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$P2Y_{13}$ RECEPTORS MEDIATE PRESYNAPTIC INHIBITION OF ACETYLCHOLINE RELEASE INDUCED BY ADENINE NUCLEOTIDES AT THE MOUSE NEUROMUSCULAR JUNCTION

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Abstract—It is known that adenosine 5′-triphosphate (ATP) is released along with the neurotransmitter acetylcholine (ACh) from motor nerve terminals. At mammalian neuromuscular junctions (NMJs), we have previously demonstrated that ATP is able to decrease ACh secretion by activation of P2Y receptors coupled to pertussis toxin-sensitive $G_{i/o}$ protein. In this group, the receptor subtypes activated by adenine nucleotides are $P2Y_{12}$ and $P2Y_{13}$. Here, we investigated, by means of pharmacological and immunohistochemical assays, the P2Y receptor subtype that mediates the modulation of spontaneous and evoked ACh release in mouse phrenic nerve-diaphragm preparations. First, we confirmed that the preferential agonist for $P2Y_{12–13}$ receptors, 2-methylthioadenosine 5′-diphosphate trisodium salt hydrate (2-MeSADP), reduced MEPP frequency without affecting MEPP amplitude as well as the amplitude and quantal content of end-plate potentials (EPPs). The effect on spontaneous secretion disappeared after the application of the selective $P2Y_{12–13}$ antagonists AR-C69931MX or 2-methylthioadenosine 5′-monophosphate triethylammonium salt hydrate (2-MeSAMP). 2-MeSADP was more potent than ADP and ATP in reducing MEPP frequency. Then we demonstrated that the selective $P2Y_{13}$ antagonist MRS-2211 completely prevented the inhibitory effect of 2-MeSADP on MEPP frequency and EPP amplitude, whereas the $P2Y_{12}$ antagonist MRS-2395 failed to do this. The preferential agonist for $P2Y_{13}$ receptors inosine 5′-diphosphate sodium salt (IDP) reduced spontaneous and evoked ACh secretion and MRS-2211 abolished IDP-mediated modulation. Immunohistochemical studies confirmed the presence of $P2Y_{13}$ but not $P2Y_{12}$ receptors at the end-plate region. Disappearance of $P2Y_{13}$ receptors after denervation suggests the presynaptic localization of the receptors. We conclude that, at motor nerve terminals, the $G_{i/o}$ protein-coupled P2Y receptors implicated in presynaptic inhibition of spontaneous and evoked ACh release are of the subtype $P2Y_{13}$. This study provides new insights into the types of purinergic receptors that contribute to the fine-tuning of cholinergic transmission at mammalian neuromuscular junction.

Key words: ATP, presynaptic inhibition, 2-MeSADP, P2Y receptors, mammalian neuromuscular junction.

INTRODUCTION

It is well known that adenosine 5′-triphosphate (ATP) is released together with the main neurotransmitter at the majority of the synapses, both in the peripheral and in the central nervous system. At mammalian neuromuscular junctions (NMJs), ATP is co-released with the neurotransmitter acetylcholine (ACh), and once in the synaptic cleft, it is degraded to adenosine via the ectonucleotidase cascade (Ribeiro and Sebastião, 1987; Meriney and Grinnell, 1991; Redman and Silinsky, 1994; Cunha et al., 1996; Magalhães-Cardoso et al., 2003). Both, adenine nucleotides and adenosine, are able to modulate transmitter release operating via presynaptic P2 and P1 receptors, respectively (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975; Bennett et al., 1991; Hamilton and Smith, 1991; Giniatullin and Sokolova, 1998; Sebastião and Ribeiro, 2000; De Lorenzo et al., 2004, 2006). On the other hand, purines may also be released from activated muscle fibers (Smith, 1991; Santos et al., 2003) and from peri-synaptic Schwann cells (Liu et al., 2005; discussed in Todd and Robitaille, 2006).

Nucleotide receptors can be divided into two types: the P2X receptors, which are ligand-gated ion channels, and the P2Y receptors that are G protein-coupled receptors (Fredholm et al., 1994; Ralevic and Burnstock, 1998). At NMJs, the presence of both types of receptors was demonstrated (Choi et al., 2001; Deuchars et al., 2001; Moores et al., 2005). So, it has been shown that the slowly hydrolysable ATP analog, β,

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Abbreviations: 2-MeSAMP, 2-methylthioadenosine 5′-monophosphate triethylammonium salt hydrate; 2-MeSADP, 2-methylthioadenosine 5′-diphosphate trisodium salt hydrate; ACh, acetylcholine; ATP, adenosine 5′-triphosphate; EPPs, end-plate potentials; IDP, inosine 5′-diphosphate sodium salt; MEPPs, miniature end-plate potentials; NMJ, neuromuscular junction; PRP, platelet-rich plasma; TMR-α-BTX, α-Bungarotoxin coupled to tetramethylrhodamine.

