Chapter 28

Visualization of Endocannabinoids in the Cell

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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.

Mauro Maccarrone (ed.), *Endocannabinoid Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 1412,
DOI 10.1007/978-1-4939-3539-0_28, © Springer Science+Business Media New York 2016

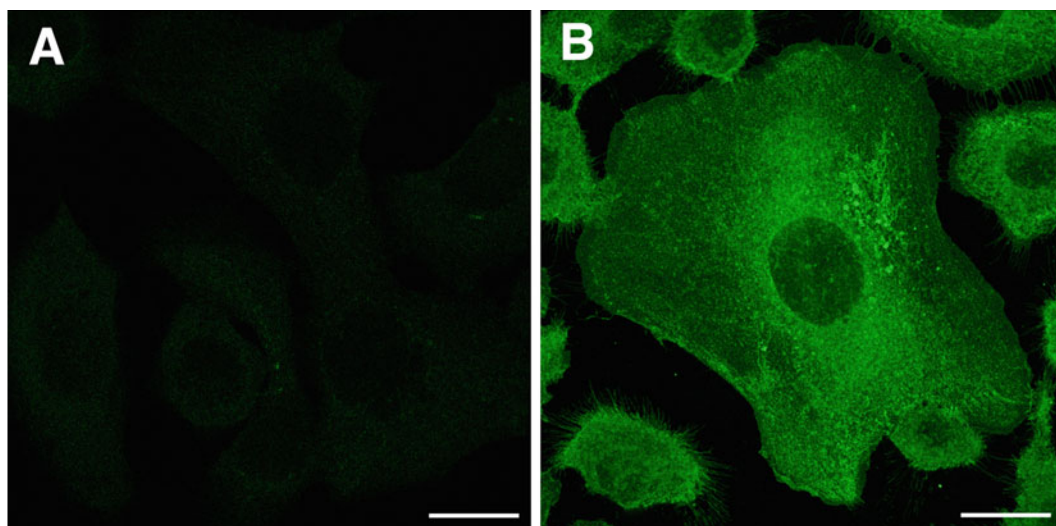

Fig. 1 Confocal microscopy images of the intracellular distribution of biotin-AEA in cultured cells. Human keratinocytes were incubated with 10 μM biotin (**a**) or with 10 μM b-AEA (**b**) for 10 min at 37 °C. Cells were washed, fixed, and stained with Alexa Fluor 488-conjugated streptavidin, and were 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.

Acknowledgements

This work was supported by the Italian Ministry of Education, University and Research (grant MIUR 2010/2011 to M.M.).

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