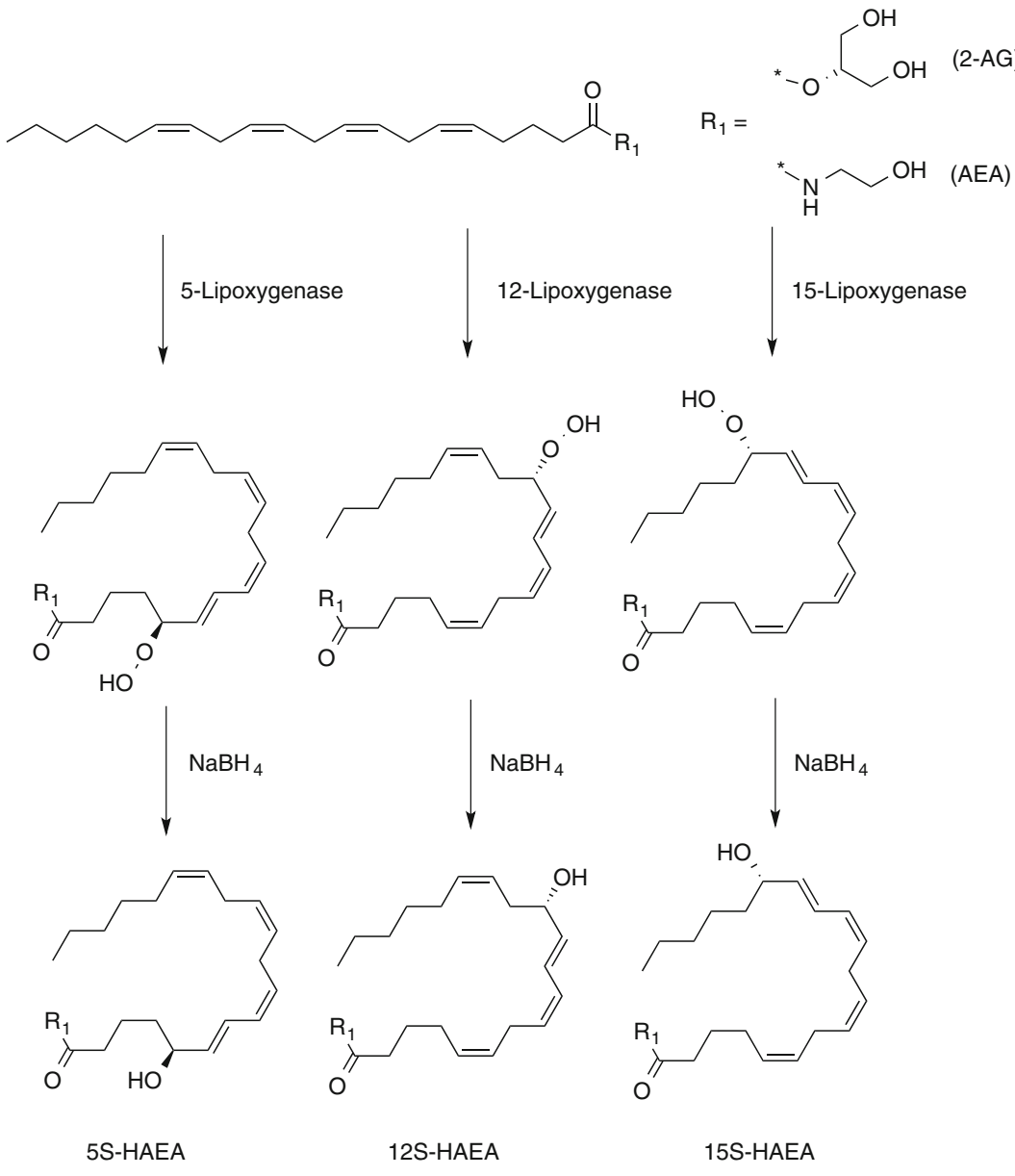

Fig. 1 Structures of oxygenated metabolites of anandamide produced by distinct lipoxygenases

3. PBS (pH 7.4): Weigh 8 g sodium chloride, 0.2 g potassium chloride, 1.80 g sodium phosphate dibasic dehydrate, and 0.24 g potassium phosphate monobasic anhydrous and transfer to a 1 l conical flask. Add 900 ml of water and dissolve the salts under magnetic stirring. Adjust the pH to 7.0 with 0.1 M HCl and bring the volume to 1 l.
4. Hypertonic PBS: Dissolve 2.7 g NaCl in 100 ml of PBS.

5. 0.05 M Sodium acetate buffer (pH 5.5), containing 134 g/l ammonium sulfate: Add 2.90 ml of glacial acetic acid to 700 ml of H_2O, and bring to pH 5.5 with 3 M NaOH. Add 134 g ammonium sulfate, dissolve, and bring volume to 1 l with H_2O.
6. 0.15 M Sodium citrate: Dissolve 22 g sodium citrate tribasic dehydrate in 500 ml of water.
7. 0.15 M Potassium phosphate (pH 6.0), containing 2 M ammonium sulfate: Dissolve 2 g potassium dihydrogen phosphate in 50 ml of water, and bring pH to 6.0 with 3 M KOH. Add 26.8 g ammonium sulfate, dissolve, and bring volume to 100 ml with H_2O.
8. 100 mM AEA: Weigh 35 mg AEA and dissolve in 1 ml of methanol in an autosampler vial.
9. Tetrahydrofuran/methanol/water/acetic acid mixture (25/30/45/0.1, vol/vol/vol/vol): Mix 250 ml of tetrahydrofuran, 300 ml of methanol, 450 ml of water, and 1 ml of glacial acetic acid. Degas the HPLC solvent just before use by purging helium through the solution for 15 min.
10. Methanol/water/acetic acid (75/25/0.1, vol/vol/vol): Mix 750 ml of methanol, 250 ml of water, and 1 ml of glacial acetic acid. Degas the HPLC solvent just before use by purging helium through the solution for 15 min.
11. Silylation reagent solution (pyridine/1,1,1,3,3,3-hexamethyldisilazane/trimethylchlorosilane, 5/1/1, vol/vol/vol): Mix 500 μl of anhydrous 99.8 % pyridine, 100 μl of 99.9 % 1,1,1,3,3,3-hexamethyldisilazane, and 100 μl of 99.0 % trimethylchlorosilane just before use in a 1 ml glass vial.

2.2 Preparation of Enzymes

Soybean lipoxygenase-1 (soyLOX, from Maple Glen cultivar) is a 15-lipoxygenase that can be obtained from Sigma-Aldrich. The protein concentration is estimated from the absorbance at 280 nm ($\epsilon_{280} = 1.6 \times 10^5\ \mathrm{l \times mol^{-1} \times cm^{-1}}$). The enzyme is stored at 4 °C at a concentration of 1.9 mg/ml (corresponding to 20 mM) in 0.05 M sodium acetate buffer (pH 5.5) containing 134 g/l ammonium sulfate. 5-Lipoxygenase, mostly isolated from potato, can be obtained from Cayman Chemicals. The enzyme is stored at 4 °C at a concentration of 1 mg/ml in 0.15 M potassium phosphate (pH 6.0) containing 2 M ammonium sulfate. Bovine lipoxygenase (bovLOX) is a 12-lipoxygenase isolated from bovine leukocytes, performing the entire purification at room temperature as follows. Bovine blood is collected in a vessel, containing 0.15 M sodium (100 ml per l) as an anticoagulant. The anticoagulated blood is centrifugated at $470 \times g$ for 20 min in a swing-out rotor, to separate blood cells from plasma: the lower part of the tube contains the red and white blood cells, and the upper part contains the platelet-rich plasma that is discarded. The leukocyte fraction is

separated from erythrocytes by lysis. To this end, 2 volumes of distilled water are added to the cell pellet and, after gently shaking for 30 s, 1 volume of hypertonic PBS is also added, to make the final suspension hypotonic. The mixture is centrifuged for 10 min at 470 × *g*, and the supernatant containing the lysed erythrocytes is removed. The pellet is resuspended in 10 ml of PBS, and the lysis procedure is repeated once more. The erythrocyte-free leukocyte pellet is suspended in 15 ml of PBS, and carefully layered on 14 ml of Ficoll-Paque plus (GE healthcare) in a 50 ml tube, and centrifuged at 400 × *g* for 40 min in a swing-out rotor. Leukocytes are found at the bottom of the tube, whereas the lymphocytes and monocytes are found on top of the Ficoll-Paque plus. Leukocytes are washed with PBS and centrifugated at 2000 × *g*, and the final cell pellet is resuspended in 10 ml of PBS.

3 Methods

3.1 Production of Hydroperoxy- AEA

1. Incubate for 45 min at room temperature 1 U enzyme (soyLOX, barLOX or bovLOX) with 5 μmol AEA (50 μl from a 100 mM stock) in 50 ml of rigorously stirred, air-saturated appropriate buffer: 0.1 M sodium borate buffer, pH 9.0 (for soyLOX), 0.1 M sodium phosphate buffer, pH 7.0 (for barLOX), or PBS, pH 7.4 (for bovLOX) (*see* **Notes 2–4**).
2. Stop reactions by acidifying the reaction mixtures to pH 4.0 with 3 M HCl.
3. Extract metabolites using 60 mg OASIS HLB (Waters) solid-phase extraction columns (or equivalent, like Bakerbond C18, J.T. Baker).Condition cartridge with 5 ml of methanol and 5 ml of H_2O, respectively. Do not allow the cartridge to run dry. Load reaction mixture into a 100 cc syringe connected to the SPE column via an SPE tube adaptor. Positive pressure is achieved by the plunger of the 100 cc syringe. Wash the column with 5 ml of H_2O until dryness, and elute the concentrated and purified reaction products with 2.5 ml of methanol.
4. Azeotropically evaporate residual water by co-evaporation with methanol under a gentle stream of nitrogen gas.
5. Dissolve products in 200 μl of methanol, and store at −25 °C until use.

3.2 Reduction of Hydroperoxy-AEA to Hydroxy-AEA

1. Reduce lipoxygenase products with a molar excess of $NaBH_4$ in 3 ml of methanol.
2. Stir reaction mixture for 30 min at 0 °C under a N_2 atmosphere.
3. Terminate reaction by adding 15 ml of water and acidify the mixture to pH 4.0 with 3 M HCl. Stir until gas production ends.

4. Extract and concentrate the reduced products with an SPE column, as described above.
5. Dissolve the metabolites in 100 µl of methanol.

3.3 Purification of Hydroxy-AEA

1. Purify the lipoxygenase metabolites with preparative HPLC on a Cosmosil 5C18-ARII (5 µm; 250×10 mm i.d.; Nacalai Tesque) column, using a tetrahydrofuran/methanol/water/acetic acid mixture (25/30/45/0.1, vol/vol/vol/vol) as the eluent, at a flow rate of 3 ml/min.
2. Dilute the collected fractions with a tenfold excess of water.
3. Concentrate the diluted fractions with an SPE column, as described above.
4. Dissolve the purified compounds in 200 µl of methanol.
5. Store compounds under N_2 at –25 °C.
6. Chiral separations of the purified sodium borohydride-reduced AEA metabolites can be carried out on a Chiralcel OD-R column (5 µm, 250×4.6 mm, Daicel) with methanol/water/acetic acid (75/25/0.1 vol/vol/vol) as the eluent, at a flow rate of 0.5 ml/min (*see* **Note 5**). Chirally pure AEA metabolites can be concentrated again with an SPE column as described above, and are stored under N_2 at –25 °C.

3.4 Analytical Characterization

1. Dry the hydroxyl-AEA derivatives under a stream of N_2 gas.
2. Dissolve them in $CHCl_3$ or $CDCl_3$, in order to perform FTIR- or ^{1}H-NMR measurements, respectively. Record IR spectra on an FTIR spectrometer from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1} and a co-addition of 60 scans in a N_2 atmosphere at 20 °C. Correct the spectra with a co-addition of 60 blank scans and a manual baseline correction.
3. ^{1}H-NMR spectra are recorded in $CDCl_3$ with a Bruker AC 300 or 500 MHz spectrometer at 27 °C.
4. For GC-MS analysis of the hydroxyl position, dry aliquots of the purified hydroxy-AEA metabolites under a stream of N_2 gas.
   (a) Redissolve in 1 ml of hexane and hydrogenate the double bonds with a catalytic amount (5 %) of palladium on calcium carbonate under a gentle stream of H_2 gas.
   (b) After 30 min, remove the catalyst by filtration over a prewashed (hexane) piece of cotton wool an empty pasteur pipette.
   (c) Evaporate hexane under N_2.
   (d) Add 50 µl of freshly prepared silylation reagent solution.
   (e) After 30 min at room temperature, evaporate the silylation reagent under a stream of N_2 and redissolve the residue in 10 µl of hexane.

(f) Analyze aliquots by GC/MS, equipped with a CP-Sil 5 CB-MS (or equivalent) column (25 m × 0.25 mm × 0.25 μm). The column temperature is held at 200 °C for 1 min, increased in 13 min to 330 °C, and held at this temperature for 2 min. Mass spectra are recorded under electron impact with an ionization energy of 70 eV.

5. Record IR spectra on an FTIR spectrometer from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} and a co-addition of 60 scans in a N_2 atmosphere at 20 °C. Correct the spectra with a co-addition of 60 blank scans and a manual baseline correction.
6. Record UV absorption spectra from 200 to 300 nm on 40 μM hydroxyl-AEA in methanol with a diode array spectrophotometer (*see* **Note 6**).
7. Record CD spectra (resolution of 1 nm and 20 scans, 10 nm/min) in a 1.0 cm cuvette at 20 °C on a CD spectrophotometer from 210 to 270 nm, using metabolites at a concentration of 40 μM in methanol (*see* **Note 7**).

3.5 Chemical Data of the Oxygenated Derivatives

5R/S-HAEA [5(R/S)-Hydroxy-eicosa-6E,8Z,11Z,14Z-tetraenoyl-N-(2-hydroxyethyl) amine]. Produced by barLOX: ^1H NMR ($CDCl_3$): δ 6.55 (dd, J = 13.6, 10.6 Hz 1H), 6.02 (t J = 10.5 1H), 5.71 (dd, J = 13.6 Hz, 1H), 5.39 (m, 5H), 4.16 (m, 1H), 3.72 (t, 2H), 3.42 (q, 2H), 2.97 (m, 2H), 2.81 (m, 2H), 2.22 (t, J = 7.8 Hz, 2H), 2.11 (m, 2H), 1.72 (q, J = 6.2 Hz 2H), 1.34 (m, 6H), 0.89 (t, J = 6.2 Hz, 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 515 [M^+], 500 [M^+–CH_3], 313 [$C_{16}H_{32}OTMS^+$], 304 [M^+–$C_{15}H_{31}$], 214 [304-TMSOH], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

11S-HAEA [11(S)-Hydroxy-eicosa-5Z,8Z,12E,14Z-tetraenoyl-N-(2-hydroxyethyl)amine]. Produced by barLOX: ^1H NMR ($CDCl_3$): δ 6.52 (dd, J = 11.0; 10.1 Hz 1H), 5.98 (t, J = 11.0 Hz 1H), 5.69 (dd, J = 15.1; 6.5 Hz 1H), 5.40 (m, 5H), 4.23 (q, 2H), 3.72 (t, J = 4.6 Hz, 2H), 3.42 (q J = 5.5 Hz; 2H), 2.81 (m, 2H), 2.32 (m, 2H), 2.22 (t, J = 7.4 Hz; 2H), 2.12 (m, 4H), 1.73 (q, 2H), 1.25 – 1.38 (m, 6H), 0.88 (t, J = 6.9 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 515 [M^+], 500 [M^+–CH_3], 388 [M^+–C_9H_{19}], 229 [$C_{10}H_{20}OTMS^+$], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

12S-HAEA [12(S)-Hydroxy-eicosa-5Z,8Z,10E,14Z-tetraenoyl-N-(2-hydroxyethyl)amine]. Produced by bovLOX: ^1H NMR ($CDCl_3$): δ 6.58 (dd, J = 14.2 Hz 1H), 5.99 (t, J = 10.5 Hz 1H), 5.74 (dd, J = 6.2 Hz 1H), 5.40 (m, 5H), 4.25 (q, 1H), 3.72 (t, J = 4.6 Hz 2H), 3.42 (q, J = 4.6 Hz 2H), 2.95 (m, 2H), 2.33 (m, 2H), 2.21 (t, J = 7.5 Hz 2H), 2.10 (m, 4H), 1.74 (q, J = 7.3 Hz, 2H), 1.28 (m, 6H), 0.89 (t, J = 6.9 Hz 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 515 [M^+], 500 [M^+–CH_3], 402

[M^+–C_8H_{17}+], 215 [$C_9H_{18}OTMS^+$], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

15S-HAEA [15(S)-Hydroxy-eicosa-5Z,8Z,11Z,13E-tetraenoyl-N-(2-hydroxyethyl)amine]. Produced by soyLOX: ^{1}H NMR ($CDCl_3$): δ 6.55 (dd, J=15.4;12,2 Hz 1H), 6.00 (t, J=10.7 Hz, 1H), 5.72 (dd, J=7.2 Hz, 1H), 5.40 (m, 5H), 4.12 (q, 1H), 3.72 (t, 2H) 3.42 (q, 2H), 2.97 (m, 2H), 2,82 (m, 2H), 2.22 (t, J=7.5 Hz, 2H), 2.11 (m, 2H), 1.72 (q, J=7.3 Hz, 2H), 1.56 (m, 2H), 1.31 (m, 6H), 0.89 (t, J=6.9 Hz, 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 515 [M^+], 500 [M^+–CH_3], 444 [M^+–C_5H_{11}], 173 [$C_6H_{11}OTMS^+$], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

5,15-diHAEA[5,15-Dihydroxy-eicosa-6E,8Z,11Z,13E-tetraenoyl-N-(2-hydroxyethyl) amine]. Produced by soyLOX: ^{1}H NMR ($CDCl_3$): δ 6.58 (m, 2H), 6.01 (m, 2H), 5.72 (m, 2H), 5.43 (m, 2H), 4.21 (m, 2H), 3.72 (t, J=4.6 Hz, 2H), 3.42 (q, J=4.6 Hz, 2H), 2.97 (m, 2H), 2.28 (t, J=6.9 Hz, 2H), 1.76 (m, 2H), 1.57 (m, 4H), 1.30 (m, 6H), 0.89 (t, 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 603 [M^+], 588 [M^+–CH_3], 532 [M^+–C_5H_{11}], 304 [M^+–$C_{15}H_{31}$], 214 [304-TMSOH], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

8,15-diHAEA[8,15-Dihydroxy-eicosa-5Z,9E,11Z,13E-tetraenoyl-N-(2-hydroxyethyl) amine]. Produced by soyLOX: ^{1}H NMR ($CDCl_3$): δ 6.70 (m, 2H), 5.97 (m, 2H) 5.74 (m, 2H), 5.46 (m,2H), 4.27 (m, 1H), 4.18 (m, 1H), 3.72 (t, J=4.6 Hz, 2H), 3.42 (q, J=4.6 Hz, 2H), 2.32 (m, 2H), 2.19 (t, J=6.9 Hz, 2H), 2.09 (q, 2H), 1.70 (m, 2H), 1.50 (m, 2H), 1.32 (m, 6H), 0.88 (t, 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 603 [M^+], 588 [M^+–CH_3], 532 [M^+–C_5H_{11}], 346 [M^+–$C_{12}H_{24}OTMS$], 173 [$C_6H_{11}OTMS^+$], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

4 Notes

1. This procedure is not suitable for the generation of lipoxygenase products of 2-AG, because of the chemical instability of this endocannabinoid that rapidly isomerizes to 1-AG during the incubation.
2. All solutions where organic solvents or reagents are involved should be in glass. Small beaker glasses (20–100 ml) or autosampler vials (1–20 ml) with PTFE septa are recommended.
3. Use 5 U soyLOX to produce 8(*S*),15(*S*)-dihydroxy AEA and 5(*S*),15(*S*)-dihydroxy-AEA.
4. Incubate 2 U barLOX with 5 μmol AEA in 50 ml of 0.1 M phosphate buffer (pH 7.4) for 60–90 min at 20 °C, to produce 5(*S*)- and 11(*S*)-hydroxy AEA.

