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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):

$m = \ln(N/n_0)$, where N is the total number of successive trials (100 at 0.5 Hz) and n_0 is the number of trials in which the response fails (absence of EPP). In this case, twitches were prevented by increasing the concentration of magnesium ($MgCl_2$ 14 mM) in the bathing solution.

All signals were amplified with Axoclamp 2A (Molecular Devices, Sunnyvale, CA, USA) and digitized with Digidata 1322 (Molecular Devices). Responses were recorded and analyzed using the pClamp 8.2 software (Molecular Devices).

Data analysis

In all cases, data are reported as mean ± SEM and n expresses number of animals (only left hemidiaphragm was used from each mouse for a given experiment). Typically, MEPP frequency or EPP amplitude was recorded in 10 different cells in each solution: control – agonist – control (washout) – antagonist – antagonist + agonist. Then, the results obtained in 3–4 experiments were averaged. Statistical comparison among three or more groups were performed using a one-way analysis of variance (ANOVA) followed by Tukey's (compare all pairs of columns) or Dunnett's (compare all columns vs. control column) post test. Two group comparisons were performed using paired Student's t test. Differences were considered to be significant when $P < 0.05$.

Immunohistochemistry

Tissues. Adult CFI mice were anesthetized with sodium thiopental (50 mg kg^{-1}) and then diaphragm or gastrocnemius muscles were dissected. In some experiments gastrocnemius muscles were previously denervated by cutting out a 0.3-cm portion of the right leg sciatic nerve. For this procedure, animals were anesthetized with ketamine 45 mg kg^{-1}/xylazine 6 mg kg^{-1} injected ip. and, after the wound was closed, the animals were allowed to recover for 10 days. At that time, mice were anesthetized with sodium thiopental (50 mg kg^{-1}) and the gastrocnemius muscles were removed. Contralateral leg muscles were used as controls.

All types of muscles were fixed for 30 min in 4% paraformaldehyde in phosphate buffer (PB, 0.1 M pH 7.4) at room temperature. Then, preparations were washed in PB for 1 min, permeabilized in 1% Triton X-100 for 5 min, washed in PB for 1 min, and finally cryoprotected in 30% sucrose in PB for no longer than 72 h. Blocks of muscle were included in a sealed plastic tube with OCT Tissue-Tek (Sakura Finetek, Inc., Torrance, CA, USA) and then frozen in isopentane precooled in dry ice.

Frozen blocks of tissue were cut transversely (8–9 μm) with a cryostat microtome, and sections were thaw-mounted into polylysine gelatin-coated slides, air dried for 15 min and stored at −20 °C.

Polyclonal antibodies and toxins. Specific primary antibody for $P2Y_{12}$ receptors was purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA, Cat. # P4871) and specific primary antibody for $P2Y_{13}$ receptors were acquired from Alomone Labs Ltd. (Jerusalem, Israel, Cat. # APR017) and Sigma–Aldrich Corp. (St. Louis, MO, USA, Cat. # P0120). Anti-$P2Y_{12}$ receptor rabbit polyclonal antibody was raised against $P2Y_{12}$ highly purified peptide corresponding to a specific epitope not present in any other known protein: residues 125-142 of human $P2Y_{12}$ receptor. Anti-$P2Y_{12}$ has been developed and validated by the Human Protein Atlas (HPA) project (www.preteinatlas.org). used specifically to recognize $P2Y_{12}$ receptors in rat brain membranes or human platelets by immunoblotting, in the epididymal portion of vas deferens by western blot (Quintas et al., 2009), and in nasal mucosa by Western blot and immunohistochemical analysis (Shirasaki et al., 2013). Anti-$P2Y_{13}$ receptor rabbit polyclonal antibody was raised against $P2Y_{13}$ highly purified peptide corresponding to amino acid residues 119-134 of human $P2Y_{13}$ receptor. Anti-$P2Y_{13}$ has been used specifically to recognize $P2Y_{13}$ receptors by western blot analysis of rat brain membranes, by immunohistochemistry in rat testis and lung paraffin sections, by immunocytochemistry in mouse hippocampal neuronal culture (del Puerto et al., 2012) and, by indirect flow cytometry in rat osteoclasts (Alvarenga et al., 2010).

Double labeling was performed using goat polyclonal anti-rabbit IgG coupled to Atto-488 (Sigma–Aldrich, Cat. # 18772) to identify the primary antibody and α-Bungarotoxin coupled to tetramethylrhodamine (TMR-α-BTX, Sigma Aldrich, Cat. # TO195) to identify the postsynaptic ACh receptors at the end-plate area. Antibody and toxin concentrations were as follows: anti-$P2Y_{12}$ receptor 1:200, anti-$P2Y_{13}$ receptor 1:200, secondary antibody 1:200, and TMR-α-BTX 1:2000. All the antibodies were diluted in 10 mM PBS containing 3% BSA, 0.1 M L-lysine and 0.075% Triton X-100, and the BgTx-R in 10 mM PBS.

Immunofluorescence. Tissue sections were processed simultaneously for double labeling by indirect immunofluorescence and direct staining with TMR-α-BTX. All incubations were performed at room temperature, using 10 mM PBS pH 7.4 except where stated.

Sections were permeabilized with 0.1% Triton X-100 in PBS for 5 min, rinsed in PBS for 15 min and then incubated with the primary antibody at 4 °C overnight (19–20 h). After being successively washed with PBS for 30 min, with higher ionic concentration PBS (in mM 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, pH 7.2) for 30 min and with PBS for 30 min, sections then were incubated simultaneously with the secondary antibody and TMR-α-BTX for 105 min, and washed in PBS for 40 min. Finally, the sections were mounted in 1:1 10 mM PBS:glycerol. The specificity of the $P2Y_{12}$-$P2Y_{13}$ signal was furthermore assessed by incubating the muscles in the absence of the primary antibody or in the presence of the corresponding control peptide antigen when it was available (supplied with anti-$P2Y_{13}$ antibody from Alomone Labs Ltd.; see Fig. 6). To verify that the $P2Y_{12}$ antibodies used were able to bind to $P2Y_{12}$ receptors

(positive control), we studied their immunoreactivity in platelet-rich plasma (PRP) from CF1 mice and healthy human volunteers. PRP obtained from blood anticoagulated with 129 mmol/l trisodium citrate, was separated by centrifugation at 200*g* for 10 min. Double labeling was performed using goat polyclonal anti-rabbit IgG coupled to Atto-488 (Sigma–Aldrich, Cat. # 18772) to identify $P2Y_{12}$ antibodies and phalloidin-tetramethylrho damine B isothiocyanate (phalloidin-TRITC, 1:200, Sigma–Aldrich, Cat. # P1951) for staining filamentous actin (F-actin) in the platelets. Anti-$P2Y_{12}$ receptor rabbit polyclonal antibody and goat polyclonal anti-rabbit IgG coupled to Atto-488 were used at the same concentrations and following the same protocol as in the muscles.

Microscopy and photography. Images were adquired with a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with an argon/HeNe-G laser which allows simultaneous scanning and acquisition of the immunofluorescent sections (Plan-Apochromat 100× oil-immersion objective, numerical aperture 1.4). Assessment of co-localization of $P2Y_{12}$ or $P2Y_{13}$ receptors and ACh receptors immunoreactivity was performed using the Zeiss LSM Image Browser 4.2 software.

We made more than 50 observations for each experimental protocol (with anti-$P2Y_{12}$ or anti $P2Y_{13}$ antibodies in diaphragm muscle, innervated and denervated gastrocnemius muscles)

Chemicals

2-Methylthioadenosine 5′-monophosphate triethylammonium salt hydrate (2-MeSAMP), 2-methylthioadenosine 5′-diphosphate trisodium salt hydrate (2-MeSADP), inosine 5′-diphosphate sodium salt (IDP), and 2,2-Dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl ester (MRS-2395) were purchased from Sigma–Aldrich Corp.; 2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS-2179), and 2-[(2-Chloro-5-nitrophenyl) azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyr idinecarboxaldehyde disodium salt (MRS-2211) were obtained from Tocris Bioscience, Ellisville, MO, USA; and N-[2-(methyltthioethyl9]-2-[3,3,3-trifluoropropyl]thio-5'-adenylic acid, monoanhydride with dichloromethylenebiphosphonic acid, tetrasodium salt (AR-C69931MX, 1 μM) was kindly provided by The Medicines Company, Massachusetts, USA. All other reagents were of the highest purity available. Aqueous dilutions of the stock solutions were made daily, and appropriate solvent controls were done.

