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Coronaridine congeners modulate mitochondrial α3β4* nicotinic acetylcholine receptors with different potency and through distinct intra-mitochondrial pathways

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ABSTRACT

In contrast to plasma membrane-expressed nicotinic acetylcholine receptors (nAChRs), mitochondrial nAChRs function in an ion-independent manner by triggering intra-mitochondrial kinases that regulate the release of cytochrome c (Cyt c), an important step in cellular apoptosis. The aim of this study is to determine the structural requirements for mitochondrial α3β4* nAChR activation by measuring the modulatory effects of two noncompetitive antagonists of these receptors, (+)-catharanthine and (±)-18-methoxycoronaridine [(±)-18-MC], on Cyt c release from wild-type and α7−/− mice mitochondria. The sandwich ELISA results indicated that α3β4* nAChRs are present in liver mitochondria in higher amounts compared to that in brain mitochondria and that these receptors are up-regulated in α7−/− mice. Correspondingly, (±)-18-MC decreased Cyt c release from liver mitochondria of wild-type mice and from brain and liver mitochondria of α7−/− mice. The effect in wild-type mice mitochondria was mediated mainly by the Src-dependent pathway, regulating the apoptogenic activity of reactive oxygen species, while in α7−/− mice mitochondria, (±)-18-MC strongly affected the calcium-calmodulin kinase II-dependent pathway. In contrast, (+)-catharanthine was much less potent than (±)-18-MC and triggered several signaling pathways, suggesting the involvement of multiple nAChR subtypes. These results show for the first time that noncompetitive antagonists can induce mitochondrial α3β4* nAChR signaling, giving a more comprehensive understanding on the function of intracellular nAChR subtypes.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels mediating fast synaptic transmission in nerve and muscle cells and regulating cell proliferation, survival or cytokine production in many, if not all, non-excitable tissues (Kalamida et al., 2007; Kawashima and Fujii, 2008). Previous studies reported that α3β2, α4β2, α7β2 and α9 nAChR subtypes are expressed in the outer membrane of mitochondria, where they regulate the internal pathway of apoptosis in an ion channel-independent manner (Gergalova et al., 2012, 2014; Lykhmus et al., 2014; reviewed in Skok et al., 2016). Both nicotinic agonists and antagonists as well as positive allosteric modulators (PAMs) with selectivity for α7-or β2-containing nAChRs attenuated cytochrome c (Cyt c) release from isolated mitochondria (Gergalova et al., 2014; Uspenska et al., 2017), an important step in the process of cellular apoptosis. Our laboratory has hypothesized that the observed decrease is triggered by ligand-induced conformational changes in the receptor (reviewed in Skok et al., 2016). As an attempt to understand such novel molecular mechanisms, we contrasted the previous results using selective agonists and PAMs with that gathered in this work on noncompetitive antagonists (NCAs) such as (±)-18-methoxycoronaridine [(±)-18-MC] and (+)-catharanthine (see Fig. 1 for the molecular structures).

Pharmacologically, (±)-18-MC and (+)-catharanthine behave as NCAs of several neuronal nAChR subtypes with preferential

Abbreviations: α3β4* nicotinic acetylcholine receptors, α3β4-containing nAChRs; NCA, noncompetitive antagonist; WT, wild-type; RT, room temperature; (±)-18-MC, (±)-18-methoxycoronaridine; (+)-catharanthine, (+)-3,4-didehydrocoronaridine; Cyt c, cytochrome c; PAM, positive allosteric modulator; CaKMII, Src; MHb, medial habenula; apparent IC_{50}, ligand concentration that produces half-maximal inhibition.

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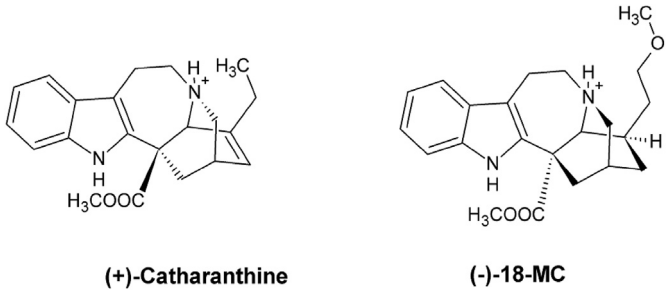

Fig. 1. Molecular structure of (−)-18-MC [(−)-18-methoxycoronaridine] and (+)-catharanthine [(+)-3,4-didehydrocoronaridine] in the protonated state (i.e., at physiological pH).

selectivity toward α3β4* nAChRs (Glick et al., 2002; Pace et al., 2004; Arias et al., 2010, 2015; 2017). Since this nAChR subtype, in the brain, is mainly expressed in the habenulo-interpeduncular cholinergic pathway (Grady et al., 2009), the observed selectivity for coronaridine congeners has been ascribed as responsible for the anti-addictive efficacy and safer toxicological profile of these compounds, especially for (±)-18-MC (McCallum et al., 2012; Glick et al., 2002, 2011; Maisonneuve and Glick, 2003).