5. Incubate 2 U bovLOX with 5 μmol AEA in 50 ml of 0.1 M phosphate buffer (pH 7.4) for 60–90 min at 20 °C, to produce 12S-hydroxy-AEA. Skip **step 2**, because the products are already reduced.
6. Alternatively, hydroxyl-AEAs can be separated with a 20-min linear gradient of methanol/water/acetic acid, from 60/40/0.1 (vol/vol/vol) to 95/5/0.1 (vol/vol/vol), at 1 ml/min on an analytical Cosmosil 5C18 ARII (5 μm, 250×4.6 mm) column.
7. A molar absorbance of 23,000 M^{-1} cm^{-1} at 236 nm is used to quantify hydroxy-AEAs. For 8,15- and 5,15-dihydroxy-AEAs, molar absorbances of 40,000 M^{-1} cm^{-1} at 269 nm and of 33,500 M^{-1} cm^{-1} at 243 nm are used, respectively.

References

1. Devane WA, Hanus L, Breuer A et al (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258:1946–1949
2. Sugiura T, Kondo S, Sukagawa A et al (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:89–97
3. Di Marzo V (2011) Endocannabinoid signaling in the brain: biosynthetic mechanisms in the limelight. Nat Neurosci 14:9–15
4. Piomelli D (2014) More surprises lying ahead. The endocannabinoids keep us guessing. Neuropharmacology 76:228–234
5. Cravatt BF, Giang DK, Mayfield SP et al (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384:83–87
6. Kohnz RA, Nomura DK (2014) Chemical approaches to therapeutically target the metabolism and signaling of the endocannabinoid 2-AG and eicosanoids. Chem Soc Rev. doi:10.1039/c4cs00047a
7. Hampson AJ, Hill WA, Zan-Phillips M et al (1995) Anandamide hydroxylation by brain lipoxygenase:metabolite structures and potencies at the cannabinoid receptor. Biochim Biophys Acta 1259:173–179
8. Ueda N, Yamamoto K, Kurahashi Y et al (1995) Oxygenation of arachidonylethanolamide (anandamide) by lipoxygenases. Adv Prostaglandin Thromboxane Leukot Res 23:163–165
9. Edgemond WS, Hillard CJ, Falck JR et al (1998) Human platelets and polymorphonuclear leukocytes synthesize oxygenated derivatives of arachidonylethanolamide (anandamide): their affinities for cannabinoid receptors and pathways of inactivation. Mol Pharmacol 54:180–188
10. Yu M, Ives D, Ramesha CS (1997) Synthesis of prostaglandin E2 ethanolamide from anandamide by cyclooxygenase-2. J Biol Chem 272:21181–21186
11. Kozak KR, Rowlinson SW, Marnett LJ (2000) Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. J Biol Chem 275:33744–33749
12. Urquhart P, Nicolaou A, Woodward DF (2015) Endocannabinoids and their oxygenation by cyclo-oxygenases, lipoxygenases and other oxygenases. Biochim Biophys Acta 1851:366–376
13. Van der Stelt M, van Kuik JA, Bari M et al (2002) Oxygenated metabolites of anandamide and 2-arachidonoylglycerol: conformational analysis and interaction with cannabinoid receptors, membrane transporter, and fatty acid amide hydrolase. J Med Chem 45:3709–3720
14. Veldhuis W, van der Stelt M, Wadman MW et al (2003) Neuroprotection by the endogenous cannabinoid anandamide and arvanil against in vivo excitotoxicity in the rat: role of vanilloid receptors and lipoxygenases. J Neurosci 23:4127–4133
15. Van Zadelhoff G, Veldink GA, Vliegenthart JF (1998) With anandamide as substrate plant 5-lipoxygenases behave like 11-lipoxygenases. Biochem Biophys Res Commun 248:33–38

Chapter 23

Assay of Endocannabinoid Oxidation by Cytochrome P450

Natasha T. Snider, Vyvyca J. Walker, and Paul F. Hollenberg

Abstract

Cytochrome P450 enzymes are a large family of heme-containing proteins that have important functions in the biotransformation of xenobiotics, including pharmacologic and environmental agents, as well as of endogenously produced chemicals with broad structural and functional diversity. Anandamide and 2-arachidonoylglycerol (2-AG) are substrates for P450s expressed in multiple tissues, leading to the production of a diverse set of mono- and di-oxygenated metabolites. This chapter describes tools and methods that have been used to identify major endocannabinoid-metabolizing P450s and their corresponding products, by using subcellular tissue fractions, cultured cells, and purified recombinant enzymes in a reconstituted system.

Key words P450 epoxygenase, P450 hydroxylase, Metabolism, Oxidation, Microsomes, Liquid chromatography-mass spectrometry, Eicosanoid

1 Introduction

Due to their structural similarity to arachidonic acid, the endocannabinoids anandamide and 2-AG are substrates for the three major classes of eicosanoid-metabolizing enzymes like cyclooxygenases, lipoxygenases, and cytochrome P450 [1–3]. The P450 branch of endocannabinoid metabolism yields a vast number of hydroxylated and epoxygenated products in vitro [4–9]. Furthermore, the epoxides of anandamide are subject to secondary metabolism via P450-mediated hydroxylation and epoxide hydrolase-mediated hydrolysis [6] (Fig. 1).

The 5,6-EET-EA metabolite of anandamide has increased stability compared to anandamide itself, and binds to type-2 cannabinoid (CB_2) receptor with nanomolar affinity, leading to decreased cAMP production in CB_2-expressing cells [10]. Murine BV-2 microglial cells stimulated with the pro-inflammatory cytokine interferon-γ (IFNγ) upregulate P450 3A1, leading to increased production of 5,6-EET-EA when the cells are treated with anandamide [10]. Similarly, the 2-AG epoxides 2-11,12-epoxyeicosatrienoylglycerol (EG) and 2-14,15-EG, which are generated by P450

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DOI 10.1007/978-1-4939-3539-0_23,

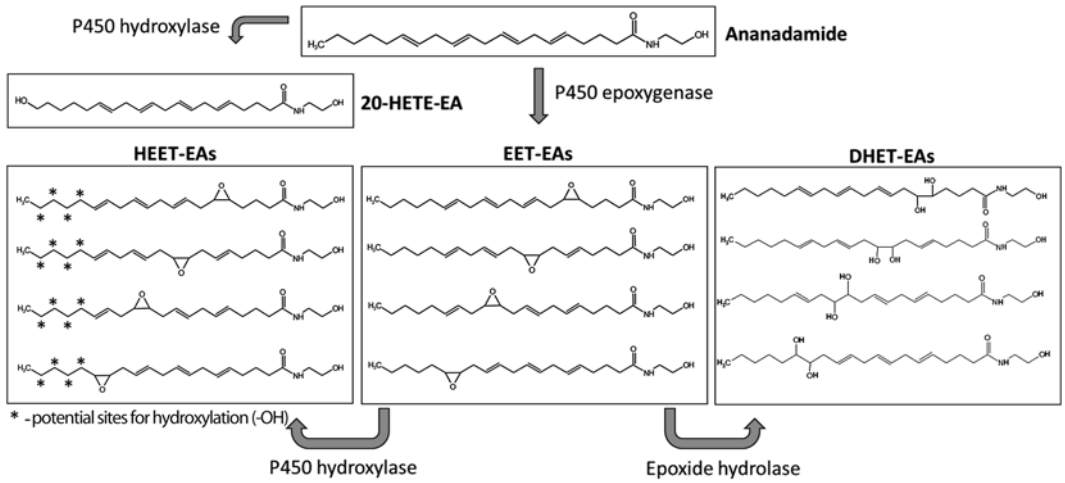

Fig. 1 Cytochrome P450 and epoxide hydrolase-derived anandamide metabolites. *HETE-EA* hydroxyeicosatetraenoic acid ethanolamide, *EET-EA* epoxyeicosatrienoic acid ethanolamide, *HEET-EA* hydroxy-epoxyeicosatrienoic acid ethanolamide, *DHET-EA* dihydroxyeicosatrienoic acid ethanolamide

epoxygenases, are ligands for the cannabinoid receptors, and are able to trigger downstream signaling events in cells (ERK signaling activation and cell migration) and physiological responses in mice (hypomotility, hypothermia, vasorelaxation) [11, 12]. Therefore, at least some of the P450-generated products of anandamide and 2-AG may be components of bioactivation pathways under specific contexts. Given that endocannabinoids are produced on demand and their concentrations are not easily quantified in tissues, studying the fate and functions of their metabolic products in vivo is a challenging task. Therefore, the physiological relevance of endocannabinoid metabolites generated by P450s, as well as by other endocannabinoid oxygenases, remains to be fully determined. In vitro P450 metabolism assays are useful to determine the contribution of specific P450 isoforms, thus elucidating the structural identity and kinetic parameters of substrate formation, and understanding the signaling pathways that may alter the P450 branch of endocannabinoid metabolism.

The three major tools to carry out in vitro metabolic assays of endocannabinoids are tissue microsomes (endoplasmic reticulum membranes enriched in P450s), purified P450s, and P450-expressing cells in culture. In general, the metabolic products are separated and analyzed by using liquid chromatography coupled with mass spectrometry (Fig. 2). Since P450 isoform-specific expression and activity change dynamically in response to (patho) physiological conditions, tissue-derived microsomes represent a valuable tool for investigating context-specific metabolic changes. Primary cells or cell lines are well suited for mechanistic studies, whereas purified enzymes are critical for performing enzyme kinetics and characterization of specific metabolites.

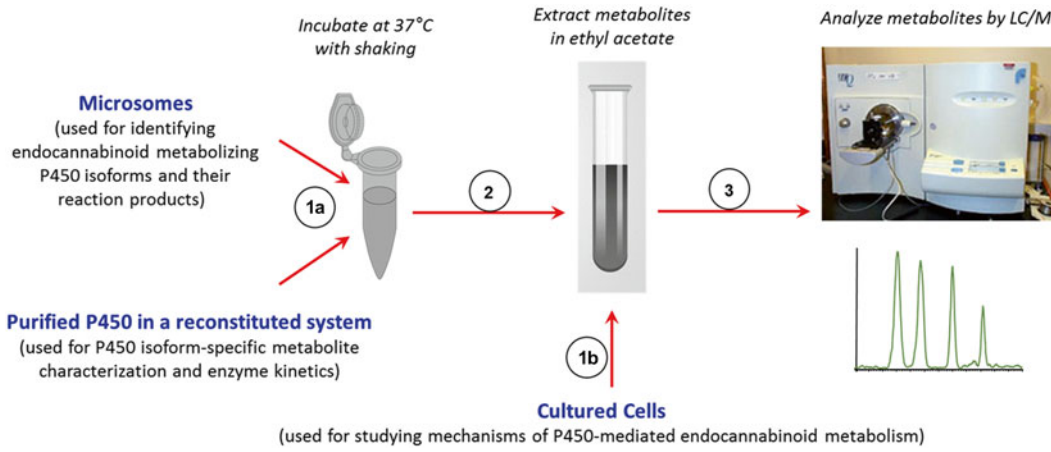

Fig. 2 General workflow for studying P450-mediated endocannabinoid metabolism in vitro

2 Materials

Microsomes should be prepared from freshly harvested tissues whenever possible, in order to minimize variability in the preparations [13]. In addition to microsomes, brain mitochondria are also enriched in P450s [14], so we recommend to analyze also this fraction in the context of brain P450s. Other sources of P450 enzymes are primary cells (most commonly hepatocytes) or recombinant enzymes that are used in a reconstituted system (*see* **Note 1**). Microsomes prepared from different human and animal tissues (generally in a pooled format), primary hepatocytes, and purified P450s are available from numerous commercial sources that are specialized in drug metabolic products (*see* **Note 2**).

2.1 Components for Metabolic Reactions

1. Source of P450: Tissue microsomes, cells, or purified P450.
2. Homogenization buffer for subcellular fractionation: 0.32 M Sucrose, 50 mM KH_2PO_4, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4.
3. Microsomal buffer: 100 mM KH_2PO_4, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20 % (vol/vol) glycerol, pH 7.4.
4. 100 mM Potassium phosphate (KPO_4) buffer, pH 7.4: Make 1 M K_2HPO_4 (dissolve 174.18 g in 1 l of water) and 1 M KH_2PO_4 (dissolve 136.09 g in 1 l of water). Mix 80.2 ml of 1 M K_2HPO_4 with 19.8 ml of 1 M KH_2PO_4, and dilute the mixture to 1 l with water to obtain 100 mM potassium phosphate buffer at pH 7.4.
5. Endocannabinoid substrate (anandamide or 2-AG): Prepare stock solutions by using nitrogen-purged ethanol, and store at −80 °C in tightly capped silanized glass vials. Stocks should be used within 4 weeks.

6. NADPH (nicotinamide adenine dinucleotide phosphate) cofactor: To make a 50 mM stock solution dissolve 42 mg β-NADPH in 1 ml of water. NADPH solutions should be prepared fresh just before use, and should be kept on ice. Use stocks at 1 mM final concentration in reaction mixtures.
7. Catalase: From bovine liver, use at 50 U per reaction for use in the reconstituted system (*see* **Note 3**).
8. Lipid mixture for reconstitution of purified P450s: Use 10 μg per reaction of a 1:1:1 mixture of L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidylserine.
9. P450 reductase: Purified recombinant enzyme, use at 50 pmol per reaction. For use in the reconstituted system.
10. Epoxide hydrolase: Purified recombinant enzyme, use at 1:1 ratio to P450.
11. P450 inhibitors: Broad and isoform-selective chemical and antibody inhibitors for various P450s are widely available from multiple commercial sources.

2.2 Components for Metabolite Extraction and Analysis

1. Ethyl acetate.
2. Methanol.
3. Acetic acid.
4. Deuterated standards for quantitation: Deuterated anandamide or 2-AG. All stock solutions, prepared using nitrogen-purged ethanol, should be stored in tightly capped silanized glass vials at −80 °C, and should be used within 4 weeks.
5. EET-EA and HETE-EA standards for generating standard curves (*see* **Note 4**).
6. Reversed-phase liquid chromatography column.
7. HPLC system.
8. Solvent A: 0.1 % Acetic acid in water.
9. Solvent B: 0.1 % Acetic acid in methanol.
10. Mass spectrometer.

3 Methods

3.1 General Protocol for the Preparation of Tissue Mitochondria and Microsomes

1. Fresh or frozen tissue samples are placed on ice in 2–4 volumes of homogenization buffer (0.32 M sucrose, 50 mM KH_2PO_4, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4), and are homogenized (100 strokes) to a suspension by using a Potter-Elvehjem polytetrafluoroethylene (PTFE) pestle and glass tube homogenizer. It is best to start with at least 0.25–0.50 g tissue, which may require pooling of small-size samples (e.g., patient-derived material).

2. The starting tissue homogenate is centrifuged for 8 min at 1500 × *g*, the pellet is discarded, and the supernatant is collected and centrifuged again for 20 min at 12,000 × *g*.
3. After an additional wash in homogenization buffer and centrifugation for 20 min at 12,000 × *g*, the pellet from **step 2** is resuspended in 2–4 volumes of homogenization buffer (this is the mitochondrial fraction).
4. The supernatant from **step 2** is centrifuged at 105,000 × *g* for 75 min using a table-top or preparative ultracentrifuge.
5. The supernatant is discarded and the pellet from **step 4** is washed once in microsomal buffer (100 mM KH_2PO_4, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20 % (vol/vol) glycerol, pH 7.4), and centrifuged again for 75 min at 105,000 × *g*. The washed pellet is resuspended in buffer B (this is the microsomal fraction).
6. Protein concentration of the mitochondrial and microsomal fractions is measured using a reducing agent-compatible protein assay kit.
7. Protein samples are analyzed for the presence of specific organelle marker proteins (e.g., mitochondrial cytochrome c and microsomal cytochrome P450 reductase) by Western blot, as a quality control of the fractionation procedure.
8. The fractions are aliquoted and stored at –80 °C for long-term use in metabolic reactions.

3.2 Metabolic Reactions

1. *Microsomes*: Incubation mixtures generally contain 75–100 μg microsomal protein, endocannabinoid substrate (0.1–100 μM dose range), and 100 mM KPO_4 buffer pH 7.4 in a final volume of 0.5 ml. The reactions are incubated for 10 min (*see* **Note 5**), and are initiated by the addition of 1 mM NADPH. A negative control (without NADPH) should always be included. Prior to the addition of NADPH, microsomes may be preincubated for 5 min with chemical or antibody inhibitors, in order to assay the relative contribution of specific P450 isoforms to endocannabinoid metabolism (*see* **Note 6**).
2. *Cells in culture*: For cell-based studies endocannabinoid substrate (20 μM starting concentration) in serum-free medium is added to the cells (plated onto 10 cm dishes), and incubation follows for 45 min (*see* **Note 7**).
3. *Purified P450s*: For metabolic reactions with purified P450s in an in vitro-reconstituted system mixtures contain 25–50 pmol of purified enzyme, 2 molar excess of P450 reductase, 10 μg of lipid mixture, and 50–500 U of catalase in 100 mM potassium phosphate buffer. After 45-min incubation on ice, substrate and NADPH are added, and the reactions are stopped after

10 min (*see* **Note 8**). To assay secondary metabolism of epoxides, purified recombinant microsomal epoxide hydrolase may be added to the reconstitution reaction during the 45-min incubation, prior to the addition of substrate and NADPH.

3.3 Extraction of Metabolites and Known Standards

1. After incubations are completed, all reactions containing either microsomal or purified P450s are spiked with 100 pmol of deuterated endocannabinoid standard (internal control for extraction efficiency), are immediately stopped by the addition of 4 reaction volumes (2 ml) of nitrogen-purged ethyl acetate, and are vortexed for 1–2 min.
2. Cultured cells are scraped into their medium and subjected to several freeze-thaw cycles to induce lysis. Samples are spiked with deuterated internal standard and extracted as above.
3. For quantitation studies, standard curves are generated by extracting known amounts of synthetic standards (EET-EAs and HETE-EAs) from the same matrix components as the reactions (e.g., potassium phosphate buffer or serum-free culture medium).
4. Samples are centrifuged at $1200 \times g$ to separate the top organic ethyl acetate layer from the bottom aqueous layer.
5. The organic layer is carefully collected, dried under a gentle stream of nitrogen, and resuspended in 100 μl of 100 % methanol (*see* **Note 9**).

3.4 Liquid Chromatography-Mass Spectrometry Analysis

1. Samples are loaded onto autosampler vials, and 10 μl is injected onto the appropriate reversed-phase column. We use a 4.6×100 mm Hypersil ODS column, 5 μm particle size (Thermo Scientific).
2. The column is pre-equilibrated with 75 % Solvent B and 25 % solvent A. Metabolites are resolved through the following gradient: 0–5 min, 75 % B; 5–20 min, 75–100 % B; 20–25 min, 100 % B; 25–26 min, 100–75 % B; and 26–30 min, 75 % B. The flow rate is 0.3 ml/min.
3. The mass spectrometer conditions for anandamide metabolism on an LCQ mass analyzer (Thermo Scientific) are as follows: sheath gas, 90 U; auxiliary gas, 0 U; capillary temperature 200 °C, and spray voltage 4.5 V. Data are acquired in positive ion mode by using one full scan from 300 to 500 mass-to-charge (m/z) ratios and one data-dependent scan of the most intense ion.