RESULTS

Our first aim was to confirm that $P2Y_{12–13}$ receptors were involved in presynaptic inhibition of cholinergic secretion. So, we studied the effect of 2-MeSADP, a preferential agonist for $P2Y_{12-13}$ receptors (Takasaki et al., 2001; Marteau et al., 2003) on MEPP frequency and compared its concentration–response curve to those obtained with ATP and ADP. Fig. 1B shows that 2-MeSADP was the most potent agonist (EC_{50}: 2-MeSADP 0.08 μM, ADP 4.15 μM, ATP 5.07 μM). 2-MeSADP-induced inhibition on MEPP frequency was maximal at 150 nM (Control 1.05 ± 0.02 s^{-1}, 2-MeSADP 0.56 ± 0.02 s^{-1}: 53.9 ± 1.6% of control values, $n = 4$, $P < 0.001$) within 20–30 min after the application of the drug, and without any change in MEPP amplitude (control 1.23 ± 0.02 mV; 2-MeSADP 1.10 ± 0.07 mV, $n = 4$). This effect was reversible upon 20–30-min washout (Fig. 1A, C). The action of 150 nM 2-MeSADP was not observed when preparations were previously incubated with 1 μM AR-C69931MX or 30 μM 2-MeSAMP, antagonists of both, $P2Y_{12}$ and $P2Y_{13}$ receptors (Takasaki et al., 2001; Marteau et al., 2003; Fumagalli et al., 2004): AR-C69931MX 94.1 ± 2.1% of control values, AR-C69931MX + 2MeSADP 95.05 ± 1.9%, $n = 4$; 2-MeSAMP 92.8 ± 2.6% of control values, 2-MeSAMP + 2MeSADP 94.3 ± 1.2%, $n = 4$ (Fig. 2A, B). Moreover, considering that 2-MeSADP is also a full agonist for $P2Y_1$ receptors (Léon et al., 1997; Waldo and Harden, 2004), we tested its action on MEPP frequency in the presence of 10 μM MRS-2179, a $P2Y_1$ selective antagonist (Camaioni et al., 1998; Boyer et al., 1998; Zizzo et al., 2007). We found that MRS-2179 did not modify the modulatory action of 150 nM 2-MeSADP (MRS-2179 106.7 ± 5.5% of control values, MRS-2179 + 2-MeSADP 62.4 ± 3.6%, $n = 4$, $P < 0.001$, Fig. 2C). All these results confirmed that the receptors involved in presynaptic modulation were $P2Y_{12}$ and/or $P2Y_{13}$.

In an attempt to elucidate the subtype of P2Y receptors mediating the presynaptic inhibition of spontaneous and evoked ACh release, we tested more selective receptor agonists and antagonists. Taking into account that the P2 receptor antagonist PPADS has been shown to have no effect on the $P2Y_{12}$ receptor (Takasaki et al., 2001; Kulick and von Kügelgen, 2002; Unterberger et al., 2002), whereas it behaves as a low potency antagonist for the $P2Y_{13}$ receptor (Marteau et al., 2003), in the last years PPADS analogs were designed in an effort to identify more potent and/or selective $P2Y_{13}$ receptor antagonists. Among them, MRS-2211 resulted to be 45-fold more potent than PPADS and displayed > 20-fold selectivity as antagonist for $P2Y_{13}$ receptors in comparison to $P2Y_1$ and $P2Y_{12}$ receptors (Kim et al., 2005). In our experiments we found that 10 μM MRS-2211 completely prevented the inhibitory effect of 150 nM 2-MeSADP on MEPP frequency (MRS-2211 99.8 ± 3.1%; MRS-2211 + 2Me-SADP: 106.00 ± 7.3%, $n = 7$, Fig. 3A).

Although $P2Y_{12}$ and $P2Y_{13}$ receptor interaction with nucleotide analogs reveals a similar pharmacological profile, it was found that IDP is about five-fold more potent for human $P2Y_{13}$ than for $P2Y_{12}$ receptors. Moreover, IDP is especially more potent for murine $P2Y_{13}$ than for human $P2Y_{13}$ and $P2Y_{12}$ receptors since their EC_{50} were 9.2, 552, and 3180 in nM, respectively (Zhang et al., 2002) and, in a recent report, IDP is considered as potent as ADP to activate $P2Y_{13}$ receptors (Malin and Molliver, 2010). Thus, we studied the effect of 100 μM IDP on spontaneous neurotransmitter secretion. As

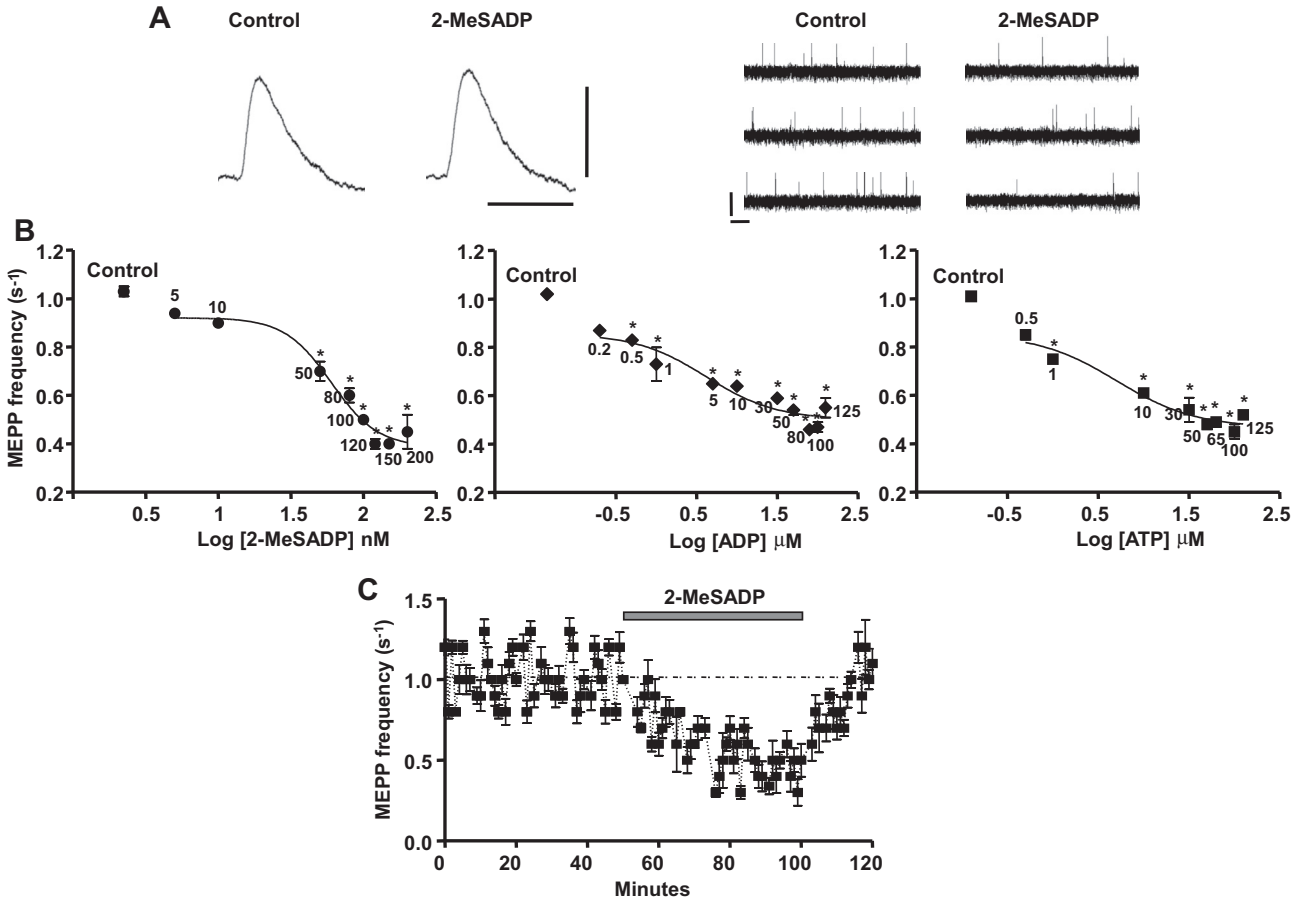

Fig. 1. (A) Effect of 2-MeSADP on spontaneous ACh secretion at the mouse NMJs. On the left, superimposed averaged MEPPs obtained during 100 s in control (Vm: −73.8, amplitude 1.12 ± 0.09 mV) and in 150 nM 2-MeSADP solutions (Vm: −74.1 mV, amplitude 1.19 ± 0.10 mV). Recordings were made from the same diaphragm preparation. Calibration: 1 mV, 5 ms. On the right, representative MEPPs recorded during 30 s from diaphragm muscle fibers bathed with control solution (Vm: −74.2 mV), and with 150 nM 2-MeSADP (Vm: −73.9 mV) after a 30 min-incubation. Recordings were made from the same diaphragm preparation. Calibration: 1 mV, 1 s. (B) Effect of 2-MeSADP, ADP and ATP on MEPP frequency (s^{-1}) as a function of its concentration. Each point represents mean ± SEM, $n = 3$. $^{*}P < 0.05$ versus control, ANOVA followed by Dunnett's test; 2-MeSADP EC_{50}: 0.076 μM; ADP EC_{50}: 4.152 μM; ATP EC_{50}: 5.067 μM. (C) Time course of 2-MeSADP effect on MEPP frequency. 2-MeSADP (150 nM) was applied as indicated by the bar. Symbols are mean ± SEM values of individual cells from a representative muscle.

