ISSN 1990-7478, Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology, 2018, Vol. 12, No. 1, pp. 70–73. © Pleiades Publishing, Ltd., 2018.
Original Russian Text © O.V. Krestinina, I.V. Odinokova, Yu.L. Baburina, T.S. Azarashvili, 2017, published in Biologicheskie Membrany, 2017, Vol. 34, No. 5, pp. 42–47.

ARTICLES

Detection of Protein Kinase A and C Target Proteins in Rat Brain Mitochondria

O. V. Krestinina*, I. V. Odinokova, Yu. L. Baburina, and T. S. Azarashvili

Institute of Theoretical and Experimental Biophysics Russian Academy of Science, Pushchino, Moscow oblast, 142290 Russia

**e-mail: ovkres@mail.ru*

Received April 7, 2017; in final form, May 31, 2017

Abstract—Phosphorylation of some membrane-bound proteins in the mitochondria of rat liver and brain is regulated by Ca^{2+} and cAMP acting as secondary messengers. These proteins are the main myelin components: 46 kDa 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) and two isoforms of the myelin basic protein (MBP) with molecular weights of 17 and 21.5 kDa, which we have identified previously and found outside myelin in rat brain mitochondria. The phosphorylation level of CNP and both MBP isoforms increases when the mitochondrial permeability transition pore (mPTP) is opened. It is known that protein kinases A and C in heart mitochondria are directly bound to mPTP regulator proteins and are able to modulate the pore function. It is shown in this study that the inhibitors of protein kinases A (H-89) and C (staurosporin, Go 6976, and GF 109203 X) decrease the phosphorylation level of CNP and two MBP isoforms allowing us to assume that they are the targets of the signaling protein kinases A and C.

Keywords: brain mitochondria, permeability transition pore, protein kinase A, protein kinase C

DOI: 10.1134/S1990747818010087

INTRODUCTION

Brain ischemia and other neurodegenerative disorders initiate abnormal metabolic stress leading to an increase in the cytosolic calcium concentration, which activates the enzymes of degradation (proteases, endonucleases, and phospholipases) and the disintegration of membranes. A key event in the intracellular signaling, responsible for regulation of various enzyme systems and for many cellular functions, is a phosphorylation cascade of membrane receptors, ion channels, and other factors. The degree of protein phosphorylation depends on the activity of specific protein kinases and phosphatases. In the central nervous system, protein kinases A (PKA) and C (PKC) are expressed at a high level; they are translocated from the cytosol to the plasma membranes, nuclei, the periphery of cell, and mitochondria. There they control a number of functions, including gene transcription, translation of proteins, membrane transport, and signal transduction in the cell. It is now established that mitochondria play a crucial role in the survival of brain cells; a high percentage of damaged mitochondria is detected in neurodegeneration [1, 2]. In the mitochondria of eukaryotes, PKA and PKC, which may phosphorylate a number of membrane-bound proteins in the mitochondrial matrix, have been discovered and identified. Recently, we have established that the degree of phosphorylation of certain rat brain mitochondrial proteins is changed upon opening of the Ca^{2+}-dependent and CsA-sensitive permeability transition pore, mPTP, in the inner membrane [3, 4]. It is known that the mPTP opening occurs in response to Ca^{2+} overload, i.e., achieving Ca^{2+} concentrations exceeding the threshold level, or to oxidative stress [5]; the mPTP opening is characterized by a drop in membrane potential, swelling of mitochondria, and release from the mitochondrial matrix to cytosol of proapoptotic factors, such as cytochrome *c*, apoptosis-inducing factor, endonuclease G, and others [5]. It is believed that mPTP participates in the initial stage of cell death (apoptosis or necrosis). Mitochondria play an important role in intracellular signaling through the formation of reactive oxygen species and regulation of intracellular calcium signaling. The data have been obtained indicating that the mPTP components can interact with PKCε during ischemia/reperfusion in heart mitochondria, and this interaction can serve as a signaling mechanism to modulate the pore functioning [6]. 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP, 46 kDa [7]) and two isoforms of the myelin basic protein (MBP, 17 and 21.5 kDa) have been identified in the rat brain mitochondria [8], the phosphorylation status of which depended on the pore state. These proteins have the ability to bind firmly to mitochondria. Upon addition of the brain myelin fraction containing CNP and MBP to liver mitochondria,

where these proteins are absent, mitochondrial resistance to the initiation of the mPTP opening has been observed [9]. Since the signal protein kinases PKA and PKC are expressed at a high level in CNS, we hypothesize that they are involved in the CNP and MBP phosphorylation. The aim of this study was to investigate the involvement of PKA and PKC in phosphorylation of 2',3'-cyclic-nucleotide-3'-phosphodiesterase and two isoforms of myelin basic protein using specific inhibitors of these protein kinases.

