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Mitochondria express several nicotinic acetylcholine receptor subtypes to control various pathways of apoptosis induction

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ABSTRACT

Nicotinic acetylcholine receptors control survival, proliferation and cytokine release in non-excitable cells. Previously we reported that α7 nicotinic receptors were present in the outer membranes of mouse liver mitochondria to regulate mitochondrial pore formation and cytochrome *c* release. Here we used a wide spectrum of nicotinic receptor subunit-specific antibodies to show that mitochondria express several nicotinic receptor subtypes in a tissue-specific manner: brain and liver mitochondria contain α7β2, α4β2 and less α3β2 nicotinic receptors, while mitochondria from the lung express preferentially α3β4 receptor subtype; all of them are non-covalently connected to voltage-dependent anion channels and control cytochrome *c* release. By using selective ligands of different nicotinic receptor subtypes (acetylcholine (1 μM) or dihydro-β-erythroidine (1 μM) for α4β2), conotoxin MII (1 nM) for α3β2, MLA (50 nM) for α7β2 and acetylcholine (10 μM) for all subtypes) and apoptogenic agents triggering different mitochondrial signaling pathways (1 μM wortmannin, 90 μM Ca^{2+} or 0.5 mM H_2O_2) it was found that α7β2 receptors affect mainly PI_3K/Akt pathway, while α3β2 and α4β2 nAChRs also significantly influence CaKMII- and Src-dependent pathways. It is concluded that cholinergic regulation in mitochondria is realized through multiple nicotinic receptor subtypes, which control various pathways inducing mitochondrial type of apoptosis.

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1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels mediating fast synaptic transmission in muscle and autonomic ganglia, controlling transmitter release in the central nervous system and regulating proliferation, survival, adhesion and cytokine secretion in non-excitable cells. Structurally, nAChRs are homo- or heteropentamers composed of ten types of α-subunits (α1–α10) and four types of β-subunits (β1–β4); muscle receptors contain also γ, δ and ε subunits. Various established subunit combinations result in different nAChR subtypes varying in the kinetic parameters and selectivity of the ion channel, as well as in ligand specificity, signaling pathways and functions performed in different tissues (Changeux, 2012). Classically, functional nAChRs have been attributed exclusively to the cell plasma membrane. We have found that nAChRs composed of α7 subunits were present in the outer membrane of mouse liver mitochondria to regulate mitochondrial pore formation by affecting intramitochondrial kinases (Gergalova et al., 2012, 2014). In another study we demonstrated an overlapping binding of α3-specific antibody and mitochondrial markers in several human cell lines (Kalashnyk et al., 2012). In the present paper we used a wide spectrum of nAChR subunit-specific antibodies and nAChR subtype-specific pharmacological tools to show that mitochondria express multiple nAChR subtypes in a tissue-specific manner to effectively sustain apoptosis induced by various stress factors.

2. Materials and methods

2.1. Animals

We used age-matched male wild-type and mutant, lacking either α7 or β2 nicotinic receptor subunit (Orr-Urtreger et al., 1997;

Abbreviations: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; cyt *c*, cytochrome *c*; DhβE, dihydro-β-erythroidine; MLA, methyllycaconitine; MPTP, mitochondria permeability transition pore; CaMKII, calcium-calmodulin-dependent kinase; PI_3K, phosphatidylinositol-3-kinase.

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Picciotto et al., 1998) mice with common C57BL/6J background. The mice were kept in the animal facilities of Pasteur Institute, Paris and Palladin Institute of Biochemistry, Kyiv. They were housed in a quiet, temperature-controlled room (22–23 °C) and were provided with water and dry food pellets *ad libitum*. Before removing the liver, brain or lungs mice were sacrificed by cervical dislocation. All procedures conformed to the guidelines of the Centre National de la Recherche Scientifique or IACUC of Palladin Institute. Before starting the experiments, the protocols were approved by the Animal Care and Use Committee of Palladin Institute of Biochemistry (Protocol 1/7-421).

2.2. Reagents

All reagents were of chemical grade and were purchased from Sigma–Aldrich unless specially indicated. Antibodies against α7(1-208), α7(179–190), α3(181–192), α4(181–192), α9(11–23), β2(190–200) and β4(190–200) nAChR fragments were obtained and characterized by us previously (Skok et al., 1999; Lykhmus et al., 2010; Koval et al., 2004, 2011). Generation of rabbit cyt *c*-specific antibodies has been described before (Gergalova et al., 2014). Antibodies against voltage-dependent anion channels (VDAC) and against TOM22 were from Sigma–Aldrich. Conotoxin MII was synthesized as described previously (Surin et al., 2012).

2.3. Mitochondria purification and fractionation

Mitochondria isolation from the mouse liver, brain and lungs and purification of their outer membranes were performed by differential ultracentrifugation according to standard procedure (Sottocasa et al., 1967) and as described previously (Gergalova et al., 2012). To prepare detergent lysates, the membranes or whole mitochondria were freezed at −70 °C, thawed and treated with the lysing buffer (0.01 M Tris–HCl, pH 8.0; 0.14 M NaCl; 0.025% NaN_3; 1% Tween 20 and protease inhibitors cocktail) for 2 h on ice upon intensive stirring. The resulting lysate was cleared by centrifugation (20 min at 25,000 × *g*) and dialysed against PBS containing 0.025% NaN_3 and protease inhibitors. To destroy non-covalent protein interactions within the lysate the samples were additionally supplemented with 2% sodium dodecylsulfate (SDS) and heated at 90 °C for 5 min in the water bath. The protein concentration was established by BCA assay (Thermo Scientific).