shown in Fig. 3B, IDP decreased MEPP frequency to values similar to those observed with 2-MeSADP (IDP: 49.8 ± 2.3% of control values, $P < 0.001$, $n = 4$, compare Fig. 3A, B). The modulatory effect of IDP was not found when preparations were previously incubated with the $P2Y_{13}$ antagonist (MRS-2211 90.3 ± 8.0% of control values; MRS-2211 + IDP 88.4 ± 5.7%, $n = 4$). MEPP amplitude was not affected by IDP (control 1.27 ± 0.08 mV; IDP 1.26 ± 0.10 mV, $n = 4$). On the other hand, IDP might be metabolized into inosine, which we demonstrated is able to activate A_3 adenosine receptors and decrease spontaneous and evoked ACh release at mammalian MNJs (Cinalli et al., 2013). So, to rule out the possibility that IDP may exert its inhibitory effect through its metabolite inosine, we studied its action in the presence of the specific A_3 receptor antagonist MRS-1191 (5 μM; Jiang et al., 1996; Jacobson et al., 1997). In these conditions, the effect of 100 μM IDP upon MEPP frequency remained unchanged (MRS-1191 99.5 ± 0.7% of control values, MRS-1191 + IDP 59.1 ± 1.5%, $n = 3$, $P < 0.001$, Fig. 3C), suggesting that IDP modulates spontaneous secretion by activating $P2Y_{13}$ receptors.

We then investigated the effect of MRS-2395, selective antagonist for $P2Y_{12}$ receptors (Xu et al., 2002). We found that 10 μM MRS-2395 (Sttaford et al., 2007; Quintas et al., 2011) was not able to reverse the inhibitory action of 150 nM 2-MeSADP on MEPP frequency (MRS-2395 96.6 ± 1.0% of control values, MRS-2395 + 2-MeSADP 64.1 ± 2.1%, $P < 0.001$; 2-MeSADP *vs.* 10 μM MRS-2395 + 2-MeSADP ns, $n = 4$). The same result was observed in the presence of 30 μM MRS-2395 (Quintas et al., 2009): MRS-2395 108.6 ± 7.0% of control values; MRS-2395 + 2-MeSADP 68.0 ± 2.7%, $P < 0.001$; 2-MeSADP *vs.* 30 μM MRS-2395 + 2-MeSADP ns, $n = 6$). At higher concentrations of MRS-2395 (50 and 70 μM, Wurm et al., 2010), the effect of 150 nM 2-MeSADP was abolished (50 μM MRS-2395 107.5 ± 1.1% of control values, 50 μM MRS-2395 + 2-MeSADP 99.0 ± 9.4%, 2-MeSADP *vs.* 50 μM MRS-2395 + 2-MeSADP $P < 0.001$, $n = 4$; 70 μM MRS-2395 106.5 ± 3.3%, 70 μM MRS-2395 + 2-MeSADP 111.3 ± 5.0%, 2-MeSADP *vs.* 70 μM MRS-2395 + 2-MeSADP $P < 0.001$, $n = 3$, Fig. 4A, right set of bars in the graph). However, similar experiments using IDP as agonist

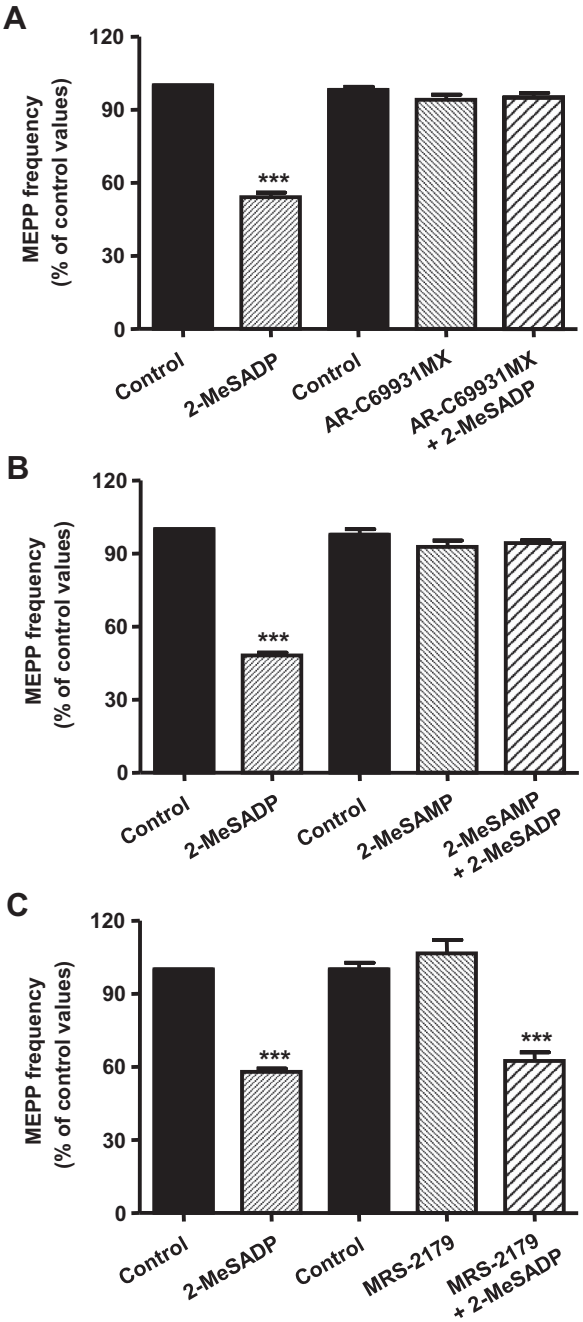

Fig. 2. Effect of 2-MeSADP in the presence of $P2Y_{12–13}$ and $P2Y_1$ antagonists. (A and B) Summary bar graphs show the modulatory effect of 150 nM 2-MeSADP on MEPP frequency and its inhibition by the $P2Y_{12–13}$ antagonists, AR-C69931MX (1 μM, $n = 4$) and 2-MeSAMP (30 μM, $n = 4$), respectively. (C) The $P2Y_1$ antagonist MRS-2179 (10 μM, $n = 4$) did not occlude 150 nM 2-MeSADP-mediated presynaptic inhibition. In A, B, and C data (mean ± SEM) are expressed as percentage of control values (black bar). Second control corresponds to values obtained after washout of the agonist. $^{***}P < 0.001$. ANOVA followed by Tukey's test.