Both α3 and β4 nAChR subunits are found in mitochondria in relatively lower amount compared to other nAChR subtypes. Since a significant up-regulation of liver mitochondrial β4 subunits has been observed in mice lacking the α7 and/or β2 subunits (Lykhmus et al., 2014), an up-regulation of mitochondrial α3β4* nAChRs was expected in α7−/− mice mitochondria. In this regard, the aims of this study were: (1) to compare the content of α3β4* nAChRs between liver and brain mitochondria obtained from wild-type (WT) and α7−/− mice by means of sandwich ELISA, and (2) to determine the modulatory effects of (+)-catharanthine and (±)-18-MC on these receptors by measuring Cyt c release from mitochondria of WT and α7−/− mice.

The results from this study show for the first time that noncompetitive antagonists such as (+)-catharanthine and (±)-18-MC modulate mitochondrial α3β4* nAChRs with different potency. This activity sheds light on the function of different mitochondrial nAChR subtypes, and suggests additional plausible mechanisms underlying the anti-addictive activities of coronaridine congeners.

2. Material and methods

2.1. Materials

Tween 20, H_2O_2, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The NeutrAvidin-peroxidase conjugate and BCA protein assay kit were obtained from Thermo Fisher Scientific (Rockford, IL, USA). (±)-18-Methoxycoronaridine hydrochloride [(±)-18-MC] was purchased from Obiter Research, LLC (Champaign, IL, USA). (+)-Catharanthine (base) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). The α3(181–192)- and β4(190–200)-specific antibodies were prepared as described previously (Skok et al., 1999; Koval et al., 2004). The β4-specific antibody was biotinylated by standard procedures (Harlow and Lane, 1988). Salts were of analytical grade.

2.2. Animals

The brain and liver of female C57Bl/6 mice and knockout mice (i.e., α7−/−, β4−/−, and α3+/−) of the same background (Orr-Urtreger et al., 1997; Xu et al., 1999a, b) were used to purify mitochondria. The WT and α7−/− mice were kept in the animal facility of Palladin Institute of Biochemistry, while α3+/-and β4−/− mice mitochondria were prepared in the Institut Pasteur, Paris. The animals were fed with a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (September 22nd, 2010) on the protection of animals used for scientific purposes. The ethical policy of the Palladin Institute of Biochemistry (Ukraine) complies with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH, USA). Formal approval to conduct the described experiments was obtained from the Animal Use and Care Committee from Palladin Institute. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Purity of mitochondrial preparations

The experiments were performed using functional mitochondria isolated from brains and livers dissected from mice (WT, α7−/−, β4−/−, and α3+/−) by differential ultracentrifugation, according to published procedures (Gergalova et al., 2012). The purity of isolated mitochondria was contrasted with that for non-mitochondrial fractions by using the sandwich ELISA method, where specific antibodies against the mitochondria-specific protein VDAC (voltage-dependent anion channel), the nuclear-specific protein α-lamin B1, and the endoplasmic reticulum-specific protein IRE-1α (inositol-resistant element), respectively, are used (Uspenska et al., 2017).

2.4. Content of α3β4* nAChRs on mitochondrial preparations obtained from wild-type and genetically modified mice

To determine the content of α3β4* nAChRs in brain and liver mitochondria prepared from WT and genetically modified mice (i.e., α7−/−, β4−/−, and α3+/−), the sandwich ELISA method was used (Lykhmus et al., 2014; Uspenska et al., 2017). To prepare detergent lysates, isolated mitochondria were first frozen (at −20 °C) and thawed, and subsequently treated with lysing buffer (0.01 M Tris-HCl, pH 8.0; 0.14 NaCl; 0.025% NaN_3; 1% Tween 20 and protease inhibitors cocktail) for 2 h on ice upon intensive stirring. The resulting lysate was pelleted by centrifugation (20 min at 20,000 × g). The protein concentration was established by using the BCA protein assay kit.

For the proper sandwich ELISA method, 96-well plates (Nunc Maxisorb, Roskilde, Denmark) were coated with α3(181–192)-specific antibody (30 μg/ml at 4 °C, overnight), subsequently blocked with 1% BSA in phosphate buffered saline, and the detergent lysates of mitochondrial fractions (100 μg/ml) were applied into coated wells for 2 h at 37 °C. Then, the plates were rinsed with water and the bound antigen was revealed with biotinylated β4(190–200)-specific antibody (10 μg/ml) applied for additional 2 h, followed by NeutrAvidin-peroxidase conjugate and *o*-phenylendiamine-containing substrate solution. The β4-specific antibody was initially titrated using the corresponding antigenic peptide, and the optimal dilution was selected according to the titration curve. The Stat-Fax2100 Microplate reader (Awarness Technology, FL, USA) was used to determine the optical density of the samples at 490 nm absorbance (OD_{490nm}).

2.5. Cytochrome c release assay in mitochondrial preparations

To determine the activity of coronaridine congeners on brain and liver mitochondria prepared from WT and α7−/− mice, purified mitochondria were incubated for 2 min at room temperature (RT) with increasing concentrations of either (±)-18-MC or