2.4. Sandwich assays

To determine the subunit composition of mitochondrial nAChRs the 96-well plates (Nunc Maxisorb, Denmark) were coated with rabbit α7(1–208)-specific antibody (40 μg/ml) and were subsequently blocked with 1% BSA/PBS. The detergent lysates of mitochondria outer membranes (100 μg/ml) were applied into the coated wells for 2 h at 37 °C and then the plates were washed with water. The bound antigen was revealed with biotinylated α3(181–192)-, α4(181–192)-, α7(179–190)-, α9(11–23)-, β2(190–200)- or β4(190–200)-specific antibodies applied for additional 2 h followed by Extravidin-peroxidase conjugate and *o*-phenylenediamine-containing substrate solution. The absorbance at 490 nm was read by the StatFax-2100 Microplate reader (Awareness Technology, USA).

To determine the β-subunits within α7-containing nAChRs, the plates were coated with α7(179–190)-specific antibody and the bound antigen was revealed with biotinylated either β2(190–200)- or β4(190–200)-specific antibodies.

To determine molecular partners of mitochondrial nAChRs, the plates were coated with VDAC- or TOM22-specific antibody (40 μg/ml) and the bound complex was revealed with biotinylated α3(181–192)-, α4(181–192)- or α7(179–190)-specific antibodies. In the "reverse" assay, the plate was coated with α7(1–208)-specific antibody and the antigen captured from the mitochondrial lysate (either treated or not with 2% SDS) was detected with biotinylated VDAC-specific antibody.

2.5. Cyt c release studies

The purified mitochondria (120 μg of protein per ml) were incubated with 90 μM $CaCl_2$, 0.5 mM H_2O_2 or 1 μM wortmannin for 2 min at room temperature and were immediately pelleted by centrifugation (10 min, 7,000 g at 4 °C). The incubation medium contained 10 mM HEPES, 125 mM KCl, 25 mM NaCl, 5 mM sodium succinate and 0.1 mM Pi(K), pH 7.4. The nAChR ligands (acetylcholine, dihydro-β-erythroidine, conotoxin MII or methyllycaconitine) were applied 2–3 min prior to apoptogenic agents. The mitochondria supernatants were collected and tested for the presence of cytochrome *c* (cyt *c*) by sandwich assay as described previously (Gergalova et al., 2012). Experimental values of optical density (OD 490 nm) shown in Fig. 4 were within the linear part of the calibration curve built with bovine cyt *c*.

3. Results

3.1. Study of nAChR subtypes and their molecular partners present in mitochondria from different tissues

First of all, we performed a sandwich ELISA with the outer membranes of liver mitochondria from the wild-type, α7−/− and β2−/− mice using the capture α7(1–208)-specific antibody and selective subunit-specific antibodies as detecting ones (Fig. 1A). The antibody generated against the whole extracellular domain (1–208) of α7 subunit could capture more or less efficiently multiple nAChR subunits due to their substantial structural homology (Kalamida et al., 2007), while antibodies generated against definite peptide epitopes of various subunits were much more specific (Skok et al., 1999; Glushakov et al., 2004; Koval et al., 2004; Shelukhina et al., 2006; Koval et al., 2011). As shown in Fig. 1B, the nAChRs captured with α7(1–208)-specific antibody in the preparations of the wild-type mitochondria were revealed with α3-, α4-, α7- and β2-, but not α9- or β4-specific antibodies. Preparations of α7−/− mitochondria provided no α7-specific signal and weaker α4- and β2-specific signals but stronger signals with α3- and β4-specific antibodies. Preparations of β2−/− mitochondria demonstrated no binding with β2-specific antibody, weaker binding with α4- and α7-specific antibodies and stronger binding with β4-specific antibody; no changes were observed with α3-specific antibody. This data indicated that the wild-type mitochondria contained α7(β2), α4β2 and α3β2 nAChR subtypes. In mice lacking α7 subunit, α7(β2) nAChRs disappeared, the number of α4β2 ones decreased, while that of α3β4 nAChRs increased. In β2−/− mice, the number of both α4- and α7-containing nAChRs decreased, that of α3 ones remained unchanged and β2 subunits were substituted with β4 ones.

α4β2/β4 and α3β2/β4 are established nAChR subunit combinations found in the brain, autonomic ganglia and some non-excitable cells (Changeux, 2012). In contrast, heteromeric α7 nAChRs were found only in certain brain regions and autonomic ganglia (Cuevas et al., 2000; Azam et al., 2003). To find out if α7 subunits were indeed combined with β2 ones in mitochondria, we designed a sandwich assay in which the receptor was captured with α7(179–190)-specific antibody and was revealed with β2- or β4-specific antibodies. As shown in Fig. 2A, combination of α7- and β2-specific antibodies gave positive signal in mitochondria of the wild-type but not α7−/− or β2−/− mice. The preparation of β2−/− mitochondria, instead, gave a strong signal with

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