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Muscarinic acetylcholine M_4 receptors play a critical role in oxotremorine-induced DARPP-32 phosphorylation at threonine 75 in isolated medium spiny neurons

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ABSTRACT

Dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) play essential roles in dopamine (DA) transmission in the striatum. It is suggested that a link exists between muscarinic acetylcholine receptors (mAChRs) and DA/DARPP-32 signaling, but the molecular mechanisms mediating this relationship have not been elucidated. The predominant mAChRs subtypes in the striatum are M_1 and M_4. In this study, we investigated the functions of these two receptors, particularly M_4, in regulating cAMP production and DARPP-32 phosphorylation in rat striatal medium spiny neurons (MSNs). We used time-resolved fluorescence resonance energy transfer, immunofluorescence confocal microscopy, and western blot assays. In cultured intact MSNs, we confirmed that muscarinic M_1 and M_4 receptors were highly expressed. Notably, M_4 receptors were co-expressed with D_1 receptors in only a portion of the cultured MSNs. The nonselective muscarinic agonist oxotremorine M (OX) slightly enhanced cAMP production, but this effect was independent of M_1 or M_4 receptors. However, OX directly participated in DARPP-32 phosphorylation, phosphorylating DARPP-32 at Thr75 (the CDK5 site) and concomitantly dephosphorylating DARPP-32 at Thr34 (the PKA site) in virtually cultured MSNs, whereas APO phosphorylated DARPP-32 at both Thr34 and Thr75. The OX-induced time-dependent increase in DARPP-32 phosphorylation at Thr75 was accompanied by increased p35 and CDK5 activity. Specifically, elevated immunoreactivity for phospho-DARPP-32-Thr75 and p35 was detected in M_4 receptor-expressing MSNs. Both genetic knockdown and pharmacologic inhibition of M_4 receptors with MT3, an M_4 receptor-selective antagonist, decreased the OX-induced DARPP-32-Thr75 phosphorylation in MSNs. These results indicate that the M_4 muscarinic receptor plays a critical role in modulating phosphorylation of DARPP-32-Thr75 in MSNs. The results suggest that M_4 receptor activation acts antagonistically with dopamine D_1-like receptors within the striatum, and indicate that M_4 receptors may be a potential target for the treatment of Parkinson's disease and other relevant central nervous system disorders.

1. Introduction

In the central basal ganglia system, the striatal acetylcholine (Ach) and dopamine (DA) systems are traditionally viewed as antagonistic, with cholinergic neurotransmission playing a critical role in the regulation of motor function by interacting with dopamine signaling (Cragg, 2006; Aosaki et al., 2010). Dysfunction of ACh–DA circuits is associated with neurological disorders, including Parkinson's and Huntington's diseases (DeLong and Wichmann, 2007; Pisani et al., 2007; Gerfen and Surmeier, 2011).

In the striatum, G protein-coupled muscarinic acetylcholine receptors (mAChRs), activated by ACh, play critical roles in the

Abbreviations: ACh, acetylcholine; APO, apomorphine; CDK5, cyclin-dependent kinase 5; CREB, cAMP response element-binding protein; DA, dopamine; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; DMEM, Dulbecco's modified Eagle's medium; KD, knockdown; mAChR, muscarinic acetylcholine receptor; MSN, medium spiny neuron; MT3, muscarinic toxin 3; MT7, muscarinic toxin 7; OX, oxotremorine; PKA, protein kinase A; shRNA, short hairpin RNA; Gpi, the internal globus pallidus; GPe, the external globus pallidus; SCH23390, (*R*)-(1)-7-chloro-8-hydroxyl-3-methyl-1-phenyl-2,3,4,5 -tetrahydro-1H-3-benzazepine,hydrochloride; raclopride, *S*(−)-raclopride (+)-tartrate salt; TR-FRET, time-resolved fluorescence resonance energy transfer.

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functional regulation of medium spiny neurons (MSNs). Receptor localization studies have shown that all five mAChRs (M_1–M_5) are expressed in the striatum. Among these mAChRs, M_1 and M_4 are the major muscarinic receptor subtypes expressed in striatal MSNs (Bernard et al., 1992; Yan et al., 2001). In contrast to M_1, M_4 receptors are co-expressed with dopamine D_1 receptors in a specific subset of striatal MSNs, giving rise to the striatonigral pathway (Bernard et al., 1992; Weiner et al., 1990; Ince et al., 1997). Moreover, the M_4 receptor is thought to play a particularly important role in controlling dopamine release and modulating DA-dependent behaviors (Gomeza et al., 1999; Tzavara et al., 2004; Brady et al., 2008; Woolley et al., 2009). Because this effect is so dominant, it has been proposed that Ach acts via M_4 receptors to modulate midbrain dopaminergic system functions. Thus, the M_4 receptor may be an attractive target for the treatment of Parkinson's disease, but the mechanisms for its actions remain unclear (Langmead et al., 2008).

In the striatum, MSNs (primarily signaling with GABA) integrate vast numbers of inputs and represent the sole output of the striatum to downstream basal ganglia nuclei (DeLong and Wichmann, 2007; Kreitzer and Malenka, 2008). DA and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) is selectively expressed in >80% of striatal MSNs and provides a mechanism for integrating dopamine and other neurotransmitter signals (Svenningsson et al., 2000; Walaas et al., 2011). Modulation of DA and protein kinase A (PKA) in MSNs exerts an important influence on DARPP-32 in the striatum. The effects of DA are mediated through protein phosphorylation catalyzed by cAMP-dependent PKA, which converts DARPP-32 into an inhibitor of protein phosphatase-1. In contrast, phosphorylation catalyzed by cyclin-dependent kinase-5 (CDK5) at Thr75 converts DARPP-32 into an inhibitor of PKA and antagonizes the PKA–DARPP-32-Thr34–protein phosphatase-1 cascade (Bibb et al., 1999; Svenningsson et al., 2000; Walaas et al., 2011). Thus, DARPP-32 can act as either a phosphatase inhibitor or a kinase inhibitor, depending on its relative state of phosphorylation at the PKA and CDK5 sites. Changes in the state of phosphorylation of DARPP-32 reinforce the behavioral effects produced by stimulation or inhibition of the cAMP pathway.