3.5 Data Analysis

1. Typical metabolic data using anandamide as substrate are shown for several purified P450s (Fig. 3), BV-2-cultured microglia cells (Fig. 4), and human liver microsomes (Fig. 5).
2. Using running conditions as specified above, m/z ratios of anandamide, EET-EAs, HETE-EAs, DHET-EAs, and HEET-EAs are 348, 364, 364, 382, and 380. The elution order is as follows:

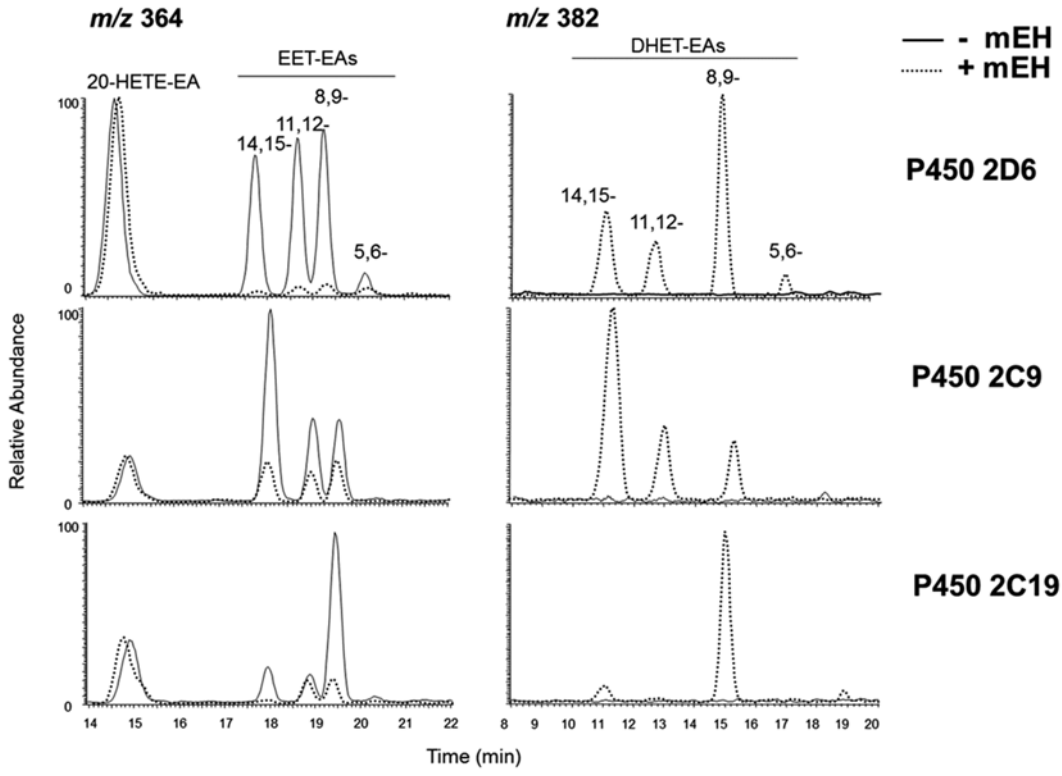

Fig. 3 Metabolism of anandamide by purified P450s 2D6, 2C9, and 2C19 in the reconstituted system, in the presence and absence of microsomal epoxide hydrolase. *Left* panels show the mono-oxygenated HETE- and EET-EAs (note the different metabolic profiles depending on the enzyme). *Right* panels show the di-oxygenated DHET-EAs, generated in the presence, but not in the absence, of microsomal epoxide hydrolase (mEH)

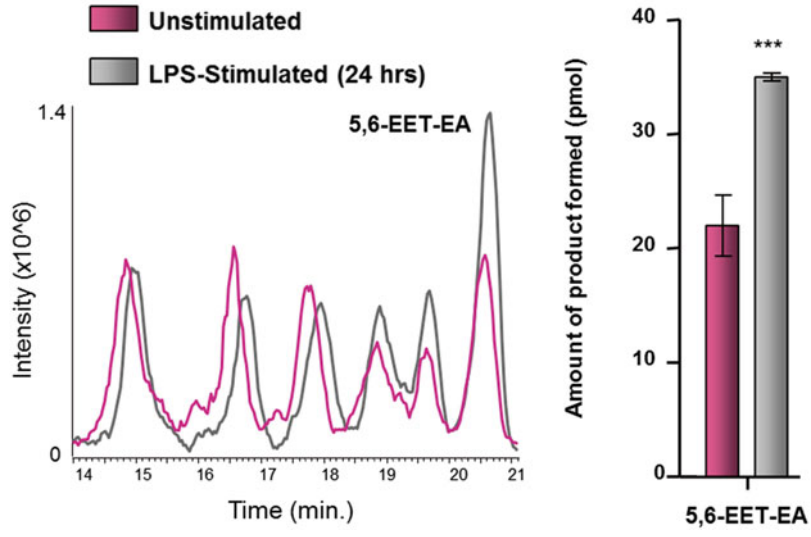

Fig. 4 P450 metabolites of anandamide formed by murine microglial BV-2 cells. The cells were either unstimulated or treated with lipopolysaccharide (LPS, 0.1 μg/ml for 24 h). Note the increased conversion of exogenously added anandamide to 5,6-EET-EA after LPS stimulation

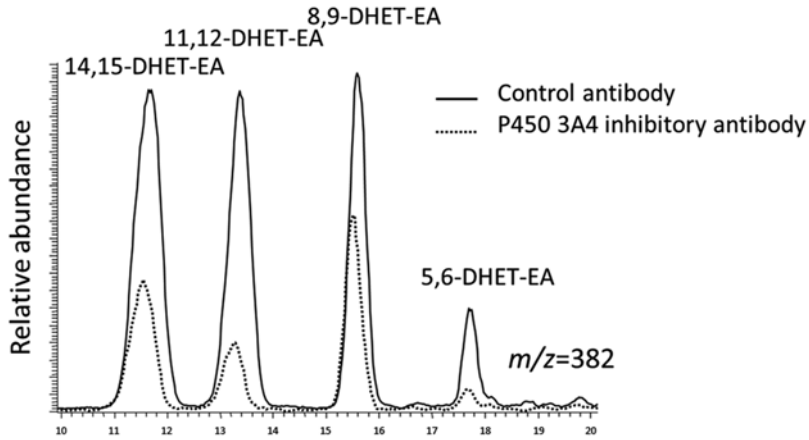

Fig. 5 Metabolism of anandamide by human liver microsomes in the presence and absence of an inhibitory antibody to P450 3A4. Shown are the di-oxygenated DHET-EA products, generated by microsomal epoxide hydrolase metabolism of the corresponding EET-EAs (extracted ion chromatogram $m/z = 382$)

HEET- EAs between 7 and 14 min; DHET-EAs between 11 and 18 min; 20-HETE-EA at 15 min; EET-EAs between 17 and 21 min; and the parent compound anandamide at 23 min.

3. Metabolite peak areas can be integrated by using an appropriate software (Xcalibur in our laboratory), and are expressed as ratios to the internal standard (d8-anandamide, m/z 356) in order to check extraction efficiency.
4. Peak areas of the synthetic metabolite standards (also normalized to an internal control) are similarly integrated to generate a standard curve (*see* **Note 10**).

4 Notes

1. Please note that primary cells should be used whenever possible, since most immortalized cell lines have generally low basal P450 expression and activity, as well as limited potential for P450 induction [15].
2. There is no standardized method for preparing tissue microsomes, and commercially available hepatic microsome preparations vary significantly in their P450 content and activity: this is a major *caveat* for these studies. We recommend to begin with a general subcellular fractionation protocol and to further optimize the specific buffer conditions. Although starting with fresh tissue is ideal, microsomes can also be prepared from snap-frozen human or animal tissues that have been properly stored for short or long term in liquid nitrogen [16].

3. Catalase is added to prevent inactivation of P450s by hydrogen peroxide. For P450 2D6 and P450 Supersomes™ assays, we add 500 U catalase. Supersomes™ Corning are commercially available microsomes from baculovirus-infected insect cells that express a specific P450 enzyme.
4. Synthetic standards for most P450-generated endocannabinoid metabolites are not commercially available. Hydroxylated and epoxygenated metabolites of anandamide (20-HETE-EA, 5,6-EET-EA, 8,9-EET-EA, 11,12-EET-EA, 14,15-EET-EA) and 14,15 epoxide of 2-AG (2-14,15-EG) can be purchased from Cayman Chemical.
5. Please note that at high substrate concentration (e.g., 100 μM) we observe enzyme inhibition. Therefore, a dose-response curve should always be performed. All quantitation experiments for kinetic purposes must be performed using substrate concentrations that are within the linear dynamic range of detection of the instrument used.
6. For chemical inhibition studies, a vehicle control should be included, and a negative control antibody (such as hen egg lysozyme) should be included in reactions containing inhibitory antibodies.
7. Cell seeding density, time of incubation, and endocannabinoid concentrations should be optimized depending on the cell type. Also note that the signal-to-noise ratios are significantly lower for whole-cell assays than for purified P450s and microsomes, due to the presence of other cellular lipid components in the organic extract.
8. Longer reaction times may be required when studying the reaction products of P450s with slower turnover rates, such as P450 2J2. This also holds true for microsomal studies.
9. If a precipitate persists after prolonged vortexing, additional solvent should be added to ensure complete solubilization prior to injection onto the column. DMSO may also be added up to 10 %, to improve solubility.
10. Since authentic standards for most P450-generated endocannabinoid metabolites are not currently available, structurally similar products may be used for generating standard curves that would allow to estimate metabolite concentrations.

Acknowledgements

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References

1. Snider NT, Walker VJ, Hollenberg PF (2010) Oxidation of the endogenous cannabinoid arachidonoyl ethanolamide by the cytochrome P450 monooxygenases: physiological and pharmacological implications. Pharmacol Rev 62:136–154
2. Zelasko S, Arnold WR, Das A (2015) Endocannabinoid metabolism by cytochrome P450 monooxygenases. Prostaglandins Other Lipid Mediat 116:112–123
3. Rouzer CA, Marnett LJ (2011) Endocannabinoid oxygenation by cyclooxygenases, lipoxygenases, and cytochromes P450: cross-talk between the eicosanoid and endocannabinoid signaling pathways. Chem Rev 111:5899–5921
4. Sridar C, Snider NT, Hollenberg PF (2011) Anandamide oxidation by wild-type and polymorphically expressed CYP2B6 and CYP2D6. Drug Metab Dispos 39:782–788
5. Snider NT, Sikora MJ, Sridar C et al (2008) The endocannabinoid anandamide is a substrate for the human polymorphic cytochrome P450 2D6. J Pharmacol Exp Ther 327:538–545
6. Snider NT, Kornilov AM, Kent UM et al (2007) Anandamide metabolism by human liver and kidney microsomal cytochrome p450 enzymes to form hydroxyeicosatetraenoic and epoxyeicosatrienoic acid ethanolamides. J Pharmacol Exp Ther 321:590–597
7. McDougle DR, Kambalyal A, Meling DD et al (2014) Endocannabinoids anandamide and 2-arachidonoylglycerol are substrates for human CYP2J2 epoxygenase. J Pharmacol Exp Ther 351:616–627
8. Stark K, Dostalek M, Guengerich FP (2008) Expression and purification of orphan cytochrome P450 4X1 and oxidation of anandamide. FEBS J 275:3706–3717
9. Bornheim LM, Kim KY, Chen B et al (1995) Microsomal cytochrome P450-mediated liver and brain anandamide metabolism. Biochem Pharmacol 50:677–686
10. Snider NT, Nast JA, Tesmer LA et al (2009) A cytochrome P450-derived epoxygenated metabolite of anandamide is a potent cannabinoid receptor 2-selective agonist. Mol Pharmacol 75:965–972
11. Chen JK, Chen J, Imig JD et al (2008) Identification of novel endogenous cytochrome p450 arachidonate metabolites with high affinity for cannabinoid receptors. J Biol Chem 283:24514–24524
12. Chen J, Chen JK, Falck JR et al (2007) Mitogenic activity and signaling mechanism of 2-(14,15-epoxyeicosatrienoyl)glycerol, a novel cytochrome p450 arachidonate metabolite. Mol Cell Biol 27:3023–3034
13. Jia L, Liu X (2007) The conduct of drug metabolism studies considered good practice (II): in vitro experiments. Curr Drug Metab 8:822–829
14. Dutheil F, Dauchy S, Diry M et al (2009) Xenobiotic-metabolizing enzymes and transporters in the normal human brain: regional and cellular mapping as a basis for putative roles in cerebral function. Drug Metab Dispos 37:1528–1538
15. Guguen-Guillouzo C, Guillouzo A (2010) General review on in vitro hepatocyte models and their applications. Methods Mol Biol 640:1–40
16. Pearce RE, McIntyre CJ, Madan A et al (1996) Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. Arch Biochem Biophys 331:145–169

Chapter 24

Assessing Gene Expression of the Endocannabinoid System

Mariangela Pucci and Claudio D'Addario

Abstract

Real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR), a major development of PCR technology, is a powerful and sensitive gene analysis technique that revolutionized the field of measuring gene expression. Here, we describe in detail RNA extraction, reverse transcription (RT), and relative quantification of genes belonging to the endocannabinoid system in mouse, rat, or human samples.

Key words Real-time quantitative polymerase chain reaction, RNA extraction, Reverse transcription, Fluorescent signals, Endocannabinoid system genes

1 Introduction

Polymerase chain reaction (PCR) has been adopted in many fields since it was first described in 1987 [1]. This three-step reaction allows the exponential increase of nucleic acid amount from complex biological materials, whose amount can be determined according to different strategies.

Reverse transcription (RT) followed by PCR monitors the increase of products generated during the reaction, by measuring in "real time" fluorescent changes on the basis of time or cycle at which amplification is first detected [2–5]. A fluorescent reporter is added to the reaction and the increase in fluorescence at each PCR cycle allows to quantify cDNA formation. The points at which fluorescent signals become detectable, or cross some arbitrary threshold value, are determined for each sample. The signals from different targets (housekeeping genes and genes under investigation) are usually amplified together, in order to make a relative quantitation. Thus, the qualitative "endpoint" assay represented by conventional PCR has been overcome by quantitative RT-PCR that following the kinetics of product formation has emerged as the most sensitive method for

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assaying gene expression levels.This is particularly valuable when transcripts are not abundant in a sample, or small changes in mRNA levels have to be detected.

Quantitative RT-PCR (qRT-PCR) assays are easy to perform and offer the advantage to combine high specificity, sensitivity, and reproducibility. However, this technique retains the typical problems of a classical PCR (e.g., amplification efficiency, amplification of unspecific products, primer-dimer and hetero-duplex formation); therefore it is crucial to recall that standardization and quality controls are always needed.

Here, we describe the quantitation of key members of the ubiquitous endocannabinoid system by qRT-PCR, an important tool to monitor their dynamic regulation in different cells and tissues, as well as under different physiological and pathological conditions.

2 Materials

Use reagenwts of high analytical grade and always prepare buffers with RNase/DNase-free water.

2.1 RNA Isolation

TRIzol Reagent or equivalent buffer containing phenol and guanidine isothiocyanate.

75 % Ethanol: To 100 ml of solution, add 75 ml of ethanol (≥99.8 %) and 25 ml of RNase/DNase-free water.

Refrigerated centrifuge and rotor capable to reach up to 12,000 × *g*.

Polypropylene microcentrifuge tubes with a capacity of 1.5 ml (sterile).

Heat block (at 60 °C).

2.2 Determination of RNA Yield and Integrity

Polypropylene microcentrifuge sterile tubes.

Spectrophotometer.

Horizontal electrophoresis system.

TAE (Tris-acetate-EDTA) buffer: Dissolve 4.85 g in 800 ml of water, and add 1.14 ml of glacial acetic acid and 2 ml of 0.5 M EDTA (pH 8.0). Add deionized water to 1 l, and store at room temperature (RT).

One % agarose gel: Weigh out 1 g of agarose into a 250 ml conical flask. Add 100 ml of TAE, and swirl to mix. Microwave for about 1 min to dissolve agarose (*see* **Note 1**), then cool it down on the bench for 5 min to about 60 °C, and add 10 μl of a 10 mg/ml solution of GelRed. Pour the gel slowly into the tank. Remove any air bubbles using a disposable tip, insert the comb, and check that it is correctly positioned.

Gel loading buffer (5×): Dissolve 25 mg bromophenol blue and 4 g of sucrose in 10 ml of H_2O (*see* **Note 2**).

DNA ladders (*see* **Note 3**).

2.3 RT Reaction

Polypropylene microcentrifuge sterile disposable plastic (tubes, strips, and caps).

cDNA Synthesis Kit.

Thermocycler.

2.4 qRT-PCR Reaction

Opaque or clear optical plastic for qPCR, based on the available RT-PCR instrument (i.e., tube and cap strips, or 96/384-well plates and adhesive films).

SYBR Green Master Mix.

Oligonucleotide forward and reverse PCR primers (*see* **Note 4** and Table 1).

RNase/DNase-free water.

RT-PCR instrument (*see* **Note 5**).

3 Methods

3.1 RNA Isolation

Homogenize the samples at RT by adding the TRIzol Reagent as suggested by the manufacturer [6] (*see* **Note 6**).

Increase the chemical/enzymatic activity with mechanical/physical methods (*see* **Note 7**).

Table 1
List of primers used for quantitative real-time RT-PCR

Gene		Human (9)	Mouse (10)	Rat (11)
CB_1	F	ccttttgctgcctaaatccac	ccaagaaaagatgacggcag	ttccaccgtaaagacagccc
	R	ccactgctcaaacatctgac	aggatgacacatagcaccag	tccacatcaggcaaaaggcc
CB_2	F	tcaaccctgtcatctatgctc	tcgcttacatccttcagacag	ttgaccgatacctatgtctgtgc
	R	agtcagtcccaacactcatc	tcttccctcccaactccttc	tgctttccagaggacataccc
"CB_3"	F	atctacatgatcaacctggc	attcgattccgtggataagc	atgctggtatgggttggaagc
	R	atgaagcagatggtgaagacgc	tgctgatgaagtagaggc	atgatgcaggctctctttcg
TRPV1	F	tcacctacatcctcctgctc	tgaactggactacctggaac	attgaacggcggaacatgacg
	R	aagttcttccagtgtctgcc	tccttgaagacctcagcatc	atctcttccagcttcagcg
FAAH	F	cccaatggcttaaaggactg	aaggcctgggaagtgaacaaagg	atggaagtcctccaagagc
	R	atgaaccgcagacacaac	aacctcctggactcttggagg	tagagctttcaggcatagcg
NAPE-PLD	F	ttgtgaatccgtggccaacatgg	aagtgtgtcttctaggttctcc	tgtcccgggttccaaagaggagc
	R	tactgcgatggtgaagcacg	ttgtcaagttcctctttggaacc	accatcagcgtcgcgtgtcc
DAGL	F	gccaccaagaggaggcagcg	aatggctatcatctggctgagc	attctctccttcctcctgc
	R	ccgcgtgcagcagaggaaca	ttccgagggtgacattcttagc	atttgggcttggtgcttcg
MAGL	F	atgcagaaagactaccctgggc	ttgtagatactggaagccc	atgttgaagaggctggacatgc
	R	ttattccgagagagcacgc	atggtgtccacgtgttgcagc	atgcagattccggattggc

Incubate the homogenate for 5–10 min at RT, and wait for total dissociation of the sample.

Add 0.1 ml of chloroform per 0.5 ml of TRIzol Reagent used in the first step, and incubate for 2–3 min at RT.

Centrifuge at 12,000 × *g* for 15 min at 4 °C (Fig. 1, *see* **Note 8**).

Without perturbing the interface, carefully recover the aqueous phase to a fresh tube (*see* **Note 9**).

Add 0.25 ml of 100 % isopropanol to the aqueous phase, and incubate at RT for 10 min.

Centrifuge at 12,000 × *g* for 10 min at 4 °C.

Remove the supernatant from the tube and wash the pellet with 0.5 ml of 75 % ethanol.

Vortex the sample and then centrifuge the tube at 7500 × *g* for 5 min at 4 °C.

Remove the supernatant and air-dry the pellet for 10 min at RT (*see* **Note 10**).

Resuspend the RNA in 30 μl of RNase/DNase-free water, and incubate in a heat block set to 60 °C for 15 min.

Samples can be stored at this stage at −80 °C for later use.

3.2 Determination of RNA Yield and Integrity

The integrity of purified RNA is critical to all techniques that assess gene expression.

In a 0.2 ml tube, dilute sample in RNase/DNase-free water (e.g., 2 μl of RNA and 98 μl of water) and measure the absorbance at 260, 280, and 230 nm (*see* **Note 11**).

Determine RNA concentration by using the formula $A_{260} \times \text{dilution} \times 40 = \mu\text{g RNA/ml}$.

Make the agarose gel as reported in Subheading 2.2. Leave to set for at least 30 min, and then pour TAE buffer into the gel tank to submerge the gel to 2–5 mm depth.

Transfer an appropriate amount of each sample (0.5–1.0 μg) to a microfuge tube, and add an appropriate amount of loading buffer into each tube (e.g., to reach a concentration of 230 μg/μl RNA = 4.3 μl of RNA + 2 μl of gel loading buffer + 2.7 μl of water).

Load the first well with marker and continue to load the samples into the other wells of the gel.

Close the gel tank, switch on the power source, and run the gel at 80 V for 20 min.

Check that current flow is regular (*see* **Note 12**).

Monitor the progress of the gel by reference to the marker dye, and stop the run when the bromophenol blue is at 3/4 of the gel length.

Switch off and unplug the gel tank and look at the gel on the UV light box (Fig. 2).