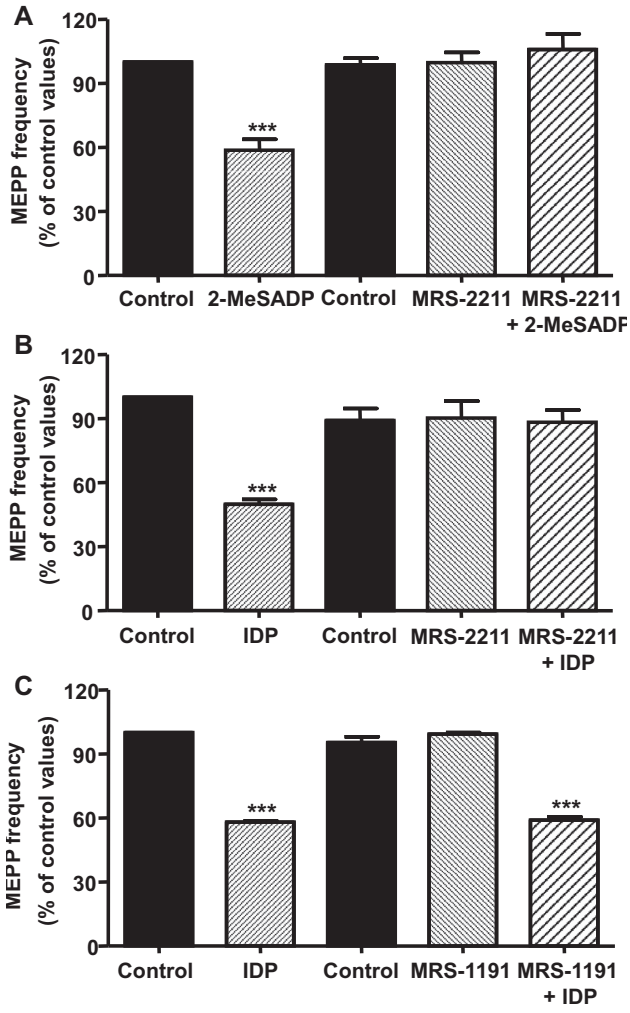

Fig. 3. The modulatory effect of 2-MeSADP upon spontaneous ACh release is mediated by $P2Y_{13}$ receptors. (A) The $P2Y_{13}$ antagonist MRS-2211 (10 μM, $n = 7$) prevented the action of 150 nM 2-MeSADP on MEPP frequency. (B) The preferential agonist of $P2Y_{13}$ receptors IDP (100 μM, $n = 4$) depressed MEPP frequency and MRS-2211 occluded this effect. (C) The inhibitory effect of IDP persisted in the presence of the A_3 receptor antagonist MRS-1191 (5 μM, $n = 3$). Data (mean ± SEM) are expressed as percentage of control values (black bar). Second control corresponds to values obtained after washout of the agonist. $^{***}P < 0.001$. ANOVA followed by Tukey's test.

(Fig. 4B) demonstrated that 50 μM MRS-2395 also prevented the action of the $P2Y_{13}$ receptor's preferential agonist (50 μM MRS-2395 101.6 ± 2.8%; 50 μM MRS-2395 + IDP 105.1 ± 2.4%, $n = 4$), suggesting that at high concentrations, MRS-2395, also binds to $P2Y_{13}$ receptors.

At amphibian NMJs, it was suggested that ATP decreased evoked ACh secretion through its own presynaptic receptors (Giniatullin and Sokolova, 1998). Here, we found that, at mammalian NMJs, 2-MeSADP also modulated evoked ACh release. In Fig. 5A, it is shown that 150 nM 2-MeSADP decreased EPP amplitude to 48.8 ± 4.9% of control values ($P < 0.001$, $n = 4$) and that this inhibition was abolished by the $P2Y_{13}$ antagonist MRS-2211 (MRS-2211 90.8 ± 7.8% of control values; MRS-2211 + 2-MeSADP 97.8 ± 3.3%). 2-MeSADP (150 nM) was also able to decrease the EPP quantal content from 3.08 ± 0.18 to 1.73 ± 0.14 (57.1 ± 6.9% of control values, $n = 4$, $P < 0.01$, Fig. 5D). On the contrary, pretreatment of the preparations with the specific $P2Y_{12}$ receptor antagonist MRS-2395 (10 μM) allowed

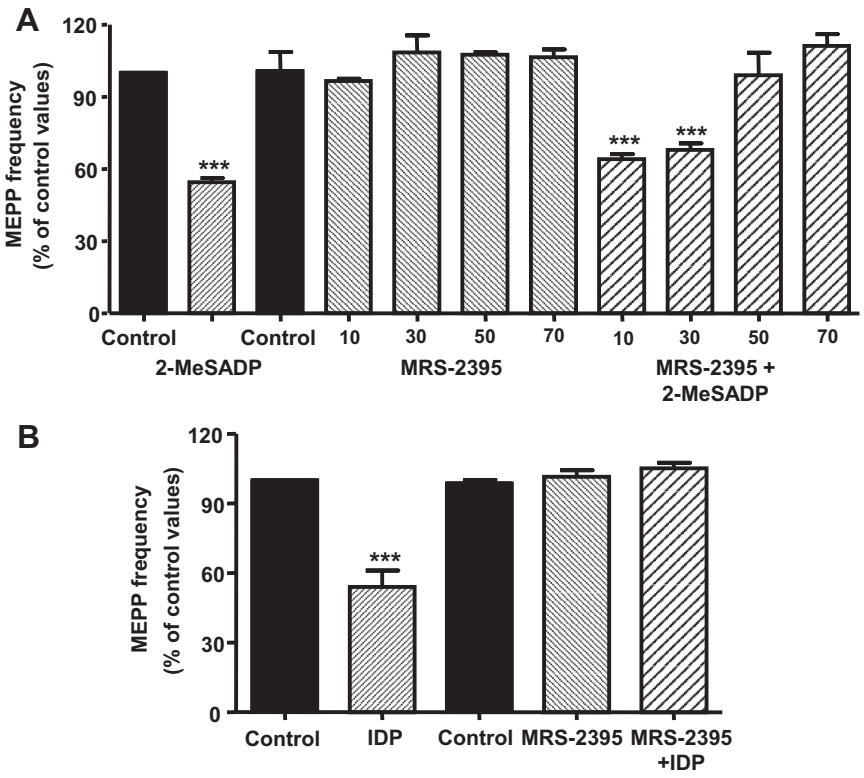

Fig. 4. The modulatory effect of 2-MeSADP upon spontaneous ACh release is not related to $P2Y_{12}$ receptors. (A) The $P2Y_{12}$ antagonist MRS-2395 did not change the action of 150 nM 2-MeSADP on MEPP frequency at 10 μM ($n = 4$) and 30 μM ($n = 6$), but at 50 μM ($n = 4$) and 70 μM ($n = 3$), MRS-2395 prevented the inhibitory effect of 2-MeSADP. (B) At 50 μM MRS-2395 ($n = 4$), the antagonist seems to be not specific for $P2Y_{12}$ receptors since it also impaired the inhibitory action of the preferential agonist of $P2Y_{13}$ receptors IDP (100 μM) on MEPP frequency. Data (mean ± SEM) are expressed as percentage of control values (black bar). Second control corresponds to values obtained after washout of the agonist. $^{***}P < 0.001$. ANOVA followed by Tukey's test.

2-MeSADP-mediated presynaptic inhibition on EPP amplitude (MRS-2395: 102.2 ± 4.8%; MRS-2395 + 2-MeSADP: 44.7 ± 3.3%, $P < 0.001$, $n = 4$, Fig. 5B).

The results obtained with 2-MeSADP on EPP amplitude were reproduced by the preferential $P2Y_{13}$ agonist IDP. Thus, 100 μM IDP decreased EPP amplitude to 51.3 ± 5.8% of control values ($P < 0.001$, $n = 3$) and the blockade of the $P2Y_{13}$ receptors with MRS-2211 prevented the inhibitory action of IDP on evoked ACh release (MRS-2211 90.9 ± 5.2% of control values; MRS-2211 + IDP 97.1 ± 6.5%, Fig. 5C). Moreover, IDP also decreased the quantal content from 2.93 ± 0.31 to 1.48 ± 0.24 (50.5 ± 7.3% of control values, $P < 0.001$, $n = 4$, Fig. 5D).

Immunohistochemical studies

$P2Y_{12}$ and $P2Y_{13}$ receptors have been described in many systems and cell types (see Burnstock, 2007), but they have not been identified at motor nerve terminals. So, to assess the specific distribution of these purinergic receptors at the neuromuscular junction, immunohistochemical studies were performed. Muscle cross-sections were dual-labeled with TMR-α-BTX to identify postsynaptic ACh receptors at the motor end-plate region and, antibodies to $P2Y_{12}$ or $P2Y_{13}$ receptors followed by staining with goat anti-rabbit IgG conjugated with Atto-488 to visualize the location of these receptors. Fig. 6 illustrates co-staining of diaphragms by TMR-α-BTX and anti-$P2Y_{13}$ (A–C) or anti-$P2Y_{12}$ (J–L) antibodies. Comparison of TMR-α-BTX binding with anti-$P2Y_{13}$ antibody labeling revealed that $P2Y_{13}$ receptors are localized at the synaptic area. In contrast, the study showed no staining with the anti-$P2Y_{12}$ antibody. No reactivity was observed in any negative control assays for $P2Y_{13}$ (D–E; G–I) or $P2Y_{12}$ (M–O) receptors. It is known that $P2Y_{12}$ receptors are expressed in platelets where they play a central role in platelet activation (Dorsam and Kunapuli, 2004; Gachet and Hechler, 2005). So, to guarantee the quality of the $P2Y_{12}$ antibody, we analyzed their expression in platelet-rich plasma (PRP) from CF1 mice and healthy human volunteers and used it as a positive control. As it is observed in Fig. 6, we found immunoreactivity with the anti-$P2Y_{12}$ antibody in both samples, PRP from CF1 mice (P–R) and healthy volunteers (S–U). To elucidate whether anti-$P2Y_{13}$ antibodies bind to epitopes localized at the presynaptic membrane, immunostaining was performed in control innervated and denervated gastrocnemius muscles. Fig. 7A–C depicted specific co-staining of innervated gastrocnemius muscle by TMR-α-BTX and anti-$P2Y_{13}$ antibody, whereas in denervated muscles (Fig. 7D–F) no immunostaining with the anti-$P2Y_{13}$ antibody is observed. Disappearance of $P2Y_{13}$ receptors in these sections is coherent with degeneration of nerve

