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Positive allosteric modulators of α7* or β2* nicotinic acetylcholine receptors trigger different kinase pathways in mitochondria

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ABSTRACT

Mitochondrial nicotinic acetylcholine receptors (nAChRs) regulate the early stage of mitochondria-driven apoptosis, including cytochrome *c* release. Mitochondrial nAChR signaling is mainly mediated by intra-mitochondrial kinases, in an ion-independent manner. To determine the relationship between specific nAChR subtypes and mitochondrial kinases, the effects of a set of nAChR subtype-selective positive allosteric modulators (PAMs) on cytochrome *c* release from mouse liver mitochondria stimulated by 0.9 μM Ca^{2+}, 0.5 mM H_2O_2 or 1.0 μM wortmanin is studied. The results indicate that Ca^{2+}-stimulated cytochrome *c* release from wild-type, but not α7-/-, mice mitochondria is attenuated by the potent agonist PNU-282987 or type II PAMs (PNU-120596, 4BP-TQS, and PAM-2-4), but not by NS-1738, a type I PAM. In contrast, wortmannin-stimulated cytochrome *c* release from wild-type and, to a lesser extent, α7-/- mice mitochondria is efficiently attenuated by the β2-selective PAM desformylfrustrabromine. In conclusion, the ligand-evoked α7* nAChR conformational changes required to induce intra-mitochondrial signaling can be triggered through orthosteric (agonists) and transmembrane (type II PAMs) sites, but not by the interaction with type I PAMs. The α7 and β2 nAChR subunits are responsible for the engagement of distinct kinase pathways, supporting the concept that multiple heteromeric nAChR subtypes ensure mitochondria resistance to various exogenous and endogenous apoptogenic agents.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels widely distributed in the animal kingdom. They were initially discovered in neuromuscular junctions and autonomic ganglia, mediating fast synaptic transmission elicited by the neurotransmitter acetylcholine (ACh). Later, these receptors were found in the central nervous system and in many, if not all, non-excitable cells, where they regulate cytokine and transmitter release, cell survival, proliferation, adhesion, and angiogenesis (Changeux, 2012; Kurzen et al., 2007; Resende and Adhikari, 2009).

Recently, by using a set of nAChR subunit-specific antibodies and mitochondria of both WT and knockout mice lacking certain nAChR subunits we have found that α3β2, α4β2, α7β2 and α9 nAChR subtypes are expressed in mitochondria isolated from mouse liver and brain (Gergalova et al., 2012; Lykhmus et al., 2014; Uspenska et al., 2017). The α3β4 and α7 nAChRs were identified in mitochondria of several cell lines (Kalashnyk et al., 2012). This finding was further confirmed in the laboratory of S. Grando by visualizing the α3, α5, α7, α9, α10, β2 and β4 nAChR subunits in mitochondria of keratinocytes (Chernyavsky et al., 2015). Subsequently, we showed that the nAChRs are located in the outer membrane of mitochondria and their activation attenuates cytochrome *c* (cyt *c*) release stimulated by apoptogenic agents like Ca^{2+} or H_2O_2, i.e. regulates the early stages of mitochondria-driven apoptosis (Gergalova et al., 2012; reviewed in Skok et al., 2016). This was the first discovery of functional nAChRs in intracellular organelle, which

Abbreviations: ACh, acetylcholine; CaKMII, Ca-calmodulin-dependent kinase type II; cyt *c*, cytochrome *c*; dFBr, desformylflustrabromine; nAChR, nicotinic acetylcholine receptor; NS-1738, 1-(5-chloro-2-hydroxyphenyl)-3-(2-chloro-5-trifluoromethylphenyl)urea; PAM, positive allosteric modulator; PAM-2, (*E*)-3-(furan-2-yl)-*N*-(p-tolyl)acrylamide; PAM-3, (*E*)-3-furan-2-yl-*N*-*o*-tolylacrylamide; PAM-4, (*E*)-3-furan-2-yl-*N*-phenylacrylamide; PNU-120956, (N-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)urea); PI_3K, phosphatidylinositol-3-kinase; 4BP-TQS, 4-(4-bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[*c*]quinoline-8-sulfonamide; DhβE, dihydro-β-erythroidine; PNU-282987, (*N*-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chloro-benzamide); Src, proto-oncogene tyrosine-protein kinase Src; WT, wild-type