MATERIALS AND METHODS

Isolation of mitochondria from the brain. Rat brain mitochondria were isolated by the method of Sims [10] modified in our laboratory. Isolated brain was shredded, separated from the blood vessels and homogenized in a tenfold volume of medium containing 320 mM sucrose, 10 mM Tris-HCl (pH 7.4), 0.5 mM K^+-EDTA, 0.5 mM EGTA, and 0.2% bovine serum albumin (BSA) (all reagents from Sigma, USA) using a glass homogenizer. The homogenate was centrifuged at 2000 *g* for 3 min, the pellet was removed, and the supernatant was again centrifuged for complete removal of nuclei and damaged cells. The sediment of mitochondria obtained by centrifugation at 12500 *g* for 10 min was washed at 11500 *g* for 10 min in the isolation medium containing EGTA, EDTA, and BSA and resuspended in the same medium. All procedures were carried out at 4°C.

Phosphorylation of the brain mitochondrial proteins. Phosphorylation of the mitochondrial proteins was performed by the method developed earlier in our laboratory [3]. A mixture of "cold" ATP and [γ-^{32}P] ATP was used in the reaction. The total ATP concentration in a sample was 0.5–1 μM; the sample contained 5–7 μCi of [γ-^{32}P]ATP. In the study of protein phosphorylation upon mPTP opening, an aliquot was taken from the mitochondrial suspension sample under the control conditions and under the conditions of the mPTP opening induced by the Ca^{2+} addition at a threshold concentration. The aliquot (20 μL) was mixed with 5 μL of the labeled and "cold" ATP mixture for protein phosphorylation. The F_oF_1-ATP-ase inhibitor oligomycin (1.5 μM) was added to the sample to prevent the ATP hydrolysis. The standard phosphorylation time was 3 min; the reaction was interrupted by the addition of 20 μL of the solubilizing solution containing 350 mM Tris-HCl (pH 7.8), 10% glycerol, 15% Na dodecyl sulfate (SDS), and 25% β-mercaptoethanol. The samples were kept in a boiling bath for 3 min.

Electrophoresis in 15% PAAG. Electrophoresis under denaturing conditions was performed in a minichamber (Hoefer, Germany) according to the method of Laemmli [11]. A 10-μg sample of protein was applied to each lane of the gel. The Farmacia Biotech (USA) sets containing high-molecular weight (14.4–97 kDa) and low-molecular weight (3.46–16.9 kDa) marker proteins were used as markers. After electrophoresis, the gels were fixed, stained with Coomassie R-250, and air-dried between sheets of cellophane. The radioautograms of the gels were obtained by exposing on a Kodak X-Omat AR-5 X-ray film (Sigma, USA). The optical density of the X-ray films in the area of localization of the proteins labeled with [γ-^{32}P]ATP was determined using the program Quantity One and a BioRad scanning densitometer (USA).

RESULTS AND DISCUSSION

First of all the spectrum of phosphoproteins was determined in the isolated brain mitochondria. For phosphorylation of proteins, the mitochondrial suspension was preincubated with [γ-^{32}P]ATP and separated by electrophoresis in polyacrylamide gel (PAAG) under denaturing conditions. After the electrophoresis, the gels containing the phosphorylated polypeptides were fixed, stained, dried, and exposed on the Kodak film to obtain radioautograms. Figure 1a shows the distribution of phosphorylated proteins after their separation in PAAG. Mitochondria were preincubated with [γ-^{32}P]ATP for 3 min. As a result, ^{32}P-containing proteins with molecular weights of 67, 46, 21.5, 17, and 3.5 kDa and different levels of ^{32}P incorporation were detected. Figure 1b shows the relative levels of ^{32}P incorporation into the phosphorylated proteins. The ^{32}P content in the peptide with a molecular weight of 3.5 kDa was approximately 33%, in the peptide with a weight of 67 kDa it was 27% of the total assimilated radioactive phosphate; the relative ^{32}P incorporation into polypeptides with molecular weights of 17, 21.5, and 46 kDa was 9, 7, and 15%, respectively.

These phosphoproteins we identified as 2',3'-cyclic-nucleotide 3'-phosphodiesterase, CNPase (46 kDa) [7], two MBP isoforms (17 and 21.5 kDa) [8] and subunit *c* of the membrane sector of the ATPase with molecular weight of 3.5 kDa [3].