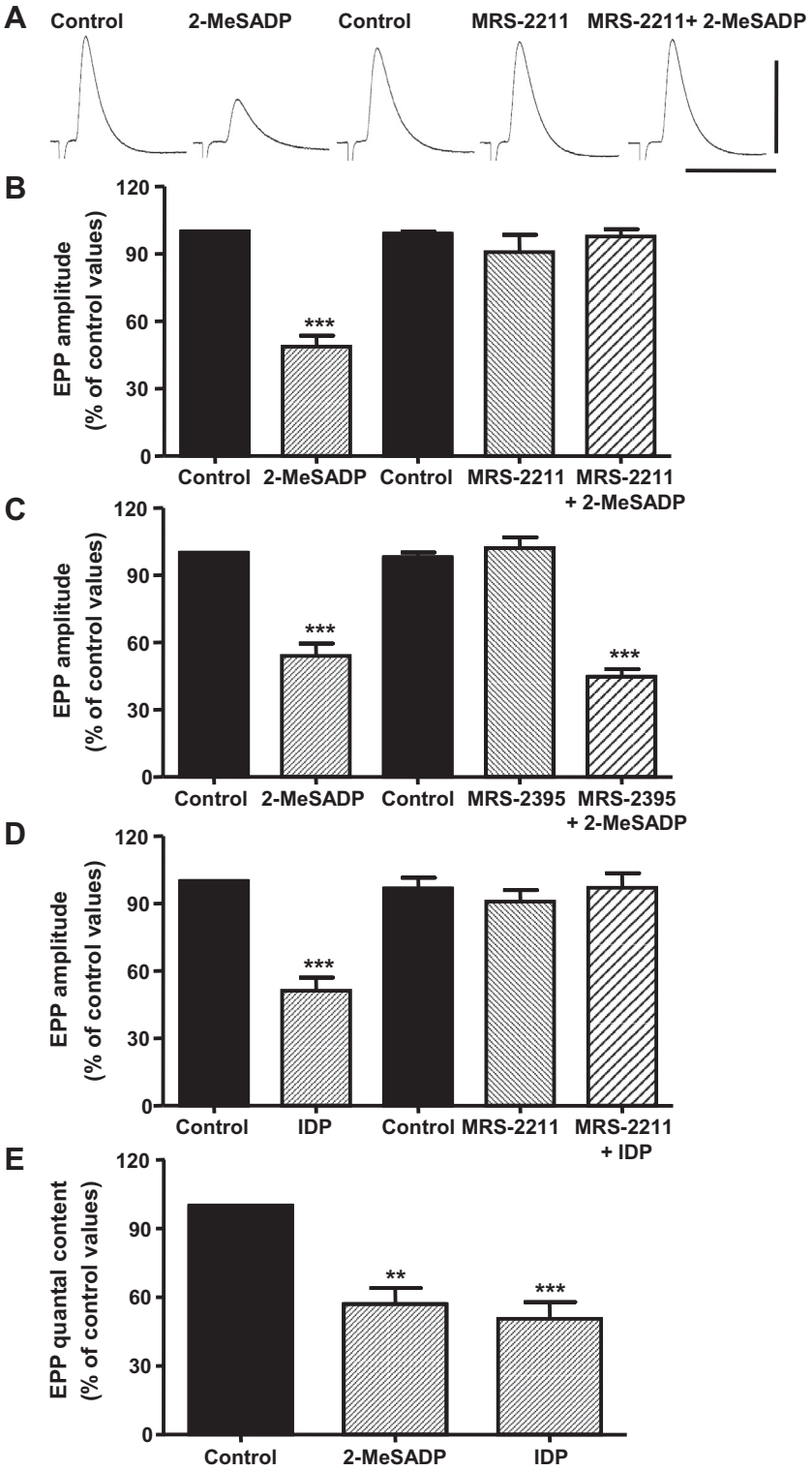

Fig. 5. The inhibitory effect of 2-MeSADP on the amplitude of EPPs is mediated by $P2Y_{13}$ receptors. (A) Each representative tracing is the average of 50 EPPs at a stimulation rate of 0.5 Hz recorded from diaphragm muscle fibers bathed with control solution (Vm: −73.2 ± 3.2 mV; EPP amplitude 3.49 ± 0.08 mV), 150 nM 2-MeSADP (Vm: −72.3 ± 1.2 mV; EPP amplitude 1.46 ± 0.06 mV), control solution (Vm: −71.1 ± 2.0 mV; EPP amplitude 3.14 ± 0.09 mV), 10 μM MRS-2211 (Vm: −72.4 ± 2.1 mV; EPP amplitude 3.32 ± 0.08 mV), and 10 μM MRS-2211 + 150 nM 2-MeSADP (Vm: −73.5 ± 3.5 mV; EPP amplitude 3.41 ± 0.09 mV). Calibration: 3 mV, 10 ms. Recordings were made from different muscle fibers at the same diaphragm preparation. (B) The $P2Y_{13}$ antagonist MRS-2211 (10 μM, $n = 4$) prevented the inhibitory action of 150 nM 2-MeSADP on evoked neurotransmitter secretion. (C) The $P2Y_{12}$ antagonist MRS-2395 (10 μM, $n = 4$) did not modify the effect of 2-MeSADP on EPP amplitude. (D) The preferential agonist of $P2Y_{13}$ receptors IDP (100 μM, $n = 3$) reduced EPP amplitude and MRS-2211 occluded this action. (E) 2-MeSADP ($n = 4$) and IDP ($n = 4$) decreased EPP quantal content. Data (mean ± SEM) are expressed as percentage of control values (black bar). In A, B, C and D, second control corresponds to values obtained after washout of the agonist. $^{***}P < 0.001$, $^{**}P < 0.01$. ANOVA followed by Tukey's test.

