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α7 nicotinic acetylcholine receptors control cytochrome *c* release from isolated mitochondria through kinase-mediated pathways

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ABSTRACT

Nicotinic acetylcholine receptors are ligand-gated ion channels found in the plasma membrane of both excitable and non-excitable cells. Previously we reported that nicotinic receptors containing α7 subunits were present in the outer membranes of mitochondria to regulate the early apoptotic events like cytochrome *c* release. Here we show that signaling of mitochondrial α7 nicotinic receptors affects intramitochondrial protein kinases. Agonist of α7 nicotinic receptors PNU 282987 (30 nM) prevented the effect of phosphatidyl inositol-3-kinase inhibitor wortmannin, which stimulated cytochrome *c* release in isolated mouse liver mitochondria, and restored the Akt (Ser 473) phosphorylation state decreased by either 90 μM Ca^{2+} or wortmannin. The effect of PNU 282987 was similar to inhibition of calcium-calmodulin-dependent kinase II (upon 90 μM Ca^{2+}) or of Src kinase(s) (upon 0.5 mM H_2O_2) and of protein kinase C. Cytochrome *c* release from mitochondria could be also attenuated by α7 nicotinic receptor antagonist methyllicaconitine or α7-specific antibodies. Allosteric modulator PNU 120526 (1 μM) did not improve the effect of agonist PNU 282987. Acetylcholine (1 μM) and methyllicaconitine (10 nM) inhibited superoxide release from mitochondria measured according to alkalization of Ca^{2+}-containing medium. It is concluded that α7 nicotinic receptors regulate mitochondrial permeability transition pore formation through ion-independent mechanism involving activation of intramitochondrial PI_3K/Akt pathway and inhibition of calcium-calmodulin-dependent or Src-kinase-dependent signaling pathways.

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1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels initially discovered in the neuromuscular junctions and fish electric organs and further found in the central and autonomic nervous system, as well as in many non-excitable cells (Changeux, 2012). The growing evidence suggests a universal role of acetylcholine (ACh), an evolutionary ancient mediator regulating vital cellular processes like proliferation, survival, adhesion and motility (Wessler and Kirkpatrick, 2008). Consequently, receptors to ACh of both nicotinic and muscarinic type were found in representatives of plant and animal kingdoms (Kawashima and Fujii, 2008; Kummer et al., 2008; Sugiyama and Tezuka, 2011), and their prototypes have been discovered in bacteria (Bocquet et al., 2007).

Structurally, the nAChRs are homo- or heteropentamers composed of various combinations of homologous subunits. Muscular receptors are composed of $(\alpha1)_2\beta1\gamma\delta(\varepsilon)$ subunits and are similar in all parts of the body. Neuronal type nAChRs, which are also expressed in non-excitable cells, are much more heterogeneous: they consist of α2–α10 and β2–β4 subunits combined either as homomers (α7, α8, α9) or heteromers (α3β2, α3(α5)β4, α4β2, *etc.*; reviewed in Kalamida et al. (2007), Albuquerque et al. (2009). Homomeric α7 nAChRs are considered to be the most evolutionary ancient (Ortells and Lunt, 1995); they are found in both neurons and non-excitable cells to control the cell viability (Parada et al., 2010; Resende and Adhikari, 2009); motility (Chernyavsky et al., 2004), activation (Koval et al., 2011), as well as angiogenesis (Arias et al., 2009) and inflammation (De Jonge and Ulloa, 2007).

Previously we reported the presence of nAChRs composed of α7 subunits in mouse liver mitochondria and in mitochondria of several cell lines. This novel and unexpected finding was confirmed by various experimental approaches including electron and confocal fluorescent microscopy, the binding of α7-specific antibodies and toxins, as well as the use of mitochondria from α7−/− animals lacking such binding (Gergalova et al., 2012; Kalashnyk et al., 2012). The nAChRs were found in

Abbreviations: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; cyt *c*, cytochrome *c*; MLA, methyllicaconitine; MPTP, mitochondria permeability transition pore; ROS, reactive oxygen species; CaMKII, calcium-calmodulin-dependent kinase; PKC, protein kinase C; PI_3K, phosphatidylinositol-3-kinase.

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the outer membrane of mitochondria and were shown to influence Ca^{2+} accumulation and early pro-apoptotic events like cytochrome *c* (cyt *c*) release. However, the mechanism of α7 nAChR signaling in mitochondria remained unclear. In the present work we addressed this question by studying the effects of various α7 nAChR ligands on cyt *c* release and related intramitochondrial signaling pathways.

2. Materials and methods

2.1. Animals

We used male C57BL/6J mice, 3–5 months of age, and Chinchilla rabbits (to produce cyt *c*-specific antibodies). Animals were kept in the animal facility of Palladin Institute of Biochemistry, 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 mice were sacrificed by cervical dislocation. All procedures conformed to the guidelines of the Animal Care and Use Committee of Palladin Institute of Biochemistry. Before starting the experiments, the protocols were approved by the IACUC (Protocol 1/7-421).