Because the relationship between the mPTP induction and the phosphorylation/dephosphorylation of the studied proteins was discovered earlier [4], we tested the involvement of PKA and PKC in the phosphorylation of mitochondrial proteins upon mPTP induction. Figure 2 shows the radioautogram of the phosphoproteins after separation by electrophoresis in 15% PAAG upon mPTP induction in the presence of H-89, a specific PKA inhibitor. It follows from Fig. 2 that H-89 (100 nM) causes a noticeable reduction of phosphorylation of the 17, 21.5, and 46 kDa phosphoproteins, i.e., MBP and CNP.

It is known that the heart mitochondrial inner membrane contains 42 and 29 kDa phosphoproteins, phosphorylation of which is regulated by cAMP-dependent PKA [12]. The TSPO (18 kDa) and VDAC (32 kDa) proteins located in the outer membrane are phosphorylated by PKA, too [13]. The obtained data indicate the involvement of this kinase in the phos-

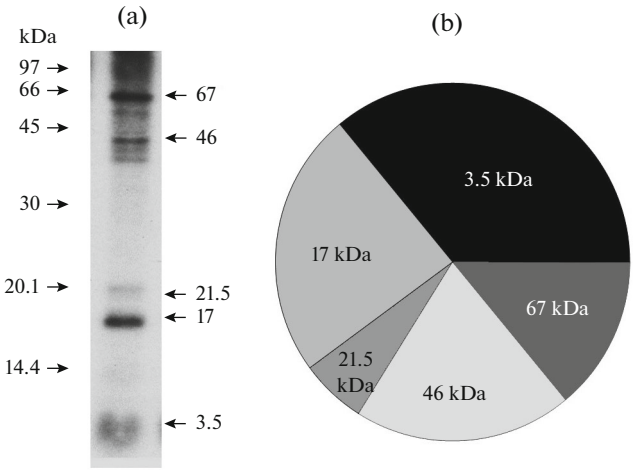

Fig. 1. Distribution of the brain mitochondrial phosphoproteins in 15% PAAG under denaturing conditions. (a) The radioautogram of phosphorylated proteins. On the left, the localization of protein markers is shown. (b) The diagram showing relative phosphorylation levels of the [γ-^{32}P]ATP labeled proteins.

phorylation of the MBP (17, 21.5 kDa) isoforms and CNP (46 kDa) as well. It should be noted that the transfer of signals in the cell requires controlled dynamic molecular interactions. A signal received by a receptor on the outer mitochondrial membrane surface is transmitted to a complex generated on a signaling platform. This complex, called signalosome, is formed by the anchor proteins for PKA (AKAP). In addition to AKAP, in mitochondria there are the regulatory PKA subunit and phosphatase/phosphoesterases, which regulate the degree of protein phosphorylation and form signaling pathways [14].

Ca^{2+}- and phospholipid-dependent protein kinase PKC carries out phosphorylation of the mitochondrial membrane proteins, too [15]. These proteins include α, β, γ, and δ subunits of the F_oF_1-ATP synthase, which are phosphorylated by PKCα. Substrates of PKSε in mitochondria are also identified: they are the respiratory chain components and cytochrome oxidase. Analysis of the PKCε subcellular distribution in the mice, in the heart of which the specifically activated PKCε is expressed, has shown that activated PKCε forms signaling complexes with mitochondrial proteins VDAC and ANT (mPTP regulators) [16].

We used the broad-spectrum inhibitor staurosporine (St) (Fig. 3) and specific PKC inhibitors, such as Go 6976 and GF 109203 X (Fig. 4), to determine the involvement of Ca^{2+}-dependent PKC in the phosphorylation of mitochondrial proteins upon the mPTP induction. For this purpose, a suspension of mitochondria was incubated in the presence of [γ-^{32}P]ATP, then separated in SDS-PAAG and exposed on the X-Ray film.

Figure 3 shows the change in the phosphorylation of polypeptides in the presence of 1 μM staurosporine upon the mPTP induction. In the presence of this inhibitor, there is a noticeable decrease in the radioactive phosphate incorporation into the phosphopeptide with a molecular weight of 46 kDa and a very strong decrease of incorporation into the 17 and 21.5 kDa phosphopeptides. These results show that MBP and CNP are potential targets of PKC.