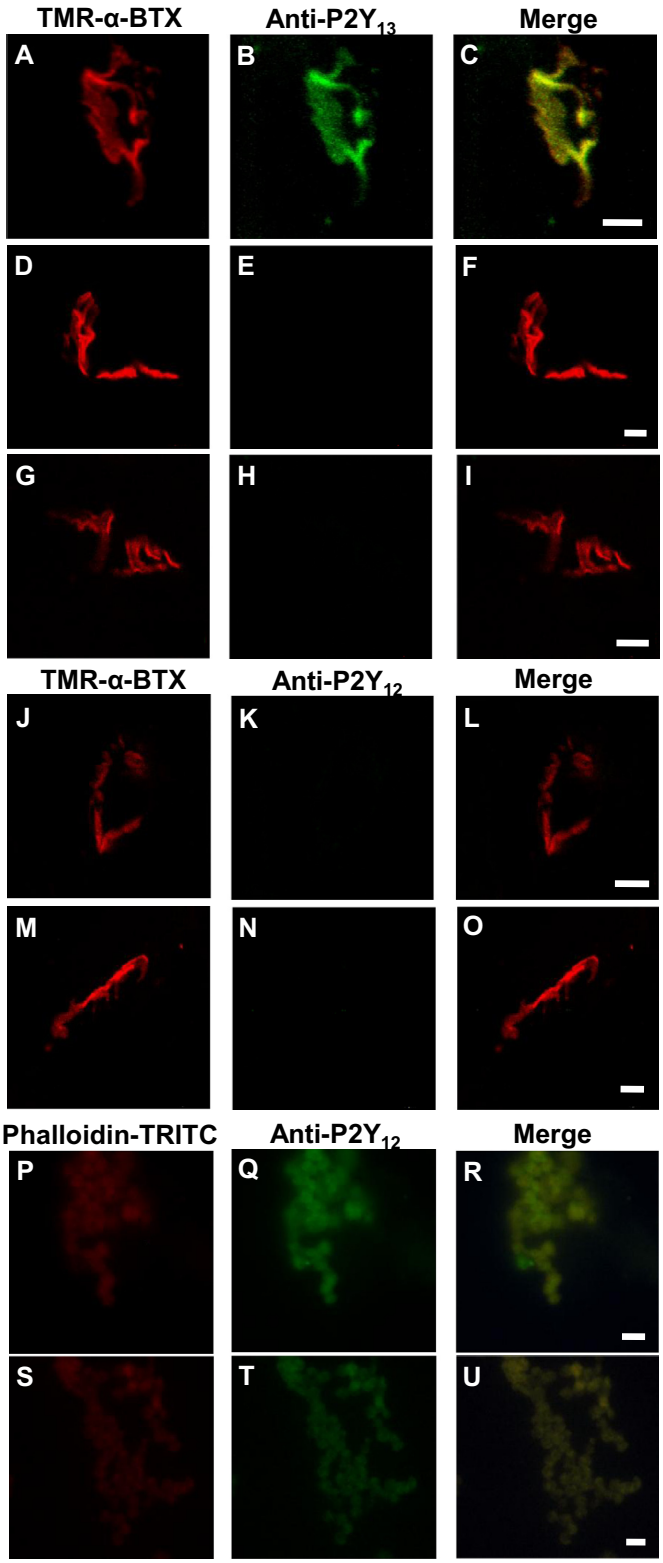

Fig. 6. Immunoreactivity for $P2Y_{13}$ receptors is present at the mouse NMJ in transverse sections of diaphragm muscles. Sections were dual-labeled with TMR-α-BTX (red) to identify ACh receptors at the end-plate regions (A, D, G, J and M) and specific $P2Y_{13}$ (B, E and H) or $P2Y_{12}$ (K and N) antibodies, visualized with Atto-488-conjugated secondary antibody (green). Negative control for the $P2Y_{13}$ receptors was assessed by omitting the primary antibody (D–F), or preincubating the primary antibody with the control peptide antigen (G–I). Negative Control for $P2Y_{12}$ receptors was analyzed by omitting the primary antibody (M–O). Positive control for the $P2Y_{12}$ antibodies: PRP from CF1 mice (P–R) and healthy volunteers (S–U) was dual-labeled with phalloidin-TRITC to stain F-actin in the platelets (red) and goat polyclonal anti-rabbit IgG coupled to Atto-488 to identify $P2Y_{12}$ antibodies (green). Scale bar = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

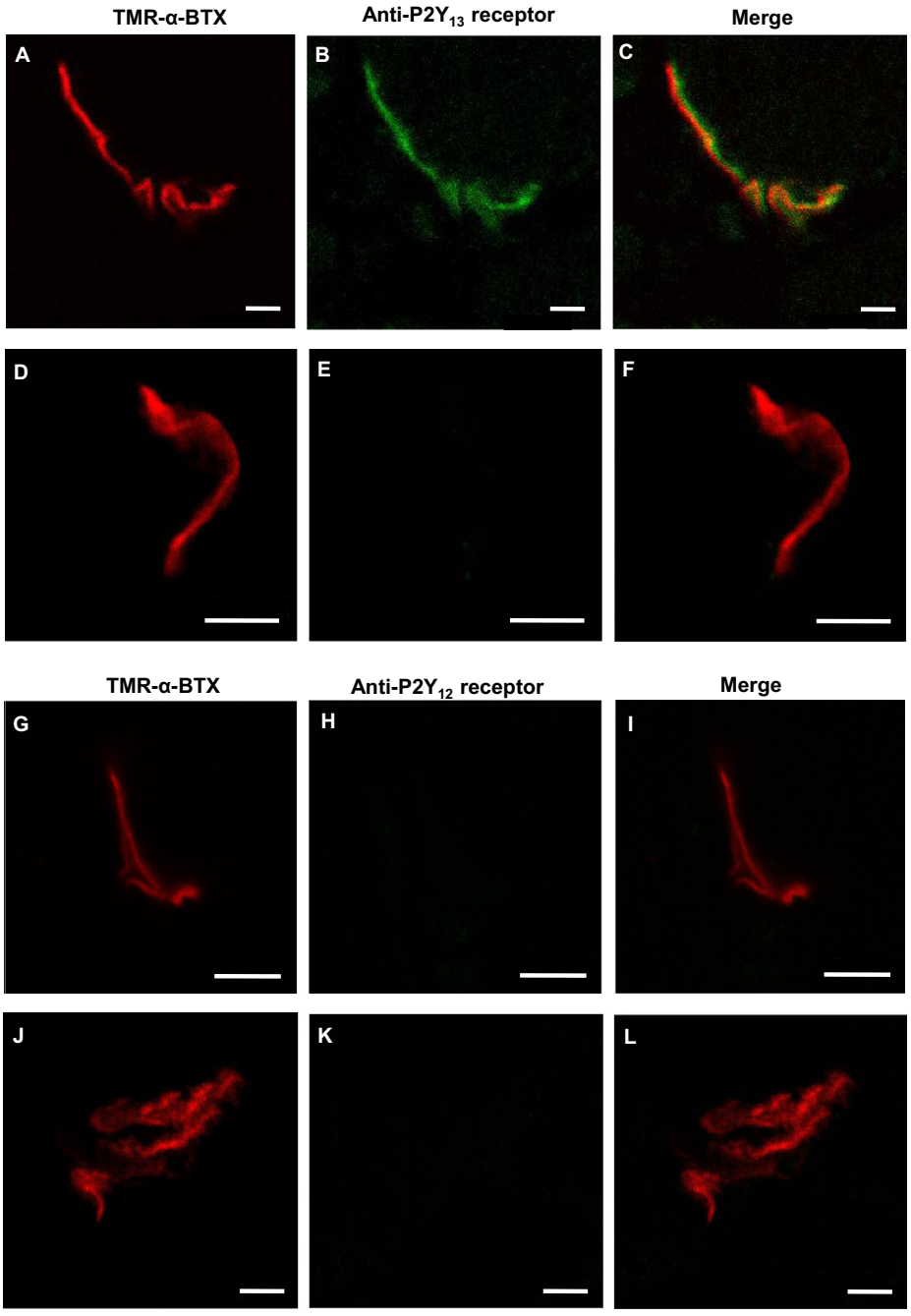

Fig. 7. Distribution of $P2Y_{13}$ receptors at the mouse NMJ in transverse sections of innervated and denervated gastrocnemius muscles. Sections were dual-labeled with TMR-α-BTX (red) to identify ACh receptors at the end-plate regions (A, D, G, and J) and specific $P2Y_{13}$ (B, C, E, and F) or $P2Y_{12}$ (H, I, K, and L) antibodies, visualized with Atto-488-conjugated secondary antibody (green). In innervated muscles, $P2Y_{13}$ (A–C) but not $P2Y_{12}$ receptors (G–I) were localized at the NMJs, whereas in denervated muscles no labeling was observed with the $P2Y_{13}$ (D–F) or $P2Y_{12}$ antibodies (J–L). Disappearance of $P2Y_{13}$ receptors in denervated muscles is consistent with retraction and degeneration of nerve terminals in response to denervation. Scale bar = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

terminals due to denervation (Miledi and Slater, 1970; Winlow and Usherwood, 1975). On the other hand, no detectable immunoreactivity for $P2Y_{12}$ receptors was observed in either innervated or denervated gastrocnemius muscles (Fig. 7G–L). All these results suggested that $P2Y_{13}$ receptors are present at the presynaptic membrane of motor nerve terminals.

DISCUSSION

In a previous report, we have found that, at the mouse neuromuscular junction, activation of P2Y receptors by ATP and βγ-imido ATP, exert an inhibitory presynaptic modulation upon cholinergic transmission and that the receptors involved were coupled to

pertussis toxin-sensitive $G_{i/o}$ protein (De Lorenzo et al., 2006). Since $P2Y_{12}$ and $P2Y_{13}$ receptors are coupled to $G_{i/o}$ protein and are activated by adenine nucleotides, we hypothesized that these receptors were the most likely candidates to mediate that inhibitory effect. In this paper, we confirmed that assumption, since the preferential agonist at these receptors, 2-MeSADP, decreased spontaneous neurotransmitter secretion and the $P2Y_{12–13}$ antagonists, AR-C69931MX and 2-MeSAMP (Takasaki et al., 2001; Marteau et al., 2003) prevented this action. It is known that ADP is the principal endogenous agonist at the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors (Palmer et al., 1998; Boeynaems et al., 2003; Marteau et al., 2003), and that 2-MeSADP displays a 10–100-fold higher potency than ADP (Zhang et al., 2001; Waldo and Harden, 2004; Hollopeter et al., 2001; Takasaki et al., 2001; Marteau et al., 2003). On the other hand, there is evidence that ATP interacts with less affinity and efficacy than ADP at $P2Y_{12}$ receptors, since ATP and other 5′-triphosphate derivatives might act even as antagonists (Hollopeter et al., 2001; Kauffenstein et al., 2004; Gachet and Hechler, 2005). At $P2Y_{13}$ receptors, both ADP and ATP might act as full agonists (see Burnstock and Verkhratsky, 2009). Consistent with these data, our experiments demonstrated that 2-MeSADP was the most potent agonist in reducing MEPP frequency when comparing to ADP and ATP (EC_{50}: 2-MeSADP 0.08 μM, ADP 4.16 μM, ATP 5.07 μM). The similar potency exhibited by ATP and ADP might suggest the involvement of $P2Y_{13}$ rather than $P2Y_{12}$ receptors. Lack of antagonism by MRS-2179 (specific antagonist of $P2Y_1$ receptors) of the depressant action caused by 2-MeSADP excluded the involvement of $P2Y_1$ receptors in the modulation of ACh secretion at the mouse NMJs. This was an expected result, since $P2Y_1$ receptors are coupled to G_q protein which is insensitive to pertussis toxin (Ralevic and Burnstock, 1998).