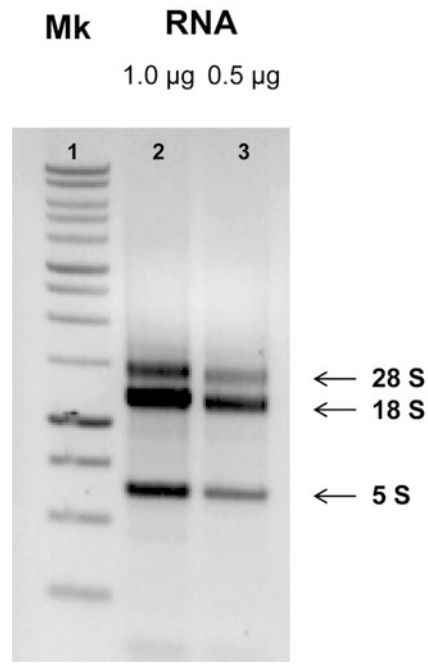

Fig. 1 Phenol-chloroform extraction used for RNA isolation. Following sample homogenization, three phases are visible: an upper aqueous phase, a lower 260 organic phase, and an intermediate phase. RNA is present in the aqueous phase, and is collected and precipitated by adding isopropanol

3.3 RT Reaction

Use 1 µg of total RNA to produce cDNA with the cDNA Synthesis Kit, following the manufacturer's instructions. Briefly: In a 0.2 ml tube, transfer 1 µg of total RNA and add 1 µl of oligo $(dT)_{18}$ primer (total volume = 12 µl).

Mix gently, centrifuge briefly, incubate at 65 °C for 5 min, and then place the vial on ice (*see* **Note 13**).

Add the following components in the indicated order (total volume 20 µl): reaction buffer, RNase inhibitor (1 U), dNTP mix (1 mM), and reverse transcriptase (20 U).

Incubate for 60 min at 42 °C, and then heat the reaction at 70 °C for 5 min to inactivate reverse transcriptase.

Each cDNA sample should be diluted between three- and ten-fold (*see* **Note 14**).

The reverse-transcribed RNA can be used immediately for second-strand cDNA synthesis reactions, or it can be stored at −20 °C for less than a week. For longer storage, −70 °C is recommended.

3.4 qRT-PCR

Set up the following PCR program on an RT-PCR instrument:

1. 95 °C	5 min	
2. 95 °C	10 s	>40 cycles
3. 55/60 °C (*see* **Note 15**)	30 s	
4. 72 °C	30 s	
5. 72 °C	5 min	
6. Melting curve (Fig. 3a): from 65 to 95 °C read every 0.5 °C and hold 00:00:02 (*see* **Note 16**)		
7. 4 °C pause		

Prepare the following mixture in each optical tube/well (final volume = 25 µl):

1. 1/2 µl of cDNA.
2. 1 µl of 10 µM primer forward.
3. 1 µl of 10 µM primer reverse.
4. 12.5 µl of SYBR Green Master Mix (2×).
5. RNase/DNase-free water to reach the final volume.

The final concentration of each primer (forward or reverse) in the mixture is 0.4 µM.

After PCR and dissociation curve analysis (melting curve) remove the tubes from the machine.

Check whether there is any bimodal dissociation curve or abnormal amplification plot.

Analyze the data with the software provided with your RT-PCR instrument.

3.4.1 Comparative C_t Method ($\Delta\Delta C_t$)

This method for relative quantitation of gene expression determines the changes in steady-state mRNA levels of a gene across multiple samples, and expresses the values relative to the levels of an internal control mRNA (*see* **Note 17**).

The most relevant parameter for quantitation is the threshold cycle C_t (*see* **Note 18** and Fig. 3b).

Differences in threshold cycle number (ΔC_t) are used to quantify the relative amount of the PCR target contained in each tube, according to the formula

$$\Delta C_t = C_t \text{ target} - C_t \text{ internal control}$$

Relative expression of different gene transcripts is calculated using the $\Delta\Delta C_t$ converted to relative expression ratio for statistical analysis, as follows:

$$\Delta\Delta C_t = \Delta C_t \text{ sample} - \Delta C_t \text{ control}$$

Comparative expression level = $2^{-\Delta\Delta C_t}$ (*see* **Note 19**).

4 Notes

1. The agarose solution can boil over very easily, so keep on checking it. It is good to stop warming up after 45 s and give it a swirl.
2. The loading buffer gives color and density to the sample, to make it easy to load it into the wells. Also, the dyes are negatively charged in neutral buffers, and thus they move in the same direction as the DNA during electrophoresis. This allows you to monitor the progress of the gel. Density is provided by glycerol or sucrose in the buffer.
3. DNA ladder is used for estimating the molecular weight of linear, double-stranded DNA fragments. The DNA ladder is composed of 20 highly purified, double-stranded DNA bands, spanning from 100 to 12,000 bp.
4. Human, mouse, and rat primers used to PCR amplification were designed using the Primer 3 software (http://primer3.ut.ee/) (Table 1).
5. DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA) was used to perform real-time PCR.

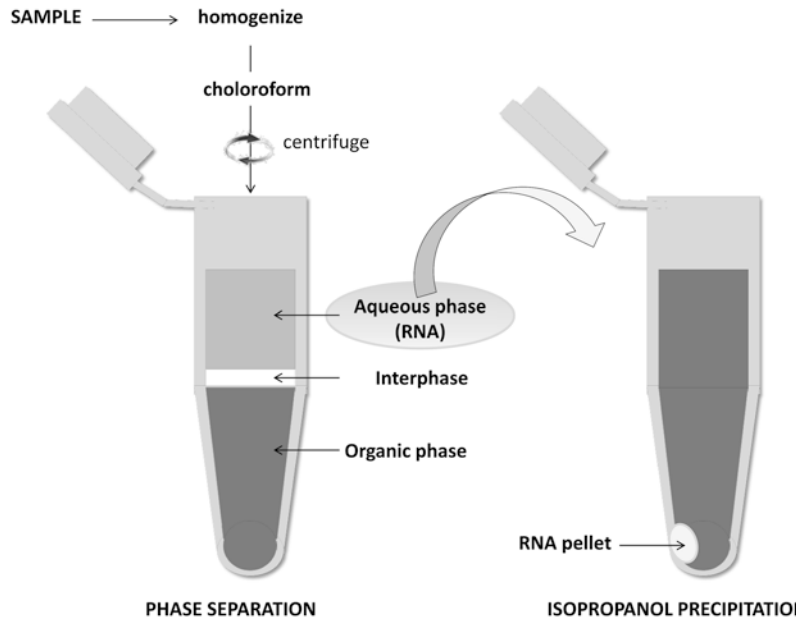

Fig. 2 Agarose gel electrophoresis. 1 and 0.5 μg of total RNA extracted from epithelial human cells run on a 1.0 % denaturing agarose gel. The 28S, 18S and 5S ribosomal RNA bands are clearly visible in the RNA sample. mRNAs run as a smear, because they represent a heterogeneously sized population. Sometimes tRNAs might be visible at the bottom of the gel. At least 200 ng of RNA must be loaded onto a denaturing agarose gel, in order to be visualized. Mk Marker

6. The sample volume should not exceed 10 % of the volume of TRIzol Reagent (e.g., to homogenize small pieces of tissue (20–25 mg) and cellular pellets ($2–3 \times 10^6$ cells) add 0.5 ml of buffer).
7. Several methods are commonly used to physically lyse samples, including mechanical disruption, liquid homogenization, freeze/thaw cycles, and manual grinding.
8. As a result of centrifugation, the mixture is separated into three phases: lower (phenol-chloroform or organic) phase containing DNA, lipids, and soluble proteins; interphase containing denatured proteins; and upper (aqueous) phase where RNA remains (Fig. 1).
9. Remove the aqueous phase by angling the tube at 45° and pipetting the solution out. Avoid contamination that occurs when pipetting the organic phase and/or the interphase.
10. Avoid drying the RNA completely, because pellet could lose solubility.
11. It is necessary to determine RNA absorbance at different wavelengths, in order to ascertain whether the extract is contaminated by unwanted molecules. The absorbance ratio at 260/280 nm is used to assess protein contamination, whereas that at 260/230 nm reveals other contaminants like phenol, guanidine, or carbohydrates. For a pure RNA preparation, the 260/280 value is ~2, and the 260/230 value is ~2.2.
12. The best way to check is to look at the electrodes and verify that they are evolving gas (i.e., bubbles).
13. RNA is first incubated with oligo$(dT)_{18}$ primer at 65 °C to denature RNA secondary structure, and then it is quickly chilled on ice to let the primer anneal to the RNA.
14. cDNA is diluted before PCR, because not-diluted cDNA might reduce reaction efficiency due to the alteration of optimum salt and pH conditions for the PCR reaction.
15. Gradient PCR is the best way to optimize the annealing temperature. Samples can be amplified in the same plate and with the same primer pair at different annealing temperatures (55, 57, and 60 °C) [7]. Melting curve and amplification plot analysis allow the selection of the optimal annealing temperature.
16. If SYBR green is used, a dissociation (melting) curve analysis should be performed. Ideally, the experimental samples should yield a sharp peak (first derivative plot) at the melting temperature of the amplicon (always >80 °C). These data indicate that the products are specific, and that SYBR Green I fluorescence is a direct measure of the accumulation of the product

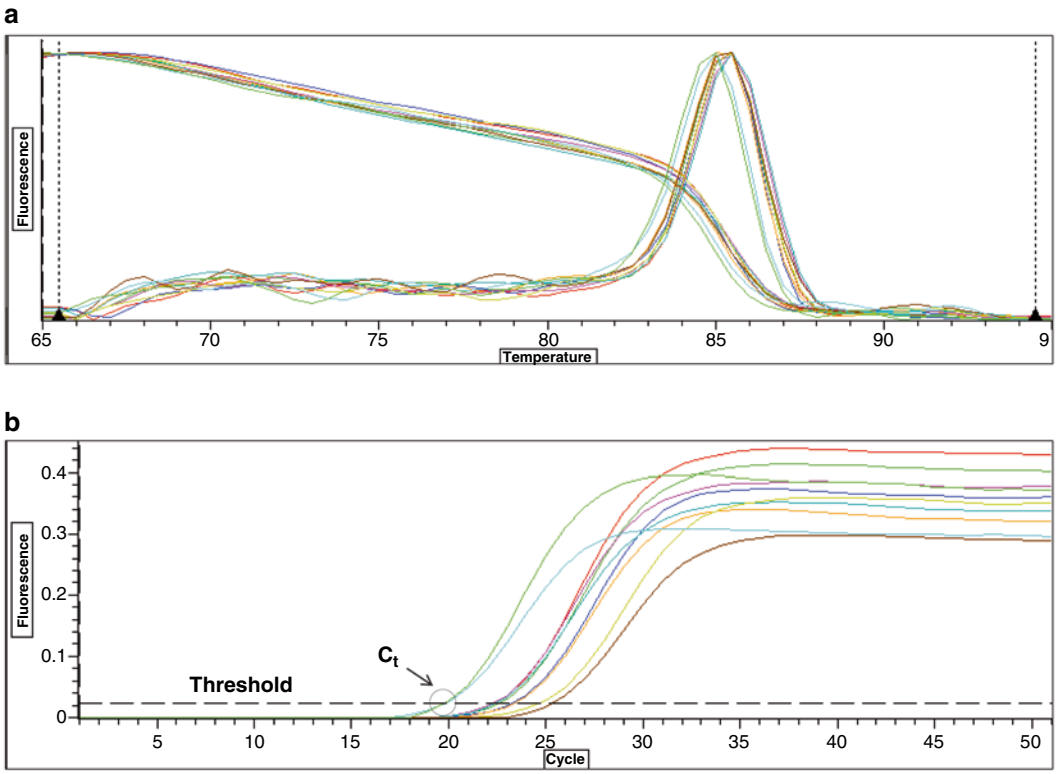

Fig. 3 Real-time PCR. (**a**) Typical derivative melting curves of amplicons after real-time PCR of RNA extracted from four different samples, each assayed in two replicates (showed by distinct colors). (**b**) Curves generated by SYBR Green Real-Time PCR for four samples, each assayed in two replicates

of interest. If the dissociation curve has a series of peaks (usually <80 °C), there is not enough discrimination between specific and nonspecific reaction products. To obtain meaningful data, optimization of the RT-PCR is necessary (Fig. 3a).

17. Gene expression of the chosen endogenous/internal controls should be more abundant and should remain constant, in proportion to total RNA, among the samples. By using an invariant endogenous control as an active reference, quantitation of an mRNA target can be normalized for differences in the amount of total RNA added to each reaction. To this aim, the most common reference genes are β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S rRNA [8]. The use of at least three internal controls is strongly recommended [9].
18. C_t (threshold cycle) is the intersection between an amplification curve and a threshold line (Fig. 3b). The higher is the initial amount of cDNA, the sooner the accumulated

product is detected in the PCR process, and the lower is the C_t value. This threshold should be placed above any baseline activity and within the exponential increase phase [10, 11].

19. Example of a comparative analysis of the expression level of a target gene between two hypothetical samples X (control) and Y. Target gene expression in sample Y is analyzed versus that in sample X.

	Target gene C_t	Internal control average C_t
Sample Y	27.4	15.2
Sample X	28.9	15.6
Sample Y ΔC_t	27.4 – 15.2 = 12.2	
Sample X ΔC_t	28.9 – 15.6 = 13.3	
Sample Y $\Delta\Delta C_t$	12.2 – 13.3 = –1.1	
Sample X $\Delta\Delta C_t$	13.3 – 13.3 = 0	
	Comparative expression level	
Sample Y	$2^{\wedge -(-1.1)} = 2.1$	
Sample X	$2^{\wedge -(0)} = 1$	

References

1. Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155:335–350
2. Orlando C, Pinzani P, Pazzagli M (1998) Developments in quantitative PCR. Clin Chem Lab Med 36:255–269
3. Lockey C, Otto E, Long Z (1998) Real-time fluorescence detection of a single DNA molecule. Biotechniques 24:744–746
4. Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193
5. Pfaffl MW, Hageleit M (2001) Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol Lett 23:275–282
6. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
7. Ishii K, Fukui M (2001) Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. Appl Environ Microbiol 67:3753–3755
8. Radonić A, Thulke S, Mackay IM et al (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 313:856–862
9. Pucci M, Pasquariello N, Battista N et al (2012) Endocannabinoids stimulate human melanogenesis via type-1 cannabinoid receptor. J Biol Chem 287:15466–15478
10. Catanzaro G, Battista N, Rossi G et al (2011) Effect of capacitation on the endocannabinoid system of mouse sperm. Mol Cell Endocrinol 343:88–92
11. Di Francesco A, Falconi A, Di Germanio C et al (2015) Extravirgin olive oil up-regulates CB1 tumor suppressor gene in human colon cancer cells and in rat colon via epigenetic mechanisms. J Nutr Biochem 26:250–258

Chapter 25

Western Blotting of the Endocannabinoid System

Jim Wager-Miller and Ken Mackie

Abstract

Measuring expression levels of G protein-coupled receptors (GPCRs) is an important step for understanding the distribution, function, and regulation of these receptors. A common approach for detecting proteins from complex biological systems is Western blotting. In this chapter, we describe a general approach to Western blotting protein components of the endocannabinoid system using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and nitrocellulose membranes, with a focus on detecting type 1 cannabinoid (CB_1) receptors. When this technique is carefully used, specifically with validation of the primary antibodies, it can provide quantitative information on protein expression levels. Additional information can also be inferred from Western blotting such as potential posttranslational modifications that can be further evaluated by specific analytical techniques.

Key words Electrophoresis, Antibody, Electrophoretic transfer, Fluorescent detection

1 Introduction

Western blotting facilitates the quantification of protein expression in a variety of tissues and allows inferences about pre- and post-translational processing (e.g., phosphorylation or alternative splicing). While Western blotting is a mature analytical technique, care must be applied in order to avoid the many pitfalls that accompany this procedure. In this chapter, we briefly describe the basic steps in Western blotting and some of the approaches we have adopted to improve efficiency, minimize artifacts, and increase the reliability of our results when detecting components of the endocannabinoid system.

2 Materials

Use distilled, deionized water for all solutions. Use analytical grade reagents whenever practical.

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2.1 Buffers and Solutions

1. Homogenization buffer: 25 mM Hepes (pH 7.4), 1 mM EDTA, 6 mM $MgCl_2$, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin.
2. 4× Sample buffer: 200 mM 1 M Tris–HCl (pH 6.8), 8 % sodium dodecyl sulfate, 40 % glycerol, 50 mM EDTA, 0.04 % bromophenol blue. This can be stored at room temperature for several weeks. Add 5 % (50 μl/ml) of 14.7 M β-mercaptoethanol just before use (*see* **Note 1**).
3. 10× TBS: 25 mM Tris base, 150 mM NaCl, 2.7 mM KCl. (pH 7.4). Add approximately 750 ml of water to a beaker with a large stir bar. Weigh out 30.3 g Tris base, 87.7 g NaCl, and 2 g KCl, and slowly add to stirring water. Bring pH to 7.4 with concentrated HCl and bring to 1 l volume. Autoclave and store at room temperature.
4. 10× Running buffer: 250 mM Tris–HCl (Trizma Base), 1.9 M glycine, 35 mM SDS. For 1 l of 10× running buffer: 30.25 g Tris base, 142.5 g glycine, and 10 g SDS. Mix and bring up to 1 l with ultrapure water.
5. 1× Running buffer: 100 ml of 10× running buffer + ultrapure water to a final volume of 1 l.
6. 1/2× Towbin solution: 12.5 mM Tris, 96 mM glycine, 20 % (v/v) methanol. For 1 l of Towbin buffer: 1.52 g Tris, 7.2 g glycine, and 500 ml of H_2O. Mix until salts are dissolved, add 200 ml of methanol, mix again, and bring to 1 l with H_2O.
7. Tris-buffered saline, Tween-20 (TBST): Add sufficient distilled water to 100 ml of 10× TBS to bring to 1 l. Mix in 500 μl Tween 20 until it is completely dissolved. Can store at room temperature. Should be used within several days.
8. LI-COR Biosciences Odyssey Blocking Solution.

2.2 Gel Apparatus Assembly

This protocol is based on the BioRad Mini-Protean II setup (*see* **Note 2**).

1. Materials: Short and tall glass plates (1 of each/gel), spacers (2/gel), gel-casting stand (one for every 2 gels), clamp assembly (1/gel), combs (1/gel), buffer chamber with bucket and electrode lid, U-shaped gasket, ultrapure water, and resolving and stacking gel solutions. Comb and spacers need to be of the same thickness (e.g., 0.5, 0.75, or 1.5 mm).
2. Thoroughly wash and dry glass plates with water, and then with 70 % ethanol.
3. Set up the clamp assembly and plates according to the manufacturer's instructions, making certain you tighten screws in a diagonal fashion to prevent glass from breaking.
4. Make sure that the bottom of the glass plates is aligned with the clamp assembly: misalignment will cause leakage.

5. Set clamp assembly on casting stand. Orientation of the clamp assembly is such that screws face the plastic flaps on the casting stand.
6. Make up solutions for resolving and stacking gels and degas (*see* **Note 3**); add APS and TEMED to the solution only when you are ready to pour each gel.
7. Pour the resolving gel—ensure that the height of the gel is at least 5 mm below the bottom of the plastic well-combs. Carefully (to avoid mixing), add ultrapure water or water-saturated isobutanol up to the top of the short plate and let resolving gel polymerize (≥30 min).
8. Decant the water and pour the stacking gel. Carefully add the well-combs to avoid bubbles and let stacking gel polymerize (30 min).
9. Once the stacking gel has polymerized, gently remove the comb and carefully rinse the wells with 1× running buffer to remove bubbles and unpolymerized acrylamide.

Recipes for 7.5 and 10 % Tris/glycine gels for the BioRad Miniprotean system. Resolving gels of other concentrations can be produced by altering the ratios of 30 % acrylamide and water as necessary.