To determine the type of PKC involved in the phosphorylation of the studied proteins, we tested the effect of specific PKC inhibitors Go 6976 (inhibitor of Ca^{2+}-dependent PKC) and GF 109203 X (inhibitor of the mixed type PKC) at the same concentration. Figure 4 shows the radioautogram of labeled proteins after sep-

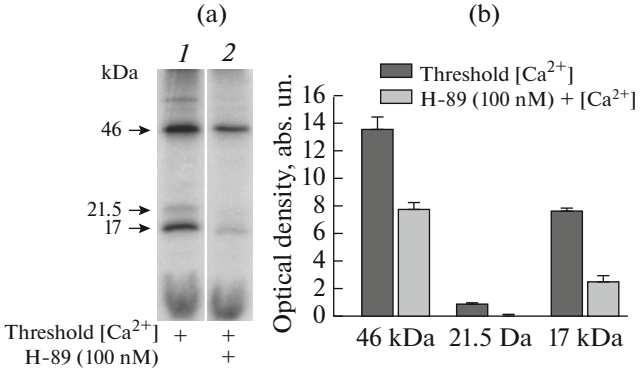

Fig. 2. The effect of the protein kinase A inhibitor H-89 on the phosphorylation of the brain mitochondrial proteins upon mPTP induction. (a) The radioautogram of phosphorylated proteins, when the pore is open without additions (*1*) and in the presence of 100 nM H-89 (*2*). (b) The diagram showing the relative levels of phosphorylation of the 46, 21.5, and 17 kDa proteins. 100% level of protein phosphorylation was taken when mPTP was open.

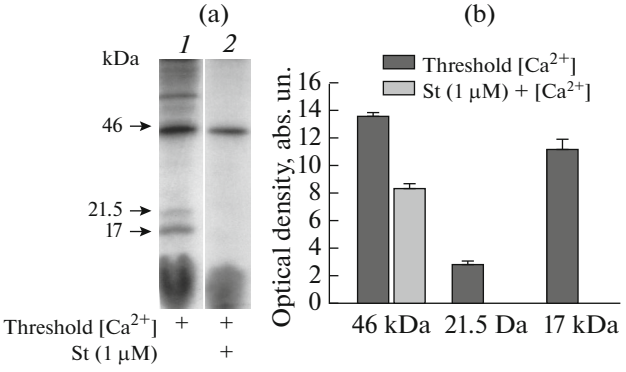

Fig. 3. The effect of protein kinase C inhibitor staurosporine. (a) The radioautogram of phosphorylated proteins, when the pore is open without additions (*1*) and in the presence of 1 μM staurosporine (*2*). (b) The diagram showing the relative levels of phosphorylation of the 46, 21.5, and 17 kDa proteins in the presence of 1 μM staurosporine, when the pore was open. The level of protein phosphorylation under open mPTP was taken as 100%.

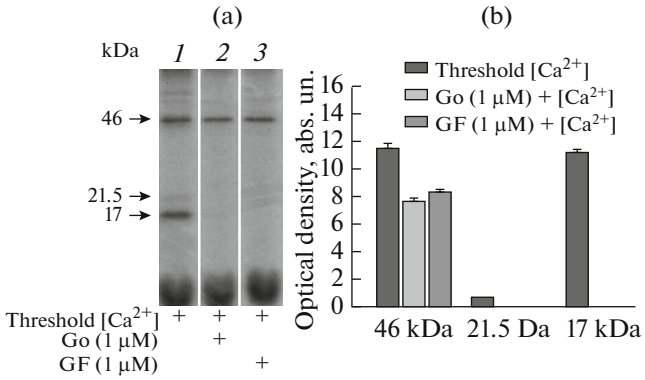

Fig. 4. The effect of protein kinase C inhibitors Go 6976 and GF 109203 X. (a) The radioautogram of phosphorylated proteins, when the pore is open without additions (*1*) and in the presence of 1 μM Go 6976 (*2*) or 1 μM GF 109203 X (*3*). (b) The diagram showing the relative phosphorylation levels of the 46, 21.5, and 17 kDa proteins in the presence of 1 μM Go 6976 (*1*) or 1 μM 109203X GF (*2*). The level of protein phosphorylation under open mPTP was taken as 100%.

aration in PAAG upon the mPTP induction (*1*) and in the presence of specific inhibitors of PKC (*2*, Go 6976; *3*, GF 109203 X). The effect similar to that of staurosporine can be seen in Fig. 4. The data suggest that Ca^{2+}-dependent PKC is involved in the phosphorylation of MBP (17 and 21.5 kDa) and CNP (46 kDa). As it was mentioned already, PKCε forms signaling complexes with VDAC and ANT in heart mitochondria [6, 16]. Interestingly, in the brain mitochondria, CNP coprecipitated with VDAC and ANT [18]. Direct interaction of the signaling kinase PKCε with mPTP regulators indicates its ability to modulate the mPTP activity. We have demonstrated previously that the association of myelin proteins with mitochondria depends on the functional state of the pore [9]. Since MBP and CNP are the substrates of PKA and PKCε, it can be assumed that the association of CNP and MBP with mitochondria and their ability to prevent the mPTP opening is regulated by these two kinases.