Thus, our next aim was to identify which of the two receptors, $P2Y_{12}$ or $P2Y_{13}$, or both, were involved in the modulatory action on neurotransmitter release. $P2Y_{12}$ and $P2Y_{13}$ receptors show a high level of similarities; both of them respond to ADP and ADP analogs with high potency, they have a high degree of amino acid identity (45–48%, Communi et al., 2001; Zhang et al., 2002) and short distance (10 kilobases) between these two syntenic genes located in tandem on 3q25 (Wittenberger et al., 2001). Therefore, identify the P2Y receptor subtype/s that mediate the physiological functions has been complicated, especially by the limited availability of P2Y receptor subtype-selective agonists and antagonists (Queiroz et al., 2003; Wang et al., 2005; Csölle et al., 2008; Heinrich et al., 2008; Carrasquero et al., 2009). So, AR-C69931MX and 2-MeSAMP, that were first reported to be selective $P2Y_{12}$ antagonists (Ingall et al., 1999; Hollopeter et al., 2001), were then demonstrated to inhibit $P2Y_{13}$ receptors as well (Marteau et al., 2003; Fumagalli et al., 2004; Wang et al., 2005; Heinrich et al., 2008; Balduini et al., 2012). Fortunately, nowadays it results easier to discriminate between $P2Y_{12}$ and $P2Y_{13}$ receptors since more selective antagonists for each receptor have been developed (Jacobson et al., 2009).

The present results provide evidence, by means of pharmacological and immunohistochemical assays that the P2Y receptors involved in the modulation of ACh release are the subtype $P2Y_{13}$. The results showed that 2-MeSADP decreased MEPP frequency without affecting MEPP amplitude and reduced EPP amplitude as well as its quantum content, confirming the presynaptic action of the nucleotide. The specific antagonist of $P2Y_{13}$ receptors, MRS-2211, completely prevented the inhibition induced by 2-MeSADP on those parameters. Consistent with these findings, IDP, a preferential agonist for $P2Y_{13}$ receptors (Zhang et al., 2002; Malin and Molliver, 2010) provoked a reduction of spontaneous and evoked ACh secretion of similar magnitude than that observed with 2-MeSADP, and, MRS-2211 occluded the modulatory action of IDP as well. It is interesting to note that MRS-2211 applied alone did not significantly modify MEPP frequency or EPP amplitude, suggesting that, at basal conditions or at low stimulation rate (0.5 Hz), endogenous nucleotide concentration at the synaptic cleft did not affect spontaneous or evoked ACh secretion.

On the contrary, the inhibitory action of 2-MeSADP on MEPP frequency and EPP amplitude could not be abolished by the $P2Y_{12}$ receptor antagonist MRS-2395 (10–30 μM). Interestingly, concentrations of the antagonist above 50 μM prevented the action of 2-MeSADP. However, this behavior could also be observed when IDP was used as agonist, suggesting that, in our experimental model, at high concentrations of MRS-2395, the drug is not able to discriminate between $P2Y_{12}$ and $P2Y_{13}$ receptors.

Contrary to our findings at mammalian NMJs, Giniatullin et al. (2015) found that, at the frog sartorius muscles, 2-MeSAMP (considered by the authors as $P2Y_{12}$-specific antagonist) eliminated the action of ATP on ACh release, while the inhibition of $P2Y_{13}$ receptors by MRS-2211 did not alter the effect of ATP. Although it was demonstrated that 2-MeSAMP inhibits not only $P2Y_{12}$, but also $P2Y_{13}$.receptors (see discussion above, Heinrich et al., 2008; Balduini et al., 2012), the main difference with our experiments is that the same concentration of MRS-2211 did not prevent the inhibitory effect of ATP, suggesting that the modulatory action of nucleotides upon ACh secretion is carried out by different P2Y subtypes depending on the species used. In this regard, it was demonstrated that extracellular ATP, acting on postsynaptic $P2Y_1$ receptors, inhibited chloride channels at mammalian, but not amphibian, skeletal muscles (Voss, 2009) indicating that $P2Y_1$ receptors are not present at the frog postsynaptic NMJs.

At mammalian NMJs, it has been demonstrated that $P2X_7$ receptors, but not any other P2X receptors, are present at motor nerve terminals and that their activation promotes vesicle release (Moores et al., 2005). On the other hand, identification of P2Y receptors at the presynaptic motor nerve terminals by immunohistochemical assays has not been reported. Our results demonstrated that $P2Y_{13}$ receptors are present at the mammalian NMJs. The disappearance of $P2Y_{13}$ immunostaining in denervated muscles suggests that

the receptors are localized at the presynaptic membrane of nerve terminals. These results reinforce the data obtained through the electrophysiological studies where 2-MeSADP demonstrated to decrease MEPP frequency and quantal content, but not MEPP amplitude. On the contrary, $P2Y_{12}$ receptors have been identified neither in innervated diaphragm or gastrocnemius muscles nor in denervated gastrocnemius ones. These findings are in concordance with experiments performed in the spinal cord by *in situ* hybridization histochemistry, where expression of $P2Y_{12}$ mRNA was observed in microglia but not in the motor neurons in the ventral horn (Kobayashi et al., 2006, 2008).

CONCLUSIONS

In summary, in this report we provide new insights into the types of purinergic receptors that contribute to the fine-tuning of cholinergic transmission at mammalian neuromuscular junction. We found, by electrophysiological and immunohistochemical assays, that the P2Y receptors implicated in presynaptic inhibition of spontaneous and evoked ACh release are those of the subtype $P2Y_{13}$.

AUTHOR CONTRIBUTIONS

JFG, ARC and VF performed the experiments and contributed to the data analysis, LIR designed the immunohistochemical studies and analyzed the data, and ASL designed the research study, analyzed and interpreted the data, and wrote the paper.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, Burnstock G (2003) Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. Trends Pharmacol Sci 24:52–55.

Alvarenga EC, Rodrigues R, Caricati-Neto A, Silva-Filho FC, Paredes-Gamero EJ, Ferreira AT (2010) Low-intensity pulsed ultrasound-dependent osteoblast proliferation occurs by via activation of the P2Y receptor: role of the P2Y1 receptor. Bone 46:355–362.

Balduini A, Di Buduo CA, Malara A, Lecchi A, Rebuzzini P, Currao M, Pallotta I, Jakubowski JA, Cattaneo M (2012) Constitutively released adenosine diphosphate regulates proplatelet formation by human megakaryocytes. Haematologica 97:1657–1665.

Bennett MR, Karunanithi S, Lavidis NA (1991) Probabilistic secretion of quanta from nerve terminals in toad (Bufo marinus) muscle modulated by adenosine. J Physiol 433:421–434.

Boeynaems JM, Wilkin F, Marteau F, Duhant X, Savi P, Gonzalez NS, Robaye B, Communi D (2003) P2Y receptors: new subtypes, new functions. Drug Dev Res 59:30–35.

Boyer JL, Mohanram A, Camaioni E, Jacobson KA, Harden TK (1998) Competitive and selective antagonism of $P2Y_1$ receptors by N6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate. Br J Pharmacol 124:1–22.

Burnstock G (2007) Purine and pyrimidine receptors. Cell Mol Life Sci 64:1471–1483.

Burnstock G, Verkhratsky A (2009) ADP-preferring P2Y receptors: P2Y1, P2Y12 and P2Y13. In: Purinergic signalling and the nervous system. Heidelberg: Springer. p. 193.

Camaioni E, Boyer JL, Mohanram A, Harden TK, Jacobson KA (1998) Deoxyadenosine-bisphosphate derivatives as potent antagonists at P2Y1 receptors. J Med Chem 41:183–190.