2.2. Reagents

All reagents were of chemical grade and were purchased from Sigma–Aldrich unless specially indicated. Antibodies against total Akt (Akt1/2/3 (H-136)) and phosphorylated Akt (p-Akt1/2/3 (Ser 473)) were from Santa-Cruz Biotechnology. Antibodies against α7(1–208) and α7(179–190) nAChR fragments were obtained and characterized by us previously (Skok et al., 1999; Lykhmus et al., 2010). To obtain cyt *c*-specific antibodies a rabbit was immunized twice with 0.3 mg of bovine cyt *c* (the first time with complete and the second one with incomplete Freund's adjuvant, with the interval of 3 weeks). A week after the second immunization, the blood was collected from the ear vein and immunoglobulins were purified from the serum using Protein A-conjugated Sepharose 4B. The purified immunoglobulins were biotinylated according to standard procedure (Harlow and Lane, 1988).

The following α7 nAChR ligands were used: acetylcholine chloride, PNU 282987, PNU 120596, methyllicaconitine (all from Sigma–Aldrich).

The following protein kinase inhibitors were used: KN62 (calcium/calmodulin-dependent kinase inhibitor); SB 202190 (p38 inhibitor); wortmannin (phosphatidylinositol-3-kinase inhibitor); PP2 (Src kinase inhibitor), all from Sigma–Aldrich; bisindolylmaleimide (protein kinase C inhibitor), from Cell Signaling Technology.

2.3. Mitochondria purification and fractionation

Mitochondria isolation from the mouse liver 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 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. The protein concentration was established by BCA assay (Thermo Scientific).

2.4. Cyt c release studies

The purified mitochondria (120 μg of protein per ml) were incubated with either 90 μM $CaCl_2$ or 0.5 mM H_2O_2 for 2 min at

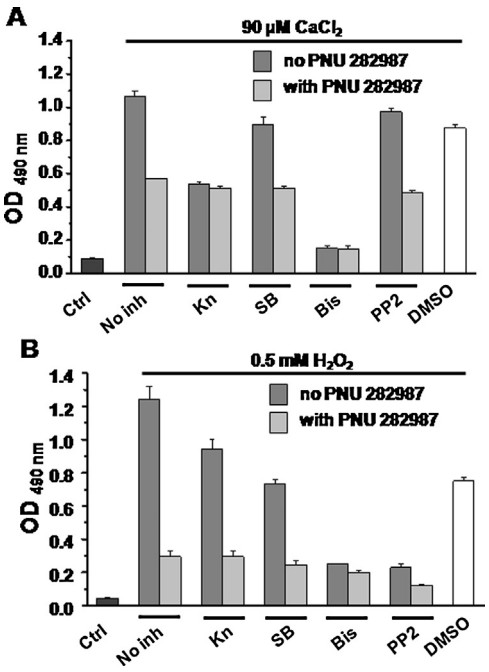

Fig. 1. The effects of kinase inhibitors and PNU 282987 on cyt *c* released in response to 90 μM Ca^{2+} (A) or 0.5 mM H_2O_2 (B). Ctrl – mitochondria without Ca^{2+} or H_2O_2; No inh – no kinase inhibitor added; Kn – KN62 (1 μM, CaMKII inhibitor); SB – SB 202190 (10 μM, p38 inhibitor); Bis – bisindolylmaleimide (50 nM, PKC inhibitor); PP2 (1 μM, Src kinase inhibitor). The inhibitors were added to mitochondria 10 min prior to Ca^{2+}; PNU 282987 (30 nM) was applied just before Ca^{2+}. Each column corresponds to mean ± S.E. ($n = 3$).

room temperature and were immediately pelleted by centrifugation (10 min, 7000 × *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 (agonists, antagonist, allosteric modulator or antibodies) were applied 2–3 min prior to apoptogenic agents. The kinase inhibitors were added either 3–10 min before or simultaneously with PNU 282987 (as specified in the figures). 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 Figs. 1 and 3 were within the linear part of the calibration curve built with bovine cyt *c*.

2.5. Superoxide release studies

The purified mitochondria were resuspended in RPMI 1640 medium and were incubated in the presence or absence of acetylcholine (ACh, 1 mM), methyllicaconitine (MLA, 10 nM), α7(179–190) antibody (6.0 μg/ml) or their combinations for 10 min. Similar medium samples without mitochondria were prepared as negative controls. The light absorbance (optical density, λ = 545 nm) of phenol red within the medium was read using StatFax-2100 Microplate reader (Awareness Technology, USA). The pH values were calculated according to calibration curve built with RPMI 1640 medium of pH established with conventional pH-meter (Orion 501, USA).

2.6. Study of Akt phosphorylation

Mitochondria were purified by usual procedure. The suspension obtained from one liver was distributed into five equal samples (100 μl) in the separation medium. The samples were treated with Ca^{2+} (90 μM); Ca^{2+} + PNU 282987 (30 nM); wortmannin (1 μM); wortmannin + PNU 282987 or PNU 282987 alone, respectively, for

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