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explained why α7 nAChR activation inhibits ethanol-induced mitochondrial dysfunction and cyt *c* release (Li et al., 2002) or mitochondrial DNA release (Lu et al., 2014). Interestingly, mitochondrial nAChR signaling appeared to be ion channel-independent; it can be triggered by agonists as well as competitive and noncompetitive antagonists and is mediated by intra-mitochondrial kinases, analogous to those found adjacent to the plasma membrane (Gergalova et al. 2014; Arias et al., 2018). However, the molecular mechanisms and structural requirements for ligand-induced mitochondrial nAChR activation are still unknown.

Positive allosteric modulators (PAMs) are small molecules which bind nAChRs at allosteric sites located apart from the orthosteric binding sites. Pharmacologically, PAMs are very different compared to agonists. For example, PAMs do not directly stimulate ion channel opening, but potentiate the activity of an agonist (*i.e.*, so-called type I PAMs), and some of them (*i.e.*, so-called type II PAMs) can delay receptor desensitization and thus, prolong the channel open time (Wang et al., 2010; Andersen et al., 2016; reviewed in Arias, 2011). This is especially important for α7 nAChRs, where a relatively fast desensitization process has been characterized (Papke, 2014). Additional studies suggested that there is a great diversity of pharmacological properties between type I and type II PAMs, and patch-clamp results indicated that both types, although with different degree, may enhance open-channel lifetime and produce episodes of successive openings (Andersen et al., 2016). In addition to α7-PAMs, other molecules are selective for non-α7 nAChRs, including α4β2 and α3β4 subtypes. For example, desformylflustrabromine (dFBr) is a very well characterized PAM with selectivity for β2-containing nAChRs (i.e., β2* nAChRs), including the α4β2 and α2β2 subtypes (Kim et al., 2007; Pandya and Yakel, 2011). We previously reported that the binding of α7- (PNU-120596) or β2-selective (dFBr) PAMs are sufficient to stimulate mitochondrial nAChR signaling and attenuate apoptogenic cyt *c* release (Uspenska et al., 2017). Here, we used a set of well-characterized PAMs (see Fig. 1) to identify structural requirements and kinase selectivity for mitochondrial nAChR activation.

2. Materials and methods

2.1. Materials

All reagents were of chemical grade and purchased from Sigma-Aldrich (Saint Louis, USA), unless specially indicated. PNU-120596, NS-1738 and desformylflustrabromine hydrochloride (dFBr), were purchased from Tocris Bioscience (Bristol, UK), Dihydro-β-erythroidine (DhβE) and PNU-280987 from Sigma-Aldrich, 4BP-TQS was kindly provided by Mark Gill. PAM-2, -3, and -4 were synthesized as previously described (Arias, 2011). Antibodies against α3(181–192), α4(181–192), α7(179-190), α9(11-23), β2(190-200) or β4(190-200) nAChR fragments and rabbit cyt *c*-specific antibodies were generated using methods previously developed in our lab (Skok et al., 1999; Koval et al., 2004; Lykhmus et al., 2010; Koval et al., 2011; Gergalova et al., 2014). The antibodies were biotinylated according to standard procedures (Harlow and Lane, 1988).