Tris/glycine				
Resolving	**10 % (1 thick)**	**10 % (2 thick)**	**7.5 % (1 thick)**	**7.5 % (2 thick)**
30 % Acryl	3.3 ml	6.6 ml	2.5 ml	5.0 ml
1.5 M Tris pH 8.8	2.5 ml	5 ml	2.5 ml	5.0 ml
H_2O	3.95 ml	7.9 ml	4.9 ml	9.8 ml
20 % SDS	50 µl	100 µl	50 µl	100 µl
10 % APS	80 µl	160 µl	100 µl	200 µl
TEMED	8 µl	16 µl	8 µl	16 µl
Stack	1 thick	2 thick	4 thick	
30 % Acryl	665 µl	1330 µl	2660 µl	
1.0 M Tris pH 6.8	625 µl	1250 µl	2500 µl	
H_2O	1.5 ml	3 ml	6 ml	
50 % glycerol	1.5 ml	3 ml	6 ml	
20 % SDS	25 µl	50 µl	100 µl	
10 % APS	20 µl	40 µl	80 µl	
TEMED	5 µl	10 µl	20 µl	
1.5 M Tris base, pH 8.8	18.17 g/100 ml			
1.0 M Tris base, pH 6.8	12.11 g/100 ml			

2.3 Gel Electrophoresis and Blotting Equipment

1. There are many gel blotting systems available that provide excellent transfer of protein from gel to support membranes such as nitrocellulose. We use a 15 × 17 cm transfer unit from Idea Scientific Company.
2. Transfer: Transfer unit complete with sponges and electrodes, square-Pyrex pan, 1/2×-Towbin solution, filter paper (2/gel), nitrocellulose membrane (1/gel).
3. Fluorescent scanner capable of dual-infrared wavelength detection (e.g., LiCor Odyssey).

3 Methods

A variety of samples can be used as starting material for Western blotting. Primarily we use tissue homogenates and cultured cell lysates in our studies. Generally, it is best to process samples from live organism/cell until separation on the gel in a single day to minimize proteolytic loss and protein aggregation. At all times, keep samples cold (e.g., on ice). If necessary, lysates can be frozen (preferably at −80 °C) in sample buffer. They should be rapidly thawed, and spun at 3000 × *g* for 10 min, and pellet discarded before separation on the gel.

3.1 Typical Tissue Preparation

1. Place 3 ml of homogenization buffer into Falcon 2059 tubes, weigh, and place on ice.
2. Sacrifice animals and transfer dissected tissue to pre-chilled tubes and immediately homogenize with Polytron homogenizer.
3. Re-weigh tubes and adjust volume of buffer to 10:1 (vol:wt). Mix gently.
4. Spin samples at 700 × *g* and 4 °C for 5 min. Transfer supernatants to sterile tubes and resuspend pellets with original 10:1 volume of buffer. Repeat homogenization and spin samples at 700 × *g* for 5 min and 4 °C.
5. Pool like supernatants and centrifuge at 16,000 *g* for 30 min and 4 °C.
6. Discard supernatants and resuspend pellets in original 10:1 volume of homogenization buffer. Triturate thoroughly to wash membranes. Centrifuge samples again at 16,000 × *g* for 30 min and 4 °C.
7. Aspirate supernatants and resuspend pellets in suitably small volumes of homogenization buffer. Quantitate protein concentrations via Bradford Assay.
8. Mix desired amount of protein with 1:3 volume of 4× sample buffer, heat for 5 min at 65 °C (be aware that *boiling causes CB1 to aggregate and the aggregated receptor separates poorly on the resolving gel*), and load onto a gel.

3.2 Preparing Cultured Cells

1. Grow cells of interest on appropriate-size culture plates. In this example, we use 6-well plates. Treat cells as required for the experiment.
2. Place plate on ice, aspirate medium, and add 250 µl of homogenization buffer containing 0.5 % CHAPS. Scrape cells and homogenize in a dounce, or polytron homogenizer. Transfer lysate to 1.5 ml microfuge tubes and spin down samples at 700 × *g* and 4 °C for 10 min. Collect supernatant and add an equal volume of 2× SDS loading buffer.
3. Incubate tubes in 65 °C water bath for 5 min (you may need to sonicate proteins prior to water bath incubation, particularly for samples that are rich in nucleic acids, which may gel and impede efficient and quantitative sample handling).
4. Spin at 100 × *g* for 2 min, transfer supernatant to a fresh Eppendorf tube, and load onto a gel.

3.3 Running the Gel

1. Load samples and molecular marker using volumes appropriate for well size and gel thickness (*see* **Note 4**).
2. Using an L-shaped glass pipette, dislodge the bubbles at the bottom of the clamp assembly with a gentle stream of 1× running buffer. This ensures even current distribution throughout the gel.
3. Run the gel at 80 V until you see higher molecular weight marker bands of the ladder begin to separate (*see* **Note 5**).
4. Increase the voltage to ~120 V and let the gel run until the blue dye front runs off (1–1.5 h for minigels).
5. Electrophoresis unit can be placed in cold room or refrigerated cabinet when running at higher voltages to minimize heat artifacts (*see* **Note 6**).

3.4 Transferring the Gel

Soak nitrocellulose membrane first in water, and then in 1/2× Towbin buffer to hydrate. Also, immerse transfer sponges and filter paper in 1/2× Towbin buffer prior to setting up the transfer.

1. Disassemble the gel unit by loosening screws on clamp assembly and gently removing the top plate and spacers to expose the gel.
2. Remove the stacking gel from the resolving gel using the long edge of a spacer.
3. Set up the transfer so the gel and nitrocellulose are sandwiched between two pieces of filter paper, which is sandwiched between two transfer sponges. The current will run from the cathode to anode plate, so the gel and nitrocellulose should be situated so negatively charged protein from the gel runs onto the nitrocellulose. Be careful to remove all air pockets between the nitrocellulose and gel (*see* **Note 7**).

4. After setting up the upright transfer unit, check the level of buffer solution. The gel and filter paper must be completely submerged in buffer during transfer.
5. Run the transfer at 30–40 V for 1 h (voltage and time depend on transfer unit used; see manufacturers' instruction for more information).

3.5 Western Blot

1. Briefly soak nitrocellulose in TBS. (*Optional: Check success of the protein transfer by soaking nitrocellulose in Ponceau-S for 15 min. Wash Ponceau-S off with reagent-grade water. Do not use Tween in wash at this point, as this will significantly increase background on Odyssey scanner.*) (*See* **Note 8**.)
2. Incubate nitrocellulose in Licor blocking solution for 1 h at room temperature.
3. Incubate nitrocellulose overnight at 4 °C in primary antibody solution (primary antibody in PBS + 1:1 (Licor block:1× PBS). Dilution of primary antibody needs to be empirically determined (*see* **Note 9**).
4. The following day, wash nitrocellulose in TBS-T with four 15-min washes.
5. Incubate nitrocellulose in secondary antibody solution (secondary antibody (1:5000) + 1:1 Licor block:1× PBS) for 1 h at room temperature.
6. Repeat the four 15-min TBS-T washes.
7. Wrap blot in Saran wrap and scan with Odyssey.

4 Notes

1. SDS binds to the protein in an amount that is typically (but not always) proportional to the protein's molecular weight, allowing proteins to migrate according to size. Glycerol increases sample density relative to the buffer, facilitating efficient loading. Beta-mercaptoethanol is a strong reducing agent, which lessens protein aggregation via disulfide bond formation.
2. Pre-poured gels can be used in systems such as the Bio-Rad Mini-Protean and Thermo Fisher Scientific XCell SureLock systems. Their use will simplify setup.
3. We use house vacuum for 10–15 min to remove excess dissolved gas from the gel solution. Polymerization requires the formation of SO_4^-. Because oxygen serves as a free radical trap, excess amounts of this gas in the gel solution can result in non-uniform polymerization of the gel.
4. The loading capacity of a well is determined by the size of the comb's teeth. For example, teeth with dimensions of

1 mm × 6 mm × 8 mm will have a capacity of 48 μl. However, we have found that banding will be tighter and more uniform using smaller volumes/well. Of course, loading volume is determined by the protein concentration of the sample. It is therefore important not to over-dilute your lysates while processing. It is also desirable that the ionic composition of samples on a single gel be similar. Marked differences in salt concentrations will result in spreading and narrowing of bands.

5. Protein ladders such as SeaBlue plus 2 from Invitrogen have dye-conjugated protein bands. These can be used to determine when proteins of a certain size have passed the interface. For instance, the SeaBlue plus 2 has an orange phosphorylase band that migrates at 148 kDa. Voltage can be increased once higher molecular weight bands have clearly separated.
6. Voltage should be kept low (5–10 V/cm) until protein bands have been compressed at the interface between stacking and resolving gels. Once higher molecular weight marker bands have separated, the voltage can be increased to 10–15 V/cm. Too high a voltage can result in overheating and a breakdown of the gel matrix. This can appear as diffuse banding or uneven migration of bands. This can be partially overcome by chilling buffers with a cooling block and/or running gels in a cold room.
7. With the gel sandwich just submerged in Towbin's buffer, roll a smooth tube over the top piece of chromatography paper with gentle pressure. Work from the center of the paper to one end, and then from the center to the other end. Do this several times to make sure that all bubbles are squeezed out.
8. Proteins on blots can be quickly quantified using Ponceau S staining. After protein transfer, the blot is briefly rinsed in TBS, and then soaked in 0.1 % (w/v) Ponceau S in 5 % acetic acid for 15 min at room temperature with gentle rocking. The blot is then rinsed several times in reagent-grade water until bands can be clearly seen. With this technique, the blot can be photographed or scanned as shown in Fig. 1. This staining can be used to normalize protein loading rather than using a housekeeping antibody, such as β-actin (Fig. 1). Using Ponceau S in this way opens up the second channel on a dual-wavelength scanner for analyzing expression levels of a second protein of interest.
9. The primary antibody is the most important element of Western blotting. A good antibody will often show strong specific bands even under suboptimal conditions, while a poor antibody will show strong nonspecific bands no matter how the procedure is optimized. Signal can be improved by adjusting protein loading and/or antibody concentrations. Antibody specificity should be determined by running negative control lysates lacking the antigen of interest. Examples are mock-

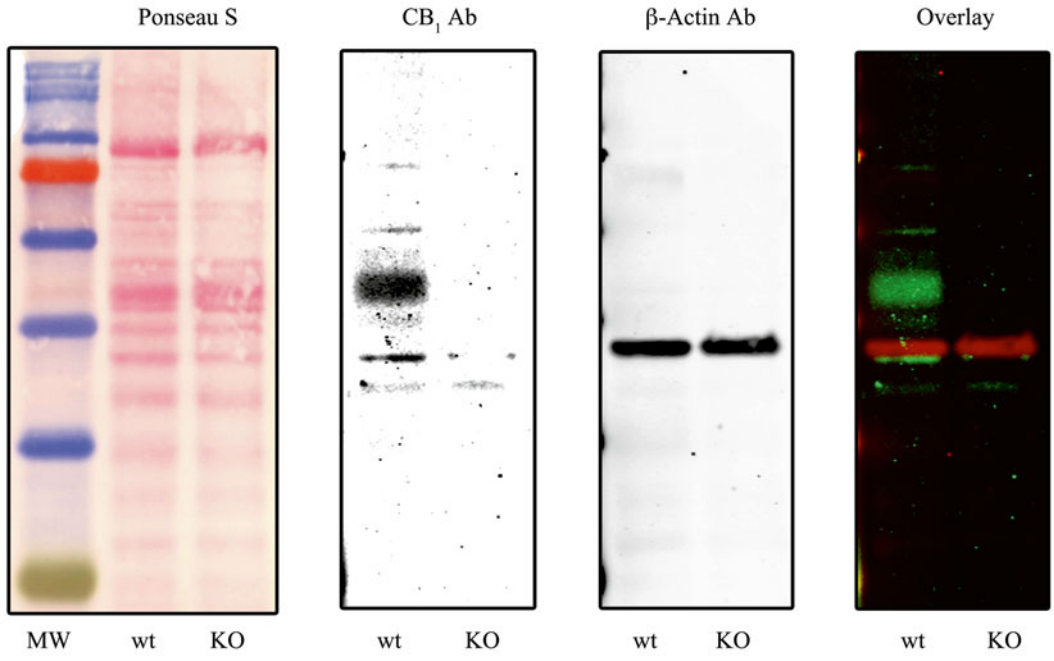

Fig. 1 Western blot showing CB_1 staining in wild-type versus CB_1 knockout mouse brain lysates. Wells were loaded with 25 μg protein (cortical homogenate). The first panel shows Ponceau-S staining of blot prior to antibody incubation. The second and third panels show grayscale images of 800 nm and 700 nm channels, respectively. The last panel shows an overlay of CB_1 staining (*green*) and actin staining (*red*). Primary antibodies used were rabbit anti-CB_1-carboxy terminus antibody (diluted 1:500), made in our laboratory, and mouse anti-actin IgM (diluted 1:5000) from Developmental Studies Hybridoma Bank (http://dshb.biology.uiowa.edu/actin). Secondary antibodies used were goat anti-mouse IgM IR_{680}RD (LI-COR Biosciences, www.licor.com), and Donkey anti-rabbit IgG IR_{800} (Rockland Inc. www.rockland-inc.com). Both secondary antibodies were diluted 1:5000

transfected cell lines and tissue lysates from knockout animals. An alternative approach is to selectively alter the mobility of the protein of interest, such as by deglycosylation. While peptide blocking is often used to show antibody specificity, this approach can lead to spurious results since many proteins share common epitopes. It is also important to note that antibody specificity needs to be demonstrated for each application. An antibody that is specific for Western blotting may not be specific for immunocytochemistry and vice versa. As discussed above, the best controls are lysates that are identical (e.g., processed at the same time and in the same fashion) to the test samples saved for the antigen of interest.

Chapter 26

Quantitation of Plasma Membrane (G Protein-Coupled) Receptor Trafficking in Cultured Cells

Jim Wager-Miller and Ken Mackie

Abstract

Measuring the functional behavior of G protein-coupled receptors (GPCRs) has been a major focus of academic and pharmaceutical research for many decades. These efforts have led to the development of many assays to measure the downstream effects of ligand binding on receptor activity. In this chapter, we describe an internalization/recycling assay that can be used to track changes in receptor number at the plasma membrane. Used in concert with other assays, this antibody-based technique can provide important information on GPCR activation by receptor-specific ligands.

Key words G protein-coupled receptor (GPCR), CB_1 cannabinoid receptor (CB_1), G protein-coupled receptor kinase (GRK), Recycling, Epitope tag, Agonist, Antagonist

1 Introduction

For many years, it has been known that GPCR trafficking to and from the plasma membrane of cells is dynamically and differentially regulated by ligands. This change in trafficking dynamics results in a loss or gain of receptors at the cell surface, which serves to "tune" receptor signaling. This phenomenon has been well characterized in the study of β-adrenergic receptors through the pioneering work of the Lefkowitz Lab, and has been observed for many other GPCRs [1]. Our lab studies how type-1 and type-2 cannabinoid (CB_1 and CB_2) receptors traffic upon activation [2–9].

Plasma membrane CB_1 and CB_2 receptors decrease following agonist exposure (Fig. 1, and refs. 5, 8). Endocytosis or internalization of receptors from the plasma membrane is thought to be one of several ways whereby cells compensate for sustained GPCR stimulation. By removing specific receptors from the surface, their signaling can be lessened. Alternatively, internalization of cell surface receptors can engage signaling pathways absent from the cell

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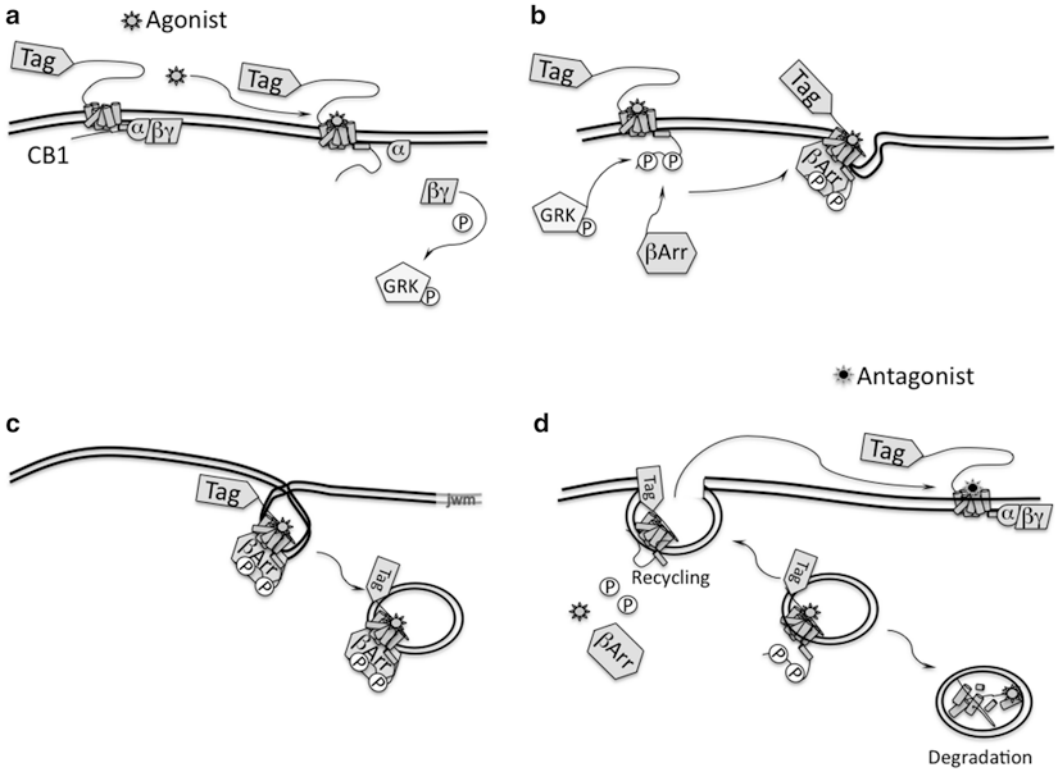

Fig. 1 The mechanisms of GPCR internalization and recycling have been well elucidated over the years. In these assays, we track the loss or gain of receptors at the surface of cells using antibodies directed against extracellular epitopes of the receptor under study. (a) As with many GPCRs, activation of CB_1 by agonist binding uncouples the receptor from G protein allowing downstream signaling through the G protein's α and βγ subunits. The latter can activate GRKs. (b) Once activated, GRKs transfer phosphate groups to intracellular targets, including serine and threonine residues of the receptor. Receptor phosphorylation acts as a signal causing β-arrestin to interact with the GPCR, subsequently directing it to clathrin-coated pits (for CB_1). (c) Through the activities of various proteins, including AP-2, dynamin, and small GTPases, clathrin-coated pits invaginate and pinch off from the plasma membrane. At this point, formerly extracellular epitopes of internalized receptors become inaccessible to extracellular antibodies. (d) With short agonist exposure, it is possible for receptors to recycle to the cell surface following the loss of dephosphorylation. For recycling experiments, cells are first incubated with translation inhibitors such as emetine or cycloheximide to impair new receptor synthesis. Receptors are then internalized via a short exposure to agonist. With the addition of antagonist, recycled receptors return to and are held at the plasma membrane

surface [10–12]. How does a cell distinguish which receptor(s) to remove and which to leave behind? It all comes down to shape.

Internalization and recycling are constitutive and dynamic processes. This is because the conformation of the receptor is in constant flux. A GPCR is constantly moving in and out of active conformations (in this case, ones that are permissive for internalization or trafficking to the surface). This leads to a portion of receptors being removed from, or returned to, the cell surface con-

tinuously over time. At steady state, this dynamic equilibrium results in no net change in receptor number. With agonist binding, the conformational population of the receptor shifts, so that more are in an internalization-competent conformation, a process that may be aided by G protein receptor kinase (GRKs) phosphorylation. Receptor phosphorylation recruits β-arrestins to interact with the receptor itself, which eventually results in receptor internalization [1]. The fate of internalized receptors depends on the receptor and the duration of agonist binding. For CB_1, following a short agonist exposure, phosphatases within endosomes will dephosphorylate the internalized receptor. This then allows its return to the plasma membrane. With longer exposures, CB_1 is shuttled to lysosomes, where it is degraded [8]. Treatment of a GPCR with an inverse agonist will favor inactive conformations. This has the effect of holding the GPCR at the plasma membrane [3].

The assay described here can detect these changes in trafficking dynamics, to probe ligand functional activity at the receptor under study (Fig. 2).

One required component for this technique is an antibody against one or more extracellular epitopes of the GPCR. This is because maintenance of cellular membrane integrity is an absolute requirement of the assay. The latter can be easily adapted to measure trafficking of any membrane receptor with high-throughput assay format, as long as a means of detecting an extracellular epitope of the receptor is available.

2 Materials

Use distilled, deionized water for all solutions. Use analytical grade reagents.