Carrasquero LM, Delicado EG, Bustillo D, Gutiérrez-Martín Y, Artalejo AR, Miras-Portugal MT (2009) P2X7 and P2Y13 purinergic receptors mediate intracellular calcium responses to BzATP in rat cerebellar astrocytes. J Neurochem 110:879–889.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S, Livi GP (2000) A G protein-coupled receptor for UDP-glucose. J Biol Chem 275:10767–10771.

Choi RC, Man ML, Ling KK, Ip NY, Simon J, Barnard EA, Tsim KW (2001) Expression of the P2Y1 nucleotide receptor in chick muscle: its functional role in the regulation of acetylcholinesterase and acetylcholine receptor. J Neurosci 2:9224–9234.

Cinalli AR, Guarracino JF, Fernandez V, Roquel LI, Losavio AS (2013) Inosine induces presynaptic inhibition of acetylcholine release by activation of A_3 adenosine receptors at the mouse neuromuscular junction. Br J Pharmacol 169:1810–1823.

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM (2001) Identification of a novel human ADP receptor coupled to G(i). J Biol Chem 276: 41479–41485.

Csölle C, Heinrich A, Kittel A, Sperlágh B (2008) P2Y receptor mediated inhibitory modulation of noradrenaline release in response to electrical field stimulation and ischemic conditions in superfused rat hippocampus slices. J Neurochem 106: 347–360.

Cunha RA, Correia-de-Sa P, Sebastiao AM, Ribeiro JA (1996) Preferential activation of excitatory adenosine receptors at rat hippocampal and neuromuscular synapses by adenosine formed from released adenine nucleotides. Br J Pharmacol 119:253–260.

De Lorenzo S, Veggeti M, Muchnik S, Losavio A (2004) Presynaptic inhibition of spontaneous acetylcholine release induced by adenosine at the mouse neuromuscular junction. Br J Pharmacol 142:113–124.

De Lorenzo S, Veggetti M, Muchnik S, Losavio A (2006) Presynaptic inhibition of spontaneous acetylcholine release mediated by P2Y receptors at the mouse neuromuscular junction. Neuroscience 142:71–85.

DelCastillo J, Katz B (1954) Quantal components of the end-plate potential. J Physiol 124:560–573.

Del Puerto A, Diaz-Hernandez JI, Tapia M, Gomez-Villafuertes R, Benitez MJ, Zhang J, Miras-Portugal MT, Wandosell F, Diaz-Hernadez M, Garrido JJ (2012) Adenylate cyclase 5 coordinate the action of ADP, P2Y1, P2Y13 and ATP-gated P2X7 receptors on axonal elongation. J Cell Sci 125:176–188.

Deuchars SA, Atkinson L, Brooke RE, Musa H, Milligan CJ, Batten TF, Buckley NJ, Parson SH, Deuchars J (2001) Neuronal P2X7 receptors are targeted to presynaptic terminals in the central and peripheral nervous systems. J Neurosci 21:7143–7152.

Dorsam RT, Kunapuli SP (2004) Central role of the $P2Y_{12}$ receptor in platelet activation. J Clin Invest 113:340–345.

Fatt P, Katz B (1951) An analysis of the end-plate potential recorded with an intracellular electrode. J Physiol 115:320–370.

Fumagalli M, Trincavelli L, Lecca D, Martini C, Ciana P, Abbracchio M (2004) Cloning, pharmacological characterisation and distribution

of the rat G-protein-coupled $P2Y_{13}$ receptor. Biochem Pharmacol 68:113–124.
Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M (1994) Nomenclature and classification of purinoceptors. Pharmacol Rev 46:143–156.
Gachet C, Hechler B (2005) The platelet P2 receptors in thrombosis. Semin Thromb Hemost 31:162–167.
Galkin AV, Giniatullin RA, Mukhtarov MR, Švandová I, Grishin SN, Vyskočil F (2001) ATP but not adenosine inhibits nonquantal acetylcholine release at the mouse neuromuscular junction. Eur J Neurosci 13:2047–2053.
Ginsborg BL, Hirst GDS (1972) The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. J Physiol 224:629–645.
Giniatullin RA, Sokolova EM (1998) ATP and adenosine inhibit transmitter release at the frog neuromuscular junction through distinct presynaptic receptors. Br J Pharmacol 124:839–844.
Giniatullin A, Petrov A, Giniatullin R (2015) The involvement of $P2Y_{12}$ receptors, NADPH oxidase, and lipid rafts in the action of extracellular ATP on synaptic transmission at the frog neuromuscular junction. Neuroscience 285:324–332.
Hamilton BR, Smith DO (1991) Autoreceptor-mediated purinergic and cholinergic inhibition of motor nerve terminal calcium currents in the rat. J Physiol 432:327–341.
Heinrich A, Kittel A, Csölle C, Sylvester Vizi E, Sperlágh B (2008) Modulation of neurotransmitter release by P2X and P2Y receptors in the rat spinal cord. Neuropharmacology 54:375–386.
Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409:202–207.
Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, Hunt SF, Kindon ND, Teobald BJ, Willis PA, Humphries RG, Leff P, Clegg JA, Smith JA, Tomlinson W (1999) Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem 42:213–220.
Jacobson KA, Ivanov AA, de Castro S, Harden K, Ko H (2009) Development of selective agonists and antagonists of P2Y receptors. Purinergic Signal 5:75–89.
Jacobson KA, Park K-S, Jiang J-L, Kim Y-C, Olah ME, Stiles GL, Ji X-D (1997) Pharmacological characterization of novel A3 adenosine receptor-selective antagonists. Neuropharmacol 36:1157–1165.
Jiang J-L, van Rhee AM, Melman N, Ji XD, Jacobson KA (1996) 6-Phenyl-1,4-dihydropyridine derivatives as potent and selective A_3 adenosine receptor antagonists. J Med Chem 39:4667–4675.
Kauffenstein G, Hechler B, Cazenave JP, Gachet C (2004) Adenine triphosphate nucleotides are antagonists at the P2Y12 receptor. J Thromb Haemost 2:1980–1988.
Kim Y-C, Lee J-S, Sak K, Marteau F, Mamedova L, Boeynaems J-M, Jacobson KA (2005) Synthesis of pyridoxal phosphate derivatives with antagonist activity at the P2Y13 receptor. Biochem Pharmacol 70:266–274.
Kobayashi K, Fukuoka T, Yamanaka H, Dai Y, Obata K, Tokunaga A, Noguchi K (2006) Neurons and glial cells differentially express P2Y receptor mRNAs in the rat dorsal root ganglion and spinal cord. J Comp Neurol 498:443–454.
Kobayashi K, Yamanaka H, Fukuoka T, Dai Y, Obata K, Noguchi K (2008) P2Y12 receptor upregulation in activated microglia is a gateway of p38 signaling and neuropathic pain. J Neurosci 28:2892–2902.
Kulick MB, von Kügelgen I (2002) P2Y-receptors mediating an inhibition of the evoked entry of calcium through N-type calcium channels at neuronal processes. J Pharmacol Exp Ther 303:520–526.
Liu GJ, Werry EL, Bennett MR (2005) Secretion of ATP from Schwann cells in response to uridine triphosphate. Eur J Neurosci 21:151–160.
Léon C, Hechler B, Vial C, Leray C, Cazenave JP, Gachet C (1997) The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. FEBS Lett 403:26–30.
Magalhães-Cardoso MT, Pereira MF, Oliveira L, Ribeiro JA, Cunha RA, Correia-de-Sá P (2003) Ecto-AMP deaminase blunts the ATP-derived adenosine A_{2A} receptor facilitation of acetylcholine release at rat motor nerve endings. J Physiol 549:399–408.
Magleby KL, Stevens CF (1972) A quantitative description of end-plate currents. J Physiol 223:173–197.
Malin SA, Molliver DC (2010) Gi- and Gq-coupled ADP (P2Y) receptors act in opposition to modulate nociceptive signaling and inflammatory pain behavior. Mol Pain 6:21–32.
Marteau F, Le Poul E, Communi D, Communi D, Labouret C, Savi P, Boeynaems JM, Gonzalez NS (2003) Pharmacological characterization of the human P2Y13 receptor. Mol Pharmacol 64:104–112.
Meriney SD, Grinnell AD (1991) Endogenous adenosine modulates stimulation-induced depression at the frog neuromuscular junction. J Physiol 443:441–455.
Miledi R, Slater CR (1970) On the degeneration of rat neuromuscular junctions after nerve section. J Physiol 207:507–528.
Moores TS, Hasdemir B, Vega-Riveroll L, Deuchars J, Parson SH (2005) Properties of presynaptic P2X7-like receptors at the neuromuscular junction. Brain Res 1034:40–50.
Palmer RK, Boyer JL, Schacter JB, Nicolas RA, Harden