REFERENCES

1. Domanska-Janik K. 1996. Protein serine/threinine kinase (PKA, PKC and CAMKII) involved in ischemic brain pathology. *Acta Neurobil. Exp.* **56**, 579–585.
2. Hansford R.G. 1994. Physiological role of mitochondrial Ca^{2+} transport. *J. Bioenerg. Biomembr.* **26** (5), 495–508.
3. Azarashvily T.S., Tyynela J., Baumann M., Evtodienko Y.V., Saris N.E. 2000. Ca^{2+}-modulated phosphorylation of a low-molecular-mass polypeptide in rat liver mitochondria: Evidence that it is identical with subunit c of F_oF_1-ATPase. *Biochem. Biophys. Res. Commun.* **270** (3), 741–744.
4. Azarashvili T., Krestinina O., Odinokova I., Evtodienko Y., Reiser G. 2003. Physiological Ca^{2+} level and Ca^{2+}-induced permeability transition pore control protein phosphorylation in rat brain mitochondria. *Cell Calcium.* **34** (3), 253–259.
5. Bernardi P. 2013. The mitochondrial permeability transition pore: A mystery solved? *Front. Physiol.* **4**, 95.
6. Baines C.P., Song C.X., Zheng Y.T., Wang G.W., Zhang J., Wang O.L., Guo Y., Bolli R., Cardwell E.M., Ping P. 2003. Protein kinase Cε interacts with and inhibits the permeability transition pore in cardiac mitochondria. *Circ. Res.* **92** (8), 873–880.
7. Azarashvili T., Krestinina O., Galvita A., Grachev D., Baburina Y., Stricker R., Reiser G. 2014. Identification of phosphorylated form of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) as 46 kDa phosphoprotein in brain non-synaptic mitochondria overloaded by calcium. *J. Bioenerg. Biomembr.* **46** (2), 135–145.
8. Krestinina, O.V., Makarov, P.R., Baburin, Y.L., Gordeeva A.E., Azarashvili T.S. 2013. Identification of phosphorylated forms of myelin basic protein associated with mitochondria. *Neyrokhimiya* (Rus.). **30** (4), 321–328.
9. Baburina, Y.L., Gordeeva A.E., Moshkov D.A., Krestinina O., Azarashvili A.A., Odinokova, I.V., And Azarashvili, T.S., 2014. Association of myelin basic protein (MBP) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) with the mitochondria. *Biochemistry (Moscow).* **79** (6), 555–565.
10. Sims N.R. 1990. Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. *J. Neurochem.* **55**, 698–707.
11. Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227** (5259), 680–685.
12. Technikova-Dobrova Z., Sardanelli A.M., Stanca M.R., Papa S. 1994. cAMP-dependent protein phosphorylation in mitochondria of bovine heart. *FEBS Lett.* **350** (2–3), 187–191.
13. Whalin M.E., Boujrad N., Papadopoulos V., Krueger K.E. 1994. Studies on the phosphorylation of the 18 kDa mitochondrial benzodiazepine receptor protein. *J. Recept. Res.* **14**, 217–228.
14. Torres-Quesada O., Mayrhofer J.E., Stefan E. 2017. The many faces of compartmentalized PKA signalosomes. *Cell Signalling.* **37** (1), 1–11.
15. Nishizuka Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature.* **308** (5961), 693–699.
16. Budas G.R., Mochly-Rosen D. 2007. Mitochondrial protein kinase Cε(PKCε): Emerging role in cardiac protection from ischaemic damage. *Biochem. Soc. Trnsactions.* **35** (5), 1052–1054.
17. Sun X., Budas G.R., Xu L., Barreto G.E., Mochly-Rosen D., Giffard R. 2013. Selective activation of PKC epsilon in mitochondria is neuroprotective *in vitro* and reduces focal ischemic brain injury in mice. *J. Neurosci. Res.* **91** (6), 799–807.
18. Azarashvili T., Krestinina O., Galvita A., Grachev D., Baburina Y., Stricker R., Evtodienko Y., Reiser G. 2009. Ca^{2+}-dependent permeability transition regulation in rat brain mitochondria by 2',3'-cyclic nucleotides and 2',3'-cyclic nucleotide 3'-phosphodiesterase. *Am. J. Physiol. Cell. Physiol.* **296**, C1428–C1439.

Translated by E. Puchkov