2.1 Plasmids and Cell Lines

1. Expression plasmid with epitope sequence (e.g., HA or FLAG) fused to the amino terminus (extracellular end) of GPCR under study. Alternatively, if a specific antibody against the amino terminus of the native receptor is available, this approach can be used to detect trafficking of natively expressed receptors (e.g., in neurons [13]). This plasmid must also contain antibiotic resistance for use in clone selection. Epitope-tagged expression plasmids are commercially available for many GPCRs at companies such as AddGene and Origene.
2. Cell lines: We have successfully measured receptor trafficking with the HEK293 and AtT20 cell lines, as well as with primary cultures of mouse hippocampal neurons using this assay. Other adherent cell lines that allow good surface expression of the GPCR should also work.

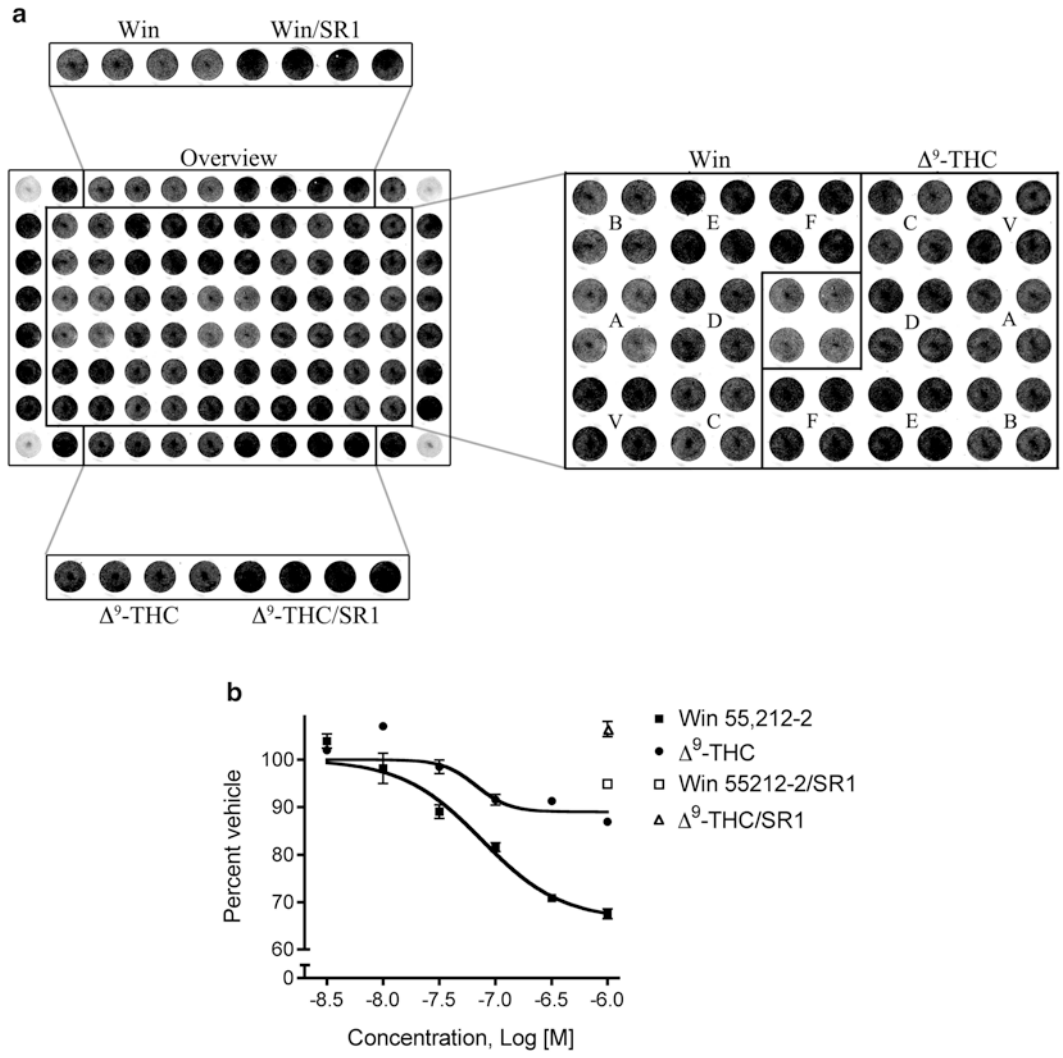

Fig. 2 Actual scan of a CB_1 internalization assay on a 96-well plate. (a) Image on the *left* is an overview of the experimental plate. The corner wells were used as a background control and were not treated with primary antibody. The integrated intensities from these wells were averaged, and this value was subtracted from those of the other wells. *Rows above* and *below* the overview plate show loss of signal in agonist-treated replicates (*left*) versus those also treated with the CB_1 antagonist SR141716A (on *right*). Panel on the *right* of the overview depicts how the center wells are treated. Quadruplicates have a letter in the *center* with "V" standing for vehicle and A–F representing decreasing concentrations of either Win55,212-2 or Δ^9-THC. Concentrations were (from A to F) 1 μM, 316 nM, 100 nM, 31.6 nM, 10 nM, and 3.16 nM. The center wells were also treated with 1 μM Win55,212-2. Plate was incubated for 1 h at 37 °C. (b) Dose–response generated with data from the plate above. Win55,212-2 is a high-efficacy agonist, while Δ^9-THC is a lower efficacy agonist. This is reflected by the differing levels of internalization for the two drugs across the dose–response. Internalization using the highest concentration of each agonist is inhibited when the antagonist (1 μM SR141716A) is also present in the medium (*open symbols*). Analysis was done using Odyssey Application Software (LiCor Biosciences version 3.0) and Prism (Graphpad Inc. version 4.0a). Images were created with Photoshop (Adobe CS5 version 12)

2.2 Buffers and Solutions

1. HBV buffer: 10 mM HEPES (pH 7.4), 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.1 % bovine serum albumin, 0.1 % drug vehicle (usually ethanol or dimethyl sulfoxide (DMSO)).
2. Agonist/antagonist: Dilute specific agonist or antagonist in HBV to two times the desired final concentration. This should be done just prior to the start of the experiment.
3. HBS buffer: Plain HBV buffer without vehicle added.
4. 10× TBS: 25 mM Tris base, 150 mM NaCl, 2.7 mM KCl, pH 7.4, add approximately 750 ml of water to a beaker with a large stir bar. Weigh out 30.3 g Tris base, 87.7 g NaCl, and 2 g KCl, and slowly add to stirring water. Bring pH to 7.4 with concentrated HCl and bring to 1 l volume. Autoclave and store at room temperature.
5. TBS-T: 1× TBS, containing 0.05 % Tween 20. Dilute 10× TBS with sterile water, and add 0.5 ml of Tween 20 per liter. Can store at room temperature. Should be used within several days.
6. 20× Poly D-lysine: Add 1 mg/ml poly D-lysine to sterile water for a 20× stock solution. Filter sterilize and store at −20 °C.
7. PBS: 20 mM monobasic sodium phosphate, 150 mM NaCl. Bring pH to 7.4 and autoclave. Can store at room temperature.
8. 100 % Ethanol.
9. Dry (anhydrous) DMSO.
10. 4 % Paraformaldehyde: Prepare in fume hood, as paraformaldehyde is a carcinogen. Add 40 g paraformaldehyde to 250 ml of 0.4 M phosphate buffer (pH 7.4). Heat with stirring until temperature reaches 65 °C. Add 750 ml of deionized water and three drops of 6 M NaOH. Continue stirring and heating until temperature is 60 °C. Run solution through filter paper into flask chilled on ice. Aliquots of 4 % PFA may be stored at −80 °C for several months. Once thawed, this solution will be good for 2 weeks if kept at 4 °C.
11. HBVI: HBV buffer with either 70 μM cycloheximide or 10 μM emetine added.
12. LI-COR Biosciences Odyssey Blocking Solution.

2.3 Plate Preparation

1. Multichannel pipette.
2. Plastic troughs (sterile).
3. Poly D-lysine-coated 96-well plates: Obtain sterile, flat-bottom, transparent tissue culture plates. Dilute 20× poly D-lysine to 1× (50 μg/ml final) with sterile water, and place 40 μl of solution into each well. Tap plate to make sure that all wells are uniformly coated. Incubate plates for 20 min to overnight at 37 °C. Wash

wells three times with PBS just prior to plating. Coated plates can be stored at room temperature for up to 2 weeks.

2.4 Cell Line Preparation

1. Cell growth media: Use media appropriate for the cell line being used. For HEK293 and AtT20 cells, a mixture of DMEM, 10 % fetal bovine serum, and 1× penicillin/streptomycin works well.
2. 6-Well culture plate.
3. 0.025 % Trypsin (wt/vol).
4. Transfection reagent, such as Lipofectamine 2000.
5. Mammalian DNA expression vector containing receptor sequence: An epitope tag should be fused in-frame with an extracellular domain of the GPCR (usually the amino terminus). However, with availability of appropriate receptor antibodies, it is possible to use this assay without an epitope-tag sequence (*see* **Note 1**).
6. Antibiotic for cell selection: Antibiotic resistance originates within the expression vector used. Many vectors contain a neomycin resistance sequence, which would require G418 or geneticin to be added to the growth medium, in order to select for gene incorporation. Check the vector map to make sure which antibiotic to use. The concentration of antibiotic must be determined empirically for each cell type by performing a dose–response, or "killing curve," on untransfected cells. The lowest concentration of antibiotic that completely kills untransfected cells is used to select for viable clones following transfection [14].
7. Anti-epitope antibody: The mouse anti-HA11 antibody works well in our hands. However, we have used antibodies against FLAG, as well as native extracellular epitopes with success.

2.5 Detection Equipment

1. Inverted fluorescent microscope.
2. Fluorescent scanner (e.g., LiCor Odyssey).

3 Methods

As mentioned above, cell lines expressing the receptor of interest must be obtained or created for this assay. The procedure that we use for generating stable cell lines is in the next section. Any cell lines used should homogeneously express the receptor, be healthy, and of low-to-moderate passage. We have found that moderate expression of receptor provides a better signal-to-noise ratio than high expression. Manipulation of cells should be done in a laminar flow cabinet and aseptic technique should be followed. During all procedures cells should be kept immersed in media to avoid drying.

3.1 Making Stable Cell Lines

1. Expand immortalized cells. The day before transfection, plate cells to a confluency of approximately 60 % on a 6-well dish. Perform transfection as per the manufacturer's protocol. Grow transfected cells overnight in normal growth medium. This allows cells to recover from transfection stress, and to begin producing resistance protein from the incorporated vector.
2. The next day, split cells and plate 50,000 and 200,000 cells onto 10 cm^2 tissue culture plates. Include appropriate antibiotic at an effective concentration in growth medium at this point. Change medium every 3–4 days and grow until visible colonies form (usually 9–14 days for HEK293 or AtT20 cells). Do not overgrow cells. Colonies must be separated in order to select homogeneous clones.

3.2 Live Cell Screening for Expression Lines

1. Once transfected cultures have formed large (>100 cells) isolated colonies, gently wash cells once with PBS, and then again with HBS. Cover cells with 4 ml of antibody diluted in HBS (*see* **Note 2**). This antibody must be against an extracellular epitope of the target receptor. We typically dilute antibody 1:500 for this procedure, but concentrations will vary depending on the antibody used and need to be determined empirically.
2. Incubate cells in antibody solution for 1 h at room temperature.
3. Wash cells very gently three times with 1× PBS. Tilt plate and stream 1× PBS on the wall of the dish so as not to dislodge colonies from the plate. Level plate out to coat cells with wash buffer.
4. Incubate cells with secondary antibody conjugated with a fluorescent tag. Again, the antibody is diluted at 1:500 in HBS. We typically use an anti-mouse IgG conjugated with Alexa 488 to recognize the monoclonal HA11 antibody.
5. Cover cells and incubate in the dark at room temperature for 45–60 min.
6. Wash cells gently three times with PBS as above.
7. Cover cells with HBS and visualize on an inverted fluorescent microscope with appropriate filters for the fluorophore. Desirable colonies will be uniformly fluorescent in appearance. Observe candidates under both fluorescent and white light, to make sure that all cells are expressing the receptor of interest. Using a low-magnification lens, circle colonies from the underside of the plate with a marking pen. After choosing 6–12 colonies, take plate back to tissue culture hood.
8. Collect each colony by depressing the plunger of a P200, and then placing the pipette tip within the circle drawn in the previous step. The pipette should be perpendicular to the surface of the plate. Releasing the plunger should pull up the colony.

9. Place each colony into separate wells of a 12-well plate containing growth media with antibiotic used for selection.
10. Grow and expand clones. Once a sufficient number of cells are present in the well, they should split and an aliquot replated, fixed, and immunostained for the receptor to ensure its uniform presence. If the staining is positive, then the remainder of the cells should be expanded and then frozen and stored in liquid nitrogen (*see* **Note 3**).

3.3 Internalization Assay

1. Wash cells stably expressing the receptor once in PBS, then cover with 0.025 % trypsin, and incubate at 37 °C until cells are detached from plate. Add growth media to inhibit trypsin, collect, and centrifuge for 5 min at $200 \times g$ and 4 °C. Resuspend cells in growth media to a density of approximately 1×10^6 cells/ml. Add 50,000–75,000 cells to each well of the poly-D-lysine-coated plates prepared above, by using a multichannel pipette. Grow under normal conditions for that cell line until cells are 90–95 % confluent.
2. When cells are ready, remove media by firmly patting plate upside-down onto paper towels (*see* **Note 4**).
3. Wash once in PBS (*see* **Note 5**).
4. Condition cells to HBV buffer by covering wells with 40 μl of HBV, by using a multichannel pipette. Incubate at 37 °C for 15 min.
5. Add 40 μl, 2× drug or vehicle in HBV to selected wells, using a multichannel pipet (*see* **Note 6**). Tap plate gently to mix and incubate for the desired time at 37 °C.
6. When drug treatment is finished, firmly pat plate dry on paper towels as above and place on ice. Immediately add ice-cold 4 % paraformaldehyde at 100 μl/well, and then move the plate to room temperature. Incubate covered at room temperature for 20 min.
7. Wash plate five times for 5 min in TBS (*see* **Note 7**).
8. Cover cells with LI-COR Bioscience Odyssey blocking buffer, and incubate at room temperature for 90 min with gentle rocking.
9. Remove blocking buffer and cover cells with 40 μl of blocking buffer containing a primary, anti-epitope antibody. Our lab routinely uses a mouse anti-HA11 antibody diluted 1:150 for HA-expressing receptors.
10. Rock cells at room temperature for 1 h, or overnight at 4 °C.
11. Remove primary antibody and wash plate five times for 5 min in TBS-T at room temperature with rocking.
12. After the last wash, cover cells with 40 μl of secondary antibody diluted in blocking buffer (*see* **Note 8**). Rock plate for 1 h at room temperature.

13. Again, wash plate five times for 5 min with TBS-T at room temperature. Briefly rinse plate with TBS and pat and wipe dry using paper towels.
14. Scan plate on the appropriate scanner (*see* **Note 9**).
15. Determine integrated intensity of each well and calculate intensity relative to percent vehicle (*see* **Note 10**).

3.4 Recycling Assay

The recycling assay is the internalization assay with some minor variations. First, protein synthesis must be inhibited in order to accurately measure receptor recycling without the complication of de novo receptor synthesis. This is accomplished by pretreating the cells with a translation inhibitor, such as emetine or cycloheximide. Either one of these reagents should be present throughout the assay.

1. Make a 2× solution of either emetine (20 μM) or cycloheximide (140 μM) in normal growth medium. Add an equal volume of the 2× solution to the wells of a plate prepared the day before. Incubate the plate for 2 h prior to the beginning of the experiment.
2. Remove growth media and replace with 40 μl of HBVI. Allow cells to equilibrate for 15 min at 37 °C.
3. Prepare HBVI and make 2× agonist preparations (*see* **Note 11**).
4. Treat wells by adding 40 μl of 2× drug preparations in HBVI, or HBVI alone, and incubate at 37 °C for the desired time (*see* **Note 11**).
5. Add 3× antagonist preparation in HBVI, or HBVI alone, to selected wells and incubate at 37 °C for an additional 60–90 min.
6. Before the end of the experiment, add agonist at a concentration and for a time that will result in complete internalization (*see* **Note 12**).
7. Process plate as in Subheading 3.3, **steps 6–15**, above.

4 Notes

1. Receptor-specific monoclonal or polyclonal antibodies will work with this assay, provided that they have high affinity and specificity for the target and that they are directed towards an extracellular domain of the receptor under study.
2. While most antibodies sold are IgG, some are not. Primary chicken antibodies are IgY, while some mammalian antibodies are IgM. The secondary antibody chosen must be targeted against the species and isotype of the primary antibody used.
3. It is a good idea to screen all cell lines collected before using any one of them in trafficking experiments. This is because known and unknown factors will affect the responsiveness and

the signal-to-noise ratio of these clones. By evaluating all lines up front, the most appropriate one for use in this assay can be selected. To this aim, the internalization assay is performed as described above, with each cell line placed in a column of a 96-well plate. Specific agonist, antagonist, or agonist/antagonist combinations are then added in selected rows. The most responsive line should be expanded and cryogenically stored in liquid nitrogen for future use. Your cell lines and detection system should also be evaluated for its *Z′*-factor [15] before beginning experiments.

4. At this point, it is no longer necessary to keep cells sterile. It should be noted that US regulations (OSHA) require human-derived cell lines such as HEK293s to be treated under the Biosafety Level 2 criterion. All reagents used on cells prior to fixation are considered biohazardous waste and must be disposed of appropriately. Check your institutional requirements for appropriate handling of these and any other biohazards used in this assay.
5. This is done by first filling a dish with PBS, TBS, or TBS-T. The dish must be large enough for the plate to fit into and deep enough to entirely submerge the wells. Before cells are fixed, the plate can be patted upside-down onto paper towels to collect the waste, and then submerged into the buffer to wash the cells. After cells are fixed, washes can be shaken into a large catch beaker. This should be done expeditiously, so as not to allow cells to dry out.
6. Each test condition should be represented by three or more replicates on the plate, if possible. Untreated control replicates must also be present. Avoid using wells along the perimeter of the plate, unless it has been determined that plate (edge) effects are minimal within these wells. For many scanner/plate combinations, counts are significantly higher on outside wells. Conversely, counts may dip in the middle of the plate in some systems. Scan untreated cell plates that have been fixed and stained on your system, to determine plate effects before running experiments. It is good practice to vary the position of drug treatments relative to wells (that is, do not always have control in one column, and ascending values from right to left across the plate).
7. No detergent is added to this wash, as cell membranes must remain intact during primary antibody incubation. This is because only surface epitopes must be accessible to the antibody, in order to measure receptor number changes on the plasma membrane. Theoretically, one could measure total receptors by permeabilizing the cells prior to antibody incubation; however we have not thoroughly evaluated this approach.

8. Concentration of secondary antibodies must be determined empirically. We typically use LI-COR Biosciences or Rockland Inc. highly immunoabsorbed antibodies with near-IR fluorophore conjugates. In our hands, secondaries conjugated with a 680 nm dye are diluted 1:200 in blocking buffer, while those conjugated with 800 nm dye are diluted 1:800.
9. We use the LI-COR Odyssey near-IR scanner; however other instruments should be compatible with this assay. The Odyssey reads from the bottom of the plate. It has two diode lasers for 700 and 800 nm emissions, allowing for detection of two target molecules on the same plate. The near-IR range also displays very low autofluorescence from the cellular monolayer. Thus, background levels are low and this improves the overall signal-to-noise ratio of the assay.
10. Most scanners contain software that will overlay a grid pattern over the final image. This places a circle on top of each well of the plate image, and is used to calculate the integrated intensity. The formula for integrated intensity is

$$a\left(\sum I_{\mathrm{i}} - Pb\right)$$

where a is the area of each pixel in mm^2, $\sum I_{\mathrm{i}}$ is the sum of all pixels with the area of interest, P is the number of pixels within each circle, and b is the mean background intensity.

Data is displayed as "percent vehicle," which is simply the integrated intensities of test samples divided by controls multiplied by 100. The signal in each well can also be normalized to cell number by staining nuclei with DRAQ5 (1:5000), and detecting the bound antibody with an anti-IgG_{800} secondary antibody. The DRAQ5 signal will appear in the 700 nm channel.
11. In working up this assay, the concentration of agonist should be near its EC_{50} value, while the time allowed for internalization should be near that at which 90 % of normal signal loss is observed. High concentrations of agonist, or extended time periods, will result in trafficking of receptors into lysosomal compartments that in turn results in a loss of signal.
12. Agonist treatment should begin at a set time before the recycling treatment is to end. The time chosen should result in maximal receptor internalization. Receptor recycling data can be compared with this signal.