The use of the non-subtype-selective muscarinic agonist oxotremorine M (OX) has shown that muscarinic receptor activation regulates the release of dopamine in cooperation with the nicotinic receptor. This, in turn, activates D_1 receptor/DARPP-32 signaling in striatonigral neurons and indicates a distinct link between muscarinic receptors and DARPP-32 signaling. These precise functional roles may be due to the differential region- or cell-specific expression of specific mAchRs. However, it is not well understood whether mAchRs regulate the phosphorylation state of DARPP-32 directly or indirectly in MSNs, and how mAchRs signaling mechanisms serve to integrate striatal inputs. Given the role of mAchRs in the control of motor function and its disruption in diseases such as Parkinson's and Huntington's diseases, understanding such processes is vital for identifying the molecular mechanisms that mediate and modulate neurotransmission within this brain region.

Thus, in this study, we investigated whether M_4 or M_1 participates in regulating cAMP signaling and DARPP-32 phosphorylation. To investigate this, we used the respective M_1 and M_4 receptor-selective antagonists MT7 and MT3 (Simon, 2001; Caulfield and Birdsall, 1998), as well as using lentivirus technology to genetically knock down the M_4 receptor. We found that muscarinic receptors indirectly modulate cAMP production but directly participate in DARPP-32 phosphorylation at Thr75 to oppose the effects of D_1 receptor/DARPP-32 phosphorylation at Thr34. Notably, pharmacologic inhibition and genetic suppression of M_4 reduced DARPP-32 phosphorylation at Thr75. These results provide new evidence that M_4 receptors modulate dopaminergic systems and indicate a potential therapeutic role for M_4 receptor antagonists in Parkinson's disease and other relevant central nervous system disorders.

2. Materials and methods

2.1. Reagents

Oxotremorine M, *R* (+)-SCH23390 ([*R*]-[1]-7-chloro-8-hydroxy-3- methyl-1-phenyl-2, 3, 4, 5-tetrahydro-1H-3-benzazepine hydrochloride), and *S* (−)-raclopride (+)-tartrate salt were purchased from Sigma-Aldrich (St. Louis, MO). Muscarinic toxin 3 (MT3) and muscarinic toxin 7 (MT7) were obtained from the Peptide Institute (Osaka, JPN). Apomorphine hydrochloride (APO) was purchased from Chinese Pharmaceutical and Biological Products (Beijing, China). All drugs were dissolved or diluted in normal saline, and the solutions were adjusted to within a pH range of 6.5–7.0.

2.2. Primary striatal MSN culture and drug administration

Male or female neonatal Sprague Dawley rat pups (12 h old) were supplied by the Animal Center of the Academy of Military Medical Science (Beijing, China). All animal experimentation was approved by the Beijing Local Committee on Animal Care and Use. All experiments were performed according to the *Guide for the Care and Use of Experimental Animals* produced by the Beijing Local Committee and the National Institutes for Health's *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1985).

Primary cultures were established according to methods described previously (Ivkovic et al., 1997). Briefly, we dissected the striata from 12-h-old neonatal Sprague Dawley rat pups. Dissected striata were chemically and mechanically dissociated into single-cell suspensions. Cells were plated onto 0.1% polyethyleneimine-coated 15-mm-diameter dishes (NEST Biotechnology Co. Ltd., Rahway, NJ, USA) and grown in DMEM (Life Technologies, Grand Island, NY, USA) with 10% horse serum (Life Technologies, Gaithersburg, MD, USA), 1% N-2, 2% B-27 serum-free supplement, and 1% L-glutamine (Invitrogen, Carlsbad, CA, USA). This was incubated at 37 °C in a 5% CO_2 atmosphere.

In some experiments, MSNs were incubated with either an activator (i.e., the nonselective mAChR agonist OX, 0.1–100 μM) or the dopamine receptor agonist APO (0.1–100 μM) for 5, 30, or 60 min, or with SKF38393 (0.1 μM). In other experiments, MSNs were pre-incubated with either an inhibitor (MT7, 10 nM; MT3, 10 nM; SCH23390, 10 mM) or raclopride (10 mM) for 60 s before the addition of OX (1 μM).

2.3. Lentivirus-mediated shRNA transfection and knockdown of M_4 gene expression

Sequences of Chrm4 (M_4) shRNA were inserted into the pGMLV-SC5 lentivirus shRNA expression system, which was obtained from Genomeditech Co., Ltd. (Shanghai, China). The shRNA-containing vectors were co-transfected into 293T cells with packaging mix to generate the respective lentiviruses. Viral stocks collected from 293T cells were used to infect MSNs. The RNA target of Chrm4 was GCAAAGTGACTCGGACAATCT. The sequence GATCCGCAAAGTGACTCGGACAATCTTTCAAGAGAAGATTGTCCGAGTCACTTTGCTTTTTTG was inserted into pGMLV-SC5 to form Chrm4 shRNA. A nonsilencing sequence (5′-TTCTCCGAACGTGTCACGT-3′) was used as a negative control.

Primary rat MSNs were plated onto 0.1% polyethyleneimine-coated 15-mm-diameter dishes or 6-well plates (NEST Biotechnology Co. Ltd.). The cells were dissected and cultured for 3 days,