2.2. Animals

Age-matched C57BL/6 J WT (WT) mice and those lacking either the α7 (α7-/-) (Orr-Urtreger et al., 1997) or β2 (β2-/-) (Picciotto et al., 1995) nAChR subunits were used. Mice were kept in the animal facilities of either Palladin Institute of Biochemistry, Kyiv or Institut Pasteur, Paris. Mice were housed in quiet, temperature-controlled rooms, and provided with water and food pellets *ad libitum*. Before removing the liver, mice were sacrificed by cervical dislocation. All procedures conformed to the guidelines of the Centre National de la Recherche Scientifique or Palladin Institute's IACUC. Before starting the experiments, the protocols were approved by the IACUC.

2.3. Mitochondria preparation

All experiments were performed with mitochondria isolated from mouse liver by differential ultracentrifugation according to standard published procedures (Sottocasa et al., 1967; Gergalova et al., 2012). To prepare detergent lysates, mitochondria were frozen at −20 °C, thawed and treated with lysing buffer (0.01 M Tris-HCl, pH 8.0; 0.14

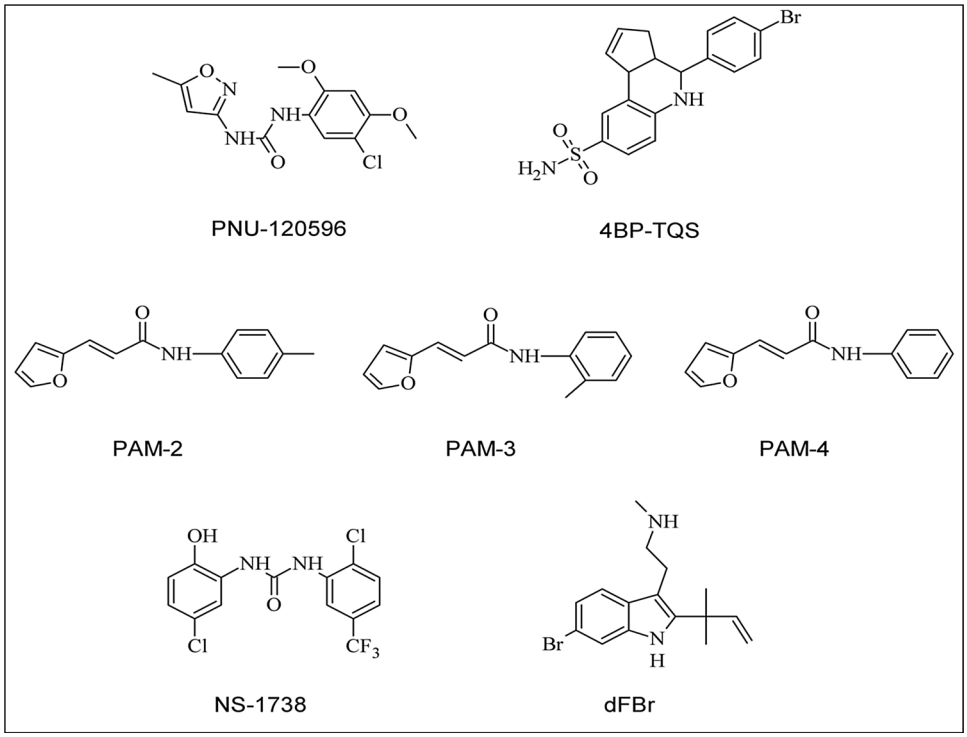

Fig. 1. Molecular structures of the PAMs used: α7-selective PAMs, including type II PAMs such as PNU-120956 (N-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl) urea), 4BP-TQS [4-(4-bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[*c*]quinoline-8-sulfonamide], PAM-2 [i.e., (*E*)-3-(furan-2-yl)-N-(p-tolyl)acrylamide], PAM-3 [(*E*)-3-furan-2-yl-*N*-*o*-tolylacrylamide], and PAM-4 [(*E*)-3-furan-2-yl-*N*-phenylacrylamide], the type I PAM NS-1738 [1-(5-chloro-2-hydroxyphenyl)-3-(2-chloro-5-trifluoromethylphenyl)urea], as well as the β2-selective PAM dFBr (desformylflustrabromine).