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References

1. Drake MT, Shenoy SK, Lefkowitz RJ (2006) Trafficking of G protein-coupled receptors. Circ Res 99:570–582
2. Petrov RR, Knight L, Chen SR et al (2013) Mastering tricyclic ring systems for desirable functional cannabinoid activity. Eur J Med Chem 69:881–907
3. Atwood BK, Wager-Miller J, Haskins C et al (2012) Functional selectivity in CB(2) cannabinoid receptor signaling and regulation: implications for the therapeutic potential of CB(2) ligands. Mol Pharmacol 81:250–263
4. Atwood BK, Huffman J, Straiker A et al (2010) JWH018, a common constituent of 'Spice' herbal blends, is a potent and efficacious cannabinoid CB receptor agonist. Br J Pharmacol 160:585–593
5. Daigle TL, Kearn CS, Mackie K (2008) Rapid CB1 cannabinoid receptor desensitization defines the time course of ERK1/2 MAP kinase signaling. Neuropharmacology 54:36–44
6. Coutts AA, Anavi-Goffer S, Ross RA et al (2001) Agonist-induced internalization and trafficking of cannabinoid CB1 receptors in hippocampal neurons. J Neurosci 21:24 25–2433
7. Roche JP, Bounds S, Brown S et al (1999) A mutation in the second transmembrane region of the CB1 receptor selectively disrupts G protein signaling and prevents receptor internalization. Mol Pharmacol 56:611–618
8. Hsieh C, Brown S, Derleth C et al (1999) Internalization and recycling of the CB1 cannabinoid receptor. J Neurochem 73:493–501
9. Jin W, Brown S, Roche JP et al (1999) Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization. J Neurosci 19:3773–3780
10. Luttrell LM, Ferguson SS, Daaka Y et al (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283:655–661
11. Miller WE, Lefkowitz RJ (2001) Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking. Curr Opin Cell Biol 13:139–145
12. Wang Q, Lu R, Zhao J et al (2006) Arrestin serves as a molecular switch, linking endogenous alpha2-adrenergic receptor to SRC-dependent, but not SRC-independent, ERK activation. J Biol Chem 281:25948–25955
13. Mikasova L, Groc L, Choquet D et al (2008) Altered surface trafficking of presynaptic cannabinoid type 1 receptor in and out synaptic terminals parallels receptor desensitization. Proc Natl Acad Sci U S A 105:18596–18601
14. Daigle TL, Kwok ML, Mackie K (2008) Regulation of CB1 cannabinoid receptor internalization by a promiscuous phosphorylation-dependent mechanism. J Neurochem 106:70–82
15. Zhang JH, Chung TD, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4:67–73

Chapter 27

Measuring ECS Interaction with Biomembranes

Clotilde B. Angelucci, Annalaura Sabatucci, and Enrico Dainese

Abstract

Understanding the correct interaction among the different components of the endocannabinoid system (ECS) is fundamental for a proper assessment of the function of endocannabinoids (eCBs) as signaling molecules. The knowledge of how membrane environment is able to modulate intracellular trafficking of eCBs and their interacting proteins holds a huge potential in unraveling new mechanisms of ECS modulation.

Here, fluorescence resonance energy transfer (FRET) technique is applied to measure the binding affinity of ECS proteins to model membranes (i.e., large unilamellar vesicles, LUVs). In particular, we describe in details the paradigmatic example of the interaction of recombinant rat FAAH-ΔTM with LUVs constituted by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).

Key words Biological membranes, Membrane proteins, Large unilamellar vesicles, FRET, Binding isotherms, Membrane affinity

1 Introduction

Membrane proteins interact preferentially with a specific lipid environment, and the enrichment of specific lipids around a protein can dramatically modulate its functional activity [1, 2]. Many cell functions are governed by the peculiar organization of lipids and proteins within biological membranes. Membrane fusion, intermembrane exchange of lipid components, formation of lipid domains in lipid mixtures, and vesicle-vesicle interactions all involve changes in the distances between different lipid components.

The plasma membrane bilayer is a closely packed and crowded environment, and fluorophores, behaving as donor and acceptor molecules with good spectral overlap, can communicate by FRET over lateral distances that span several lipid molecular diameters. FRET is one of the most useful techniques to interrogate these processes. Another major application of FRET in cell biology is the analysis of protein-protein and protein-lipid interactions.

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These are often important for intercellular communication and for successful transfer of information within a cell, leading to specific signal transduction pathways. FRET is also a useful tool for measuring both average distances of proteins from membranes and peripheral binding of proteins and other molecular components to the membrane surface [3].

FRET is a process whereby the energy of an excited state is non-radiatively transferred from an excited donor molecule to an acceptor molecule [4]. For FRET to occur, a number of conditions need to be fulfilled: (a) the emission spectrum of the donor needs to have sufficient overlap with the absorption spectrum of the acceptor; (b) the emission and absorption dipole moment of donor and acceptor must not be perpendicular; (c) donor and acceptor have to be in close proximity [4].

Förster [5, 6] was the first to establish that energy can be transferred efficiently by a resonance dipole-dipole mechanism over distances from 10 to 100 Å. Since these pioneering studies, FRET has been successfully used in different fields of research for over 40 years. The increasing availability of new synthesized/purified biological macromolecules, along with the increasing availability of fluorescent compounds, will continue to facilitate the design of innovative biotechnological applications of FRET.

One of the classical and most prevalent uses of FRET is to measure distances between defined sites within a protein structure and to study its oligomeric organization in solution. Indeed, proteins contain intrinsic fluorophores (i.e., Trp and Tyr), and they can also be covalently labeled (often specifically) with extrinsic fluorophores. Many of these extrinsic probes are commercially available and easy to use.

With the Förster equation it is possible to calculate the efficiency of the energy transfer that occurs between the donor fluorophore and the acceptor fluorophore, and the distance at which it may occur [5, 6]. However, the Förster equation can be used only if there is a single donor-acceptor pair, because the presence of more than one donor, or of more acceptors, reduces the accuracy of data acquisition. Thus, we used FRET to describe a protein-lipid interaction where the donor is a constituent of the protein (Trp), with the emission maximum from 307 to 353 nm, and the acceptor fluorophore is the synthetic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(1-pyrenesulfonyl) (PyPE), with absorption maximum at 351 nm [7, 8]. Of note, the latter compound can be embedded within synthetic membranes. In this setup, analysis of the FRET signal allows to monitor quenching of Trp fluorescence that occurs at different concentrations of vesicles containing the acceptor fluorophore (Fig. 1). From these data, binding isotherms are obtained to calculate membrane affinity of the investigated protein.

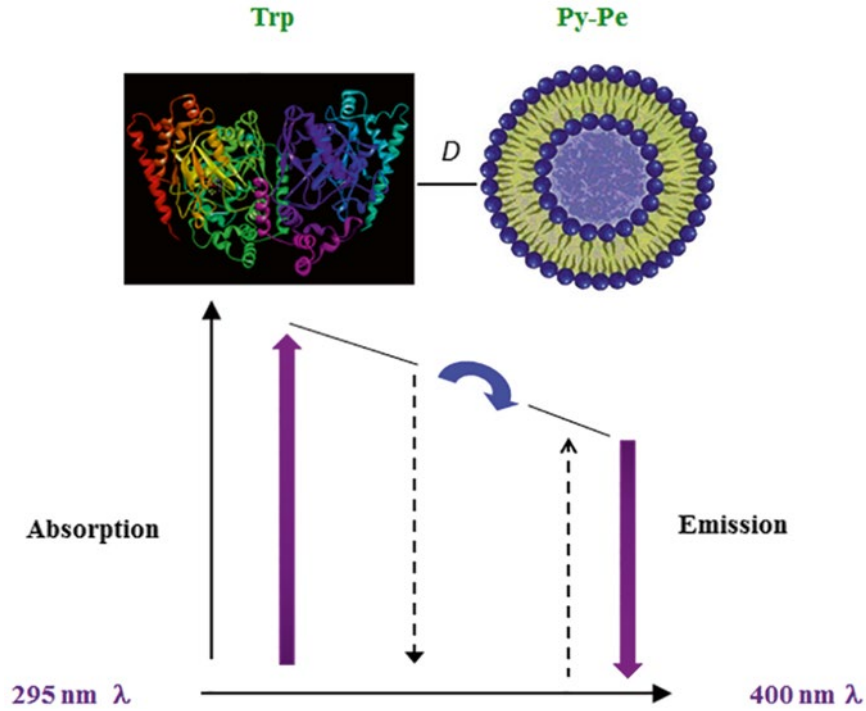

Fig. 1 Scheme of the energy transfer in a typical FRET experiment. *Trp:* intrinsic protein tryptophan donor fluorophore, *PyPE:* acceptor fluorophore embedded within the liposome bilayer, *D*: Förster distance

2 Materials

Use reagents of high analytical grade, and prepare buffers with ultrapure water.

2.1 Components for Liposome Preparation

Glass test tubes (2 cm diameter).
Ethyl alcohol (gradient grade) ≥99.8 % pure.
Chloroform.
POPC: 16:0–18:1 PC.
PyPE:18:1pyrenePE1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(1-pyrenesulfonyl), ammonium salt (*see* **Note 1**).

Buffer: 50 mM Tris–HCl, pH 7.5. In a glass beaker, weigh 6.06 g Trizma base, add 800 ml of water, and adjust pH to 7.5 with concentrated HCl. Bring to a final volume of 1 l with water in a volumetric flask. Store at 4 °C.
Nitrogen gas.
Water bath.
LiposoFast apparatus, equipped with 100 nm pore-size polycarbonate membranes and gastight syringes (500 μl volume).

2.2 Components for Isolation of Rat Liver Membranes

Potter glass (5 ml).
Protease inhibitor cocktail (IC).

Buffer A: 5 mM Tris–HCl, 10 mM KCl, 5 mM EDTA, pH 7.8. In a glass beaker, weigh 0.606 g Trizma base, 0.74 g KCl, and 1.9 g EDTA. Add 800 ml of water, adjust pH to 7.8 with concentrated HCl, and bring to a final volume of 1 l with water in a volumetric flask. Store at 4 °C.

Buffer B: Buffer A containing 1 mM DTT and IC. In a glass beaker, weigh 0.5 mg DTT and 30 μl of IC, and bring to a final volume of 3 ml with Buffer A in a volumetric flask.

Buffer A 60 % sucrose: In a glass beaker, weigh 60 g sucrose and bring to a final volume of 100 ml with Buffer A in a volumetric flask. Store at 4 °C.

Buffer A 37 % sucrose: In a glass beaker, weigh 37 g sucrose and bring to a final volume of 100 ml with Buffer A in a volumetric flask. Store at 4 °C.

2.3 Protein Solution

Recombinant rat FAAH-ΔTM is dissolved in the same buffer used for liposome preparation. Use only highly purified protein samples. Enzyme purification is a complex procedure [10, 11] that remains far from the scope of this chapter. The reader can obtain pure FAAH-ΔTM through collaboration with our group or others.

2.4 FRET Components

LS50B fluorimeter (PerkinElmer Life and Analytical Science, Boston, MA) connected to a circulating water bath. Adjust excitation and emission slits to 4 nm and 10 nm, respectively, with a scanning speed of 1500 nm/min (*see* **Note 2**).

Quartz cuvettes with a maximum volume of 250 μl (*see* **Note 3**), and a light path of 10 mm.

3 Methods

3.1 Liposome Preparation

Warm about 100 ml of buffer at 25 °C in a water bath (*see* **Note 4**).

Dissolve 0.5 mg PyPE in 100 μl of chloroform, in order to have a 5 mg/ml PyPE solution (*see* **Note 1**).

To prepare 1 ml of liposomes composed of POPC at a concentration of 2 mM, weigh 1.5 mg POPC in a glass test tube and dissolve it in 200 μl of ethanol (*see* **Note 5**).

Take 100 μl of this solution and put it in a second test tube, together with 2 μl of 5 mg/ml PyPE solution. The final lipid:fluorophore molar ratio is 100:1 (*see* **Note 6**).

Evaporate the organic solvent in the two test tubes under a N_2 stream, keeping the tubes immersed in the water bath at a controlled temperature of 25 °C (*see* **Note 4**). Gently rotate the test tubes, in order to form a monomolecular film around the walls. Once the dry lipid film is formed, add 1 ml of warm buffer in each test tube while keeping it immersed in the water bath; this proce-

dure will facilitate the emulsion of the lipid and the correct formation of liposomes (*see* **Note 7**).

In order to have homogeneous POPC LUVs in size, use the extrusion method. To this aim, use the LiposoFast extruder immersed in a water bath at 25 °C. This instrument consists of two 500 μl syringes and a steel jacket containing a polycarbonate membrane with a 100 nm pore diameter, through which lipids are fluxed. Pushing a multilamellar lipid solution 11–21 times through the membrane will produce homogeneous unilamellar vesicles with lateral dimensions of about 100 nm. The formation of LUVs is macroscopically evident as clarification of the lipid mixture (*see* **Note 8**).

To investigate lipid composition-induced preferential binding of a protein, instead of liposomes consisting of POPC alone, LUVs

Table 1
Preparation of LUVs of defined concentration

	LUV concentration in stock solution (mM)	Added volume (μl)	Total volume (μl)	Final LUV concentration (μM)	Final protein concentration (μM)
0		0	100	0	0.2
1	0.4	1	101	4	0.2
2	0.4	1	102	8	0.2
3	0.4	1	103	11	0.19
4	0.4	1	104	15	0.19
5	0.4	1	105	19	0.19
6	0.4	2	107	26	0.19
7	0.4	2	109	33	0.18
8	0.4	1	110	36	0.18
9	0.4	1	111	40	0.18
10	0.4	4	115	52	0.17
11	0.4	4	119	64	0.17
12	0.4	2	121	70	0.17
13	0.4	4	125	80	0.16
14	0.4	5	130	92	0.15
15	0.4	5	135	103	0.15
16	2	4	139	158	0.14
17	2	4	143	210	0.14
18	2	4	147	258	0.14
19	2	4	151	305	0.13

can be produced with different lipid compositions, or with lipids extracted from specific cell membranes (Table 1).

For instance, LUVs from the plasma (PM) and the endoplasmic reticulum (ER) membranes can be prepared from lipids extracted from rat hepatocytes, as follows. Disrupt liver tissue by a Potter glass, and mix with a volume of Buffer B (*see* **Note 9**). After homogenization, liver is centrifuged at 10,000 × *g* for 5 min, supernatant (~2.4 ml) is transferred to a 14 ml ultracentrifugation tube, and 9.6 ml of Buffer A containing 60 % of sucrose (final sucrose concentration: 48 %) is gently overlayed with a Pasteur pipette. Separately, prepare two tubes with 1 ml of Buffer A, and two tubes with 4.5 ml of Buffer A containing 37 % of sucrose. Then, in a 14 ml tube add 6 ml of the previously prepared solution containing 48 % of sucrose, slowly stratify over 4.5 ml of Buffer A containing 37 % of sucrose, and finally add 1 ml of buffer A. In another tube, repeat the same procedure with the remaining 6 ml of the solution containing 48 % of sucrose. Then, centrifuge the tubes at 150,000 × *g* for 2 h. In the last step, recover PM and ER lipid membranes with a glass Pasteur pipette (*see* **Note 10**), then wash with Buffer A in a new tube, and centrifuge again at 150,000 × *g* for 1 h [9]. Recover the pellet containing the two different membranes, dissolve it in 200 μl of chloroform, evaporate the organic solvent under a N_2 stream, and proceed as described for the preparation of the liposomes.

3.2 Fluorescence Measurements

In the fluorimeter, set the excitation wavelength at 292 nm (Trp excitation) and record fluorescence emission spectra between 300 and 600 nm.

Record the spectrum of the blank (i.e., 100 μl of buffer), to be subtracted from all spectra (*see* **Note 11**).

Record the value of Trp fluorescence emission intensity (FI) at its maximum (approximately 340 nm). Note that the maximum of fluorescence emission of PyPE is about 380 nm (*see* **Note 12**).

To correctly perform the procedure, it is important to set up three series of measurements of FI maximum, according to the scheme below. Of note, the first series is done for the correction of FI due to protein dilution. The second series is for eliminating the eventual contribution of quenching Trp emission due to protein/lipid interaction. The third series is the measurement of FRET.

The first step (step 0) of each series is a measure of FI spectrum of the protein solution (F0), that is, 100 μl of 0.2 μM rat FAAH-ΔTM (*see* **Note 13**).

1. In the first series, add to 100 μl of protein solution in the quartz cuvette increasing amounts of buffer, as reported below in column 2, and record Trp FI maximum ($F_{(\text{protein})}$) (*see* **Note 14**).

2. In the second series, add the same volumes indicated below in column 2 of LUV_S from the diluted or concentrated stock solutions (column 1), and record Trp FI maximum ($F_{(lipid)}$).
3. In the third series, register Trp FI maximum upon addition of increasing amounts of LUVs containing the fluorophore ($F_{(lipid/PyPE)}$) (*see* **Notes 14** and **15**).

Do the additions directly into the cuvette and mix thoroughly. Let the sample equilibrate to the cuvette temperature for 5 min, before recording emission spectra.

For each series, normalize to 1 the value of F_0, and then normalize each FI value to F_0, in order to minimize the contribution of possible experimental errors in the determination of protein concentration.

For each measure (n), calculate the fluorescence intensity variation (ΔF) with respect to the maximum fluorescence intensity value:

$$\Delta F_n = F_0 - F_n \tag{1}$$

Subsequently, correct the normalized values of FI maximum for the effect of protein dilution and of lipid addition, in order to get ΔF values that are due to FRET, as follows:

$$\Delta F = \Delta F_{(lipid/PyPe)} - \left(\Delta F_{(lipid)} - \Delta F_{(protein)}\right) \tag{2}$$

where:

$\Delta F_{(protein)}$ = FI variation in the spectra due to the addition of buffer only (series 1).

$\Delta F_{(lipid)}$ = FI variation in the spectra due to the addition of the lipid (series 2).

$\Delta F_{(lipid/PyPe)}$ = FI variation in the spectra due to the addition of the lipid in the presence of the acceptor fluorophore (series 3).

ΔF is the fluorescence intensity variation at different lipid concentrations; therefore it follows that

$$\Delta F = f\left([\mathrm{L}]\right) \tag{3}$$

where [L] is the lipid concentration.

The protein binding isotherm to the lipid is described by the following function:

$$\Delta F = \left(\Delta F_{max}\,[\mathrm{L}]\right) / \left([\mathrm{L}]_{1/2} + [\mathrm{L}]\right) \tag{4}$$

By fitting the values of ΔF as a function of lipid concentration through nonlinear regression analysis of the binding isotherm with Eq. 4 (Fig. 2), ΔF_{max} and $L_{½}$ parameters can be obtained. ΔF_{max} is the maximum value of ΔF that is obtained under conditions of saturation of the protein-vesicle binding, and $[\mathrm{L}]_{½}$ is the concentration of lipid vesicles at which $\Delta F = ½\Delta F_{max}$ (Table 2) [13].

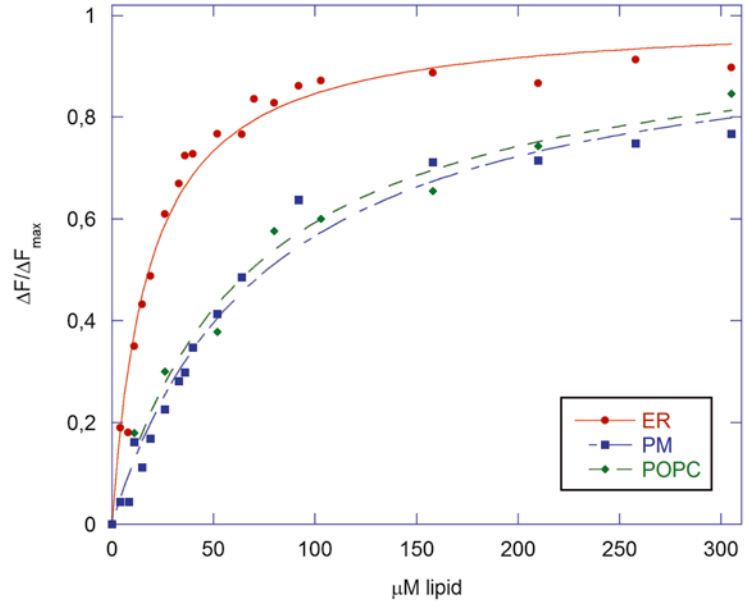

Fig. 2 Binding isotherms of rat FAAH-ΔTM as tryptophan FRET quenching at different concentrations of POPC (*green diamonds*), or of reconstituted LUVs from the ER (*red circles*) and PM (*blue squares*)

Table 2
$L_{½}$ values for the binding of rFAAH-ΔTM with membranes of different lipid compositions (modified from ref. 1)

LUV	$L_{1/2}$ (μM)
POPC	67 ± 10
Endoplasmic reticulum (ER)	18 ± 3
Plasma membrane (PM)	79 ± 8

4 Notes

1. Dissolve PyPE powder in organic solvent (chloroform) at a concentration of 5 mg/ml, prepare 100 μl aliquots, dry with N_2 flux, and keep at −20 °C until use.
2. The setup of the slits has to be optimized for each instrument.
3. We optimized the method for a quartz cuvette with a window volume of 100 μl and maximum capacity of 250 μl.
4. Temperature must be kept above the main transition temperature (T_m) of the lipid, thus favoring a high mobility degree of the acyl chains, and an easy flux through the pores of the extruder.