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γ-imido ATP facilitates [^{3}H]ACh release (37 °C, 5 Hz) from rat hemidiaphragm preparations presumably by activation of P2X receptors (Salgado et al., 2000). On the other hand, the inhibitory effect of ATP on ACh release was ascribed to P2Y receptors. Thus, at the frog NMJs, it was found that ATP decreased evoked neurosecretion by activating P2Y receptors (Giniatullin and Sokolova, 1998; Sokolova et al., 2003) and at mammalian NMJs, Galkin et al. (2001) showed that ATP and adenosine reduced MEPP frequency, while ATP, but not adenosine, suppressed the non-quantal ACh release, suggesting that ATP acts on both quantal and non-quantal release due to a direct action on presynaptic metabotropic P2 receptors. Moreover, in previous reports, we have demonstrated that ATP and βγ-imido ATP activate P2Y receptors and decrease spontaneous secretion by a mechanism that involves the reduction of Ca^{2+} entry through the calcium channels related to spontaneous secretion, L-type and N-type voltage-dependent calcium channels (De Lorenzo et al., 2006), as well as through an effect on a Ca^{2+}-independent step in the cascade of the exocytotic process (Veggetti et al., 2008). The apparent discrepancy of our findings with those by Salgado et al. (2000) might be due to differences in target species, in the recording systems, in the type of secretion analyzed (spontaneous vs. evoked ACh secretion), or in the experimental temperature (22 °C vs. 37 °C).

Very recently, Giniatullin et al. (2015) suggested that, at the frog NMJs, the inhibition of synaptic transmission by extracellular ATP was mainly mediated by metabotropic $P2Y_{12}$ receptors. However, the subtype/s of P2Y receptor/s involved in the modulation of cholinergic secretion at mammalian NMJs has not been identified so far. To date, eight different types of P2Y receptors have been identified: $P2Y_{1,2,4,6,11,12,13,}$ and $_{14}$ (Ralevic and Burnstock, 1998; Communi et al., 2001; Hollopeter et al., 2001; Zhang et al., 2001; Abbracchio et al., 2003). From a phylogenetic and structural point of view, two distinct P2Y receptor subgroups have been identified. The first one includes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors that exhibited different selectivity for adenine and uracil nucleotides, all exclusively coupled to G_q protein, except $P2Y_{11}$, which is also positively coupled to the cAMP pathway *via* G_s protein (White et al., 2003). The second subgroup is composed of $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors that form a cluster of preferentially G_i-coupled receptors (see Burnstock, 2007). Since in our experiments, we have found that pertusis toxin and N-ethylmaleimide abolished the effect of βγ-imido ATP, it was suggested that the P2Y receptors involved in the presynaptic inhibition were those coupled to $G_{i/o}$ protein (De Lorenzo et al., 2006). Among the P2Y receptors coupled to $G_{i/o}$ protein, $P2Y_{12}$ and $P2Y_{13}$ receptors are activated by adenine nucleotides, while $P2Y_{14}$ receptors are activated by UDP-glucose as well as UDP-galactose, UDP-glucuronic acid and UDP-N-acetylglucosamine (Chambers et al., 2000; Hollopeter et al., 2001; Communi et al., 2001; Abbracchio et al., 2003). Therefore, we speculated that βγ-imido ATP-induced modulation on spontaneous secretion was mediated by $P2Y_{12}$ and/or $P2Y_{13}$ receptors.

The experiments performed in this paper were designed to elucidate, by means of pharmacological and immunohistochemical assays, the P2Y receptor subtype/s that mediate the modulation of spontaneous and evoked ACh release at the mouse NMJ.

EXPERIMENTAL PROCEDURES

Preparations and solutions

Electrophysiological recordings were performed on phrenic nerve-diaphragm preparations taken from adult CF1 mice (30–40 g) of either sex. All animal procedures were performed under protocols approved by national guidelines, which are in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NIH Publications no. 80-23) revised 1996. The study was approved by the Ethics Committee of the Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires (Re. # 115).

Mice were anesthetized with sodium thiopental (50 mg kg^{-1}) intraperitoneally and left hemidiaphragms were excised and pinned in a 5 ml recording chamber superfused (3 ml min^{-1}) with Ringer Krebs solution (mM: NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 11, HEPES 5, pH 7.3-7.4, bubbled with O_2). In each experimental group, the muscles were allowed to equilibrate in the respective solution for at least 20 min before the recording of synaptic potentials. Experiments were carried out at room temperature (22–23 °C).

Electrophysiological recordings

Miniature end-plate potentials (MEPPs) or end-plate-potentials (EPPs) were recorded at the end-plate region from muscle fibers in the conventional way (Fatt and Katz, 1951), using borosilicate glass microelectrodes (WP Instruments, Sarasota, FL, USA) with a resistance of 5–10 MΩ filled with 3 M KCl. Muscle fibers with a resting membrane potential (Vm) less negative than −60 mV or MEPPs/EPPs with a rise time greater than 1 ms were rejected. We used only those recordings in which their Vm did not deviate by more than 5 mV. MEPP recordings (frequency and amplitude) were performed in control and test solutions during 100 s from at least 10 different NMJs and their values were averaged. In the experiments where the EPP amplitude was measured, the phrenic nerve was stimulated with supramaximal stimuli (pulse width 0.1 ms) applied at a frequency of 0.5 Hz, using a suction electrode placed near its entrance to the muscle. Pulses were delivered by a Grass S48 stimulator (Grass Instruments, Quincy, MA, USA) coupled to a stimulus isolation unit (Grass SIU5). Muscle twitches were prevented by a submaximal concentration (0.8–1.6 μM) of *d*-tubocurarine. MEPP/EPP amplitudes were normalized to − 75 mV, assuming 0 mV as the reversal potential for ACh-induced current (Magleby and Stevens, 1972), using the formula $V_c = [V_o \times (-75)]/E$, where V_c is the corrected MEPP/EPP amplitude, V_o is the observed MEPP/EPP amplitude, and E is the measured resting membrane potential. Quantal content of the EPP (m) was assessed using the failure method (Del Castillo and Katz, 1954):