5. Try to keep the solvent volume as low as possible, in order to reduce the time needed for evaporation.
6. In the literature a molar ratio 98:2 is reported [8], but this value can be adjusted to fit instrument characteristics.
7. During the preparation phase of the liposomes, it is appropriate to coat the tube containing the lipid/fluorophore mixture with an aluminum foil, in order to avoid photobleaching of the fluorophore.
8. The LiposoFast-Basic apparatus produces unilamellar liposomes by the manual extrusion of a multilamellar liposome suspension through a polycarbonate membrane of defined pore size. The sample is passed through the membrane by pushing it back and forth between two syringes (0.5 or 1.0 ml syringes). In order to facilitate the passage of the lipid suspension through the syringes during the extrusion process, it is important that the LiposoFast-Basic apparatus is immersed in a water bath heated at a temperature higher than the T_m of the lipid used. To obtain a homogeneous solution, it is recommended to have a minimum of 11 steps to a maximum of 21 steps, always in odd [12]. This procedure will prevent that any residual impurity can contaminate the final preparation. In order to avoid any carryover contamination of PyPE, at the end of each preparation syringes and the entire LiposoFast-Basic apparatus have to be perfectly cleaned by several rinses with hot ethanol, followed by hot distilled water.
9. For a liver of ~0.74 g, use 1.5 ml of Buffer B. Homogenize the liver, filter with a gauze, and then centrifuge at 2,000×*g* for 5 min at 4 °C. Add to the supernatant 1.5 ml of Buffer B, homogenize, and centrifuge again at 10,000×*g* for 5 min at 4 °C.
10. After centrifugation at 150,000×*g* for 2 h, stratification of the membranes can be observed in the tube from top to bottom, as follows: the first white layer is an unspecific fatty mixture, the second layer is constituted by PM, and the third layer is constituted by RE. Pellets represent cellular debris, and should be discarded.
11. If the buffer has an isotropic signal along all emission spectra, fluorescence intensity at 600 nm must be subtracted from all of them.
12. Trp maximum of fluorescence intensity is at ~340 nm. It is possible that during the binding measurements a blue or a red shift of this peak is observed, which can be due to eventual membrane-induced aggregation or dissociation of the protein sample, or variation of the chemical environment of the emitting Trp intrinsic fluorophores.
13. Protein concentration depends on the number of Trp residues in its primary structure, their solvent exposition, and the characteristics of the instrument. As a general rule, adjust protein

concentration to have a value of ~800 AFU for the measure of F_0 (at a lipid concentration = 0).

14. It is strongly recommended to store all values in a datasheet for subsequent analysis (e.g., by using Microsoft Excel or KaleidaGraph software).
15. In our method, we use liposome concentrations ranging from 10 to 300 μM. Others [13] have reported binding isotherms with different lipid concentrations (up to 1 mM) for different human enzymes. The lipid concentration range must be adjusted according to the affinity of the protein to the membrane.

References

1. Dainese E, De Fabritiis G, Sabatucci A et al (2014) Membrane lipids are key-modulators of the endocannabinoid-hydrolase FAAH. Biochem J 457:463–472
2. Dainese E, Angelucci CB, Sabatucci A et al (2010) A novel role for iron in modulating the activity and membrane binding ability of a trimmed soybean lipoxygenase-1. FASEB J 24:1725–1736
3. Clegg R (1995) Fluorescence resonance energy transfer. Curr Opin Biotechnol 6:103–110
4. Förster T (1948) Zwischenmolekulare energiewanderung und fluorescenze. Ann Phys 2:55–75
5. Förster T (1946) Energy transport and fluorescence [in German]. Naturwissenschafien 6:166–l75
6. Förster T (1951) Fluorescence of organic compounds [in German]. Vandenhoeck & Ruprecht, Gettingen, 312
7. Pande AH, Moe D, Nemec KN et al (2004) Modulation of human 5-lipoxygenase activity by membrane lipids. Biochemistry 43:14653–14666
8. Pande AH, Qin S, Tatulian SA (2005) Membrane fluidity is a key modulator of membrane binding, insertion, and activity of 5-lipoxygenase. Biophys J 88:4084–4094
9. Oddi S, Fezza F, Pasquariello N et al (2008) Evidence for the intracellular accumulation of anandamide in adiposomes. Cell Mol Life Sci 65:840–850
10. Patricelli MP, Lashuel HA, Giang DK et al (1998) Comparative characterization of a wild type and transmembrane domain-deleted fatty acid amide hydrolase: identification of the transmembrane domain as a site for oligomerization. Biochemistry 37:15177–15187
11. Di Venere A, Dainese E, Fezza F et al (2012) Rat and human fatty acid amide hydrolases: overt similarities and hidden differences. Biochim Biophys Acta 1821:1425–1433
12. MacDonald RC, MacDonald RI, Menco BP et al (1991) Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. Biochim Biophys Acta 1061:297–303
13. Qin S, Pande AH, Nemec KN et al (2004) The N-terminal α-helix of pancreatic phospholipase A_2 determines productive-mode orientation of the enzyme at the membrane surface. J Mol Biol 344:71–89

Chapter 28

Visualization of Endocannabinoids in the Cell

Sergio Oddi, Antonio Totaro, and Mauro Maccarrone

Abstract

A still unsolved, although critical, issue in endocannabinoid research is the mechanism by which the lipophilic compound anandamide (AEA) moves from its site of synthesis, crosses the aqueous milieu, and reaches the different intracellular compartments, where its metabolic and signaling pathways take place. The difficulty of studying intracellular AEA transport and distribution results from the lack of specific probes and techniques to track and visualize this bioactive lipid within the cell. Here, we describe the use of a biotinylated, non-hydrolyzable derivative of AEA (biotin-AEA, b-AEA) for visualizing the subcellular distribution of this endocannabinoid by means of confocal fluorescence microscopy.

Key words Endocannabinoids, Anandamide, Subcellular distribution, Glass cover slips, Biotinylated derivatives, Confocal microscopy

1 Introduction

The knowledge of the intracellular trafficking and distribution of endocannabinoids like AEA is a general prerequisite for a deeper understanding of the signal transduction pathways triggered by these bioactive lipids, and hence of their biological functions [1]. Getting this information requires the availability of specific probes able to reveal, by means of state-of-the-art morphological techniques, the distribution of endocannabinoids in the different membrane compartments of the cell. Unfortunately, this type of studies is experimentally hampered by the very low antigenicity of endocannabinoids, a feature shared by almost all lipids. To date, only a few compounds have been developed to investigate these aspects in relation to AEA metabolism: SKM 4-45-1 [2], and BODIPY-FL-AEA [3]. The former is a fluorescein isothiocyanate-conjugated analogue of AEA that becomes fluorescent (Ex. 495 nm/Em. 519 nm) upon hydrolysis by cytosolic esterases. The latter bears a BODIPY-FL-fluorophore (Ex. 508 nm/Em. 535 nm) that was found to be a substrate of AEA transmembrane transport machinery.

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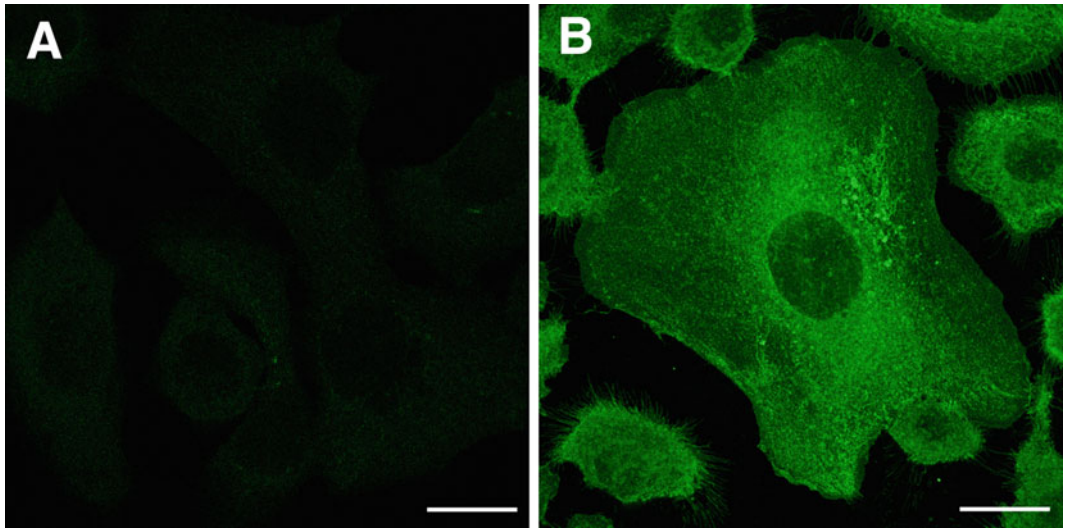

Fig. 1 Confocal microscopy images of the intracellular distribution of biotin-AEA in cultured cells. Human keratinocytes were incubated with 10 μM biotin (**a**) or with 10 μM b-AEA (**b**) for 10 min at 37 °C. Cells were washed, fixed, and stained with Alexa Fluor 488-conjugated streptavidin, and were observed at green emission wavelengths by a TCS SP confocal microscope (Leica Microsystems, Wetzlar, Germany), equipped with a 63× oil objective and a digital zoom of 2.5. Pictures were taken using the LAS AF program (Leica Microsystems), and then they were processed with Adobe Photoshop CS4 (Mountain View, CA, USA) for adjustments of brightness and contrast. Scale bars = 10 μm

However, likely because of the low degree of signal associated with direct fluorescence, the suitability of these compounds for fine morphological analysis of AEA distribution within the cell is rather limited.

In this context, we have recently described the characterization of a biotinylated derivative of AEA (b-AEA), and have shown through biochemical, morphological, and functional assays that b-AEA is a suitable probe for visualizing distribution and accumulation of this endocannabinoid in intact cells [4–7].

In this example, we used a bright, green fluorescent streptavidin (conjugated with 488-Alexa Fluor dye) to detect the distribution of b-AEA in cultured keratinocytes, by using laser scanning confocal microscopy (Fig. 1). Here, the protocol used to visualize AEA in intact cells is presented, as a prototype of live imaging of other endocannabinoids for which biotinylated probes are still missing.

2 Materials

All chemicals are of the purest analytical grade. Prepare all solutions using ultrapure water (≥18 MΩ × cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature, unless indicated otherwise.

2.1 Cell Culture

1. Primary normal human epidermal keratinocytes (ATCC, Manassas, VA, USA).
2. DMEM-K growth medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with keratinocyte growth kit (ATCC).

2.2 Cover Slips

1. 12 mm diameter borosilicate cover slips (*see* **Note 1**).
2. Collagen solution: 0.1 mg/ml Human placenta type IV collagen in 0.1 M acetic acid.
3. Sterilization: Sterilize cover slips by immersing them for 20 min in 80 % ethanol, and then rinse abundantly with sterile water.
4. Collagen coating (*see* **Note 2**): Coat with collagen the ethanol-sterilized cover slips by immersing them in collagen solution for 2 h at room temperature. Rinse cover slips in sterile water three times for 10 min. Dry cover slips in the hood for 12 h.

2.3 Solutions and Imaging Equipment

1. Biotin-AEA stock solution: 10 mM Biotin-AEA (Tocris Bioscience, Bristol, UK) in dimethylsulfoxide (DMSO). Store at –20 °C (stable for up to 1 month).
2. Biotin control stock solution: 10 mM Biotin in DMSO. Store the solution at –20 °C (stable for up to 1 month).
3. Prepare 1–10 μM b-AEA and biotin solutions by diluting 10 mM stocks in DMEM-K growth medium. Prepare at least 1 ml of each solution for each culture dish that has to be tested. Prepare immediately before use (*see* **Note 3**).
4. Wash solution: Phosphate-buffered saline (PBS).
5. Fixative solution: 3 % (wt/vol) formaldehyde solution. Dilute 10 % (wt/vol) formaldehyde solution with PBS (*see* **Note 4**), add sucrose at 4 % (wt/vol), and store in a dark bottle at 4 °C for up to 1 month.
6. PBS/glycine solution: 0.2 M Glycine in PBS. Add 0.05 % (wt/vol) sodium azide as a preservative. Store at 4 °C for up to 1 month.
7. Staining solution (*see* **Note 5**): Alexa Fluor 488-conjugated streptavidin, diluted 1:100 in PBS. Add 0.05 % (wt/vol) saponin as a membrane permeabilizer (*see* **Note 6**). Prepare immediately before use.
8. Mounting medium: ProLong Gold® antifade mountant (Thermo Fisher Scientific).
9. Clear nail polish.
10. Confocal microscope (*see* **Note 7**) with high magnification (e.g., 1000×), set up for image acquisition and processing.

3 Methods

3.1 Cell Culture

1. Culture NHEK cells onto collagen-coated glass cover slips in 24-well tissue culture plates (one cover slip per well) with 2 ml of DMEM-K for 24–48 h, until 50–70 % confluence is reached.
2. Wash cells three times with 2 ml of DMEM-K.

3.2 Incubation with b-AEA

1. Remove the DMEM-K growth medium from the cells, and add 1 ml of 1–10 μM biotin-AEA, or biotin control solution to each well (*see* **Note 8**).
2. Incubate cells from 10 to 30 min at 37 °C (*see* **Note 9**).
3. After incubation, place the plates on ice, aspire rapidly the media, and rinse the wells three times, for 3 min each, with 2 ml of ice-cold PBS to remove excess probe and to reduce background.

3.3 Labeling with b-AEA

1. Transfer the cover slips with a pair of tweezers into new multi-wells containing 0.3 ml of fixative solution per well for 20 min at room temperature.
2. Wash cells three times with 2 ml of PBS for 3 min each. Remove the excess of fixative by incubating cells for 5 min at room temperature with 2 ml of PBS/glycine solution.
3. Incubate cells in the dark with staining solution for 30 min at room temperature (*see* **Note 10**).
4. Wash cells three times (3 min each) with 2 ml of PBS.
5. By holding the cover glasses with a pair of tweezers, almost dry them with absorbent paper. Invert the cover slips with the attached cells onto a small drop (3 μl) of mounting medium.
6. Leave the mounting medium to dry overnight, and then seal the edges of the cover slips with clear nail polish (*see* **Note 11**).

3.4 Cell Examination

1. View cells under a laser scanning confocal microscope at high magnification (e.g., 1000×).
2. Acquire images with cells excited with 488 nm laser lines, collecting emissions at green (530–560 nm) wavelengths. Optimize image capture by using a minimum of optics, high-numerical aperture objectives, relatively low magnification and laser power, high-quality optical filters, and high-efficiency detectors.

Biotin-AEA is readily incorporated into plasma membranes, and reaches nearly all internal membranes within a few minutes, particularly those of the endoplasmic reticulum and those surrounding the nucleus (Fig. 1).

4 Notes

1. Modern microscope objectives are designed to be used with cover slips of 0.17 mm thickness. The quality of the cover glass, in particular the compliance with the nominal thickness, has thereby a crucial impact on the imaging quality. To this end, we recommend high-precision cover slips, featuring an exceptionally accurate thickness of 170 ± 5 μm.
2. Although it is not strictly necessary for the attachment of adherent cell lines, collagen coating of glass cover slips is performed to enhance growth and adherence of keratinocytes to the glass surface, clearly improving the fluorescence imaging results. For less adherent cells, coating is necessary to help them to stick to the glass surface. Almost any sort of extracellular matrix protein can be used to coat cover slips, including collagen, fibronectin, and laminin. Alternatively, either poly-L-lysine or gelatin from porcine skin represents excellent and inexpensive coating reagent that can be conveniently used in promoting cell adhesion.
3. Since b-AEA is not hydrolyzed by FAAH [6], the addition of specific inhibitors of this AEA-degrading enzyme in the culture medium can be omitted. The concentration of b-AEA used is an important factor for the success of cell labeling experiments. The optimal probe concentration should be determined empirically for each cell line. Therefore, it is useful to perform a preliminary study where a wide concentration range is used to optimize the signal-to-noise ratio.
4. To avoid shrinkage of the cells, it is important to use only those commercial preparations of formaldehyde that do not contain methanol. Formaldehyde is extremely toxic. Please read the material safety data sheet before working with this chemical. Gloves and safety glasses should be worn at all time, and solutions should be made inside a fume hood.
5. As an alternative to avidin-based reagents, the b-AEA tag can be detected also by high-affinity mouse monoclonal antibody against biotin. Both unlabeled and fluorescent dye-labeled versions of the anti-biotin antibody are commercially available for direct or indirect staining procedures.
6. Since b-AEA is a neutral lipid, particular attention should be paid to the choice of the appropriate membrane-permeabilizing agent. In our hands, 0.05–0.10 % digitonin or saponin permeabilize plasma membrane selectively by removing cholesterol only, and are therefore both considered gentler than other nonionic detergents, such as Triton X-100 and Nonidet P-40. The two latter substances are nonselective in nature and may extract the bulk of membrane lipids along with a significant fraction of b-AEA.

7. Samples may be examined with either a conventional epifluorescence microscope or a laser scanning confocal microscope. In our laboratory, we used confocal microscopy for imaging b-AEA distribution. Indeed, confocal microscopy offers several distinct advantages over traditional wide-field fluorescence microscopy, including an increased resolution and contrast, a better signal-to-noise ratio, and the capability to collect serial optical sections from the same specimen.
8. Serum albumin interferes with, or even abolishes, AEA uptake by the cell, possibly due to the high-affinity binding of AEA to this protein [4]. For this reason, we recommend to avoid albumin-containing media, such as those supplemented with fetal sera.
9. The time to reach an adequate level of b-AEA incorporation should be determined empirically for each cell line, and typically ranges from 10 to 30 min.
10. Streptavidin concentration should be varied in pilot experiments, to maximize signal while minimizing background. The time to reach an adequate level of staining should be determined empirically for each cell line, and typically ranges from 10 to 30 min.
11. After being mounted with the mounting medium, cover slips can be stored in a covered slide box at 4 °C. Sealing the edges with clear nail polish delays the oxidation and extends the lifespan of the specimens up to several months.

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References

1. Maccarrone M, Dainese E, Oddi S (2010) Intracellular trafficking of anandamide: new concepts for signaling. Trends Biochem Sci 35:601–608
2. Muthian S, Nithipatikom K, Campbell WB et al (2000) Synthesis and characterization of a fluorescent substrate for the N-arachidonoylethanolamine (anandamide) transmembrane carrier. J Pharmacol Exp Ther 293:289–295
3. Gretskaya NM, Akimov MG, Bezuglov VV (2014) A new fluorescent analogue for the studies of anandamide transport in cell cultures. Russ J Bioorg Chem 40:228–232
4. Oddi S, Fezza F, Catanzaro G et al (2010) Pitfalls and solutions in assaying anandamide transport in cells. J Lipid Res 51:2435–2444
5. Oddi S, Fezza F, Pasquariello N et al (2009) Molecular identification of albumin and Hsp70 as cytosolic anandamide-binding proteins. Chem Biol 16:624–632
6. Fezza F, Oddi S, Di Tommaso M et al (2008) Characterization of biotin-anandamide, a novel tool for the visualization of anandamide accumulation. J Lipid Res 49:1216–1223
7. Oddi S, Fezza F, Pasquariello N et al (2008) Evidence for the intracellular accumulation of anandamide in adiposomes. Cell Mol Life Sci 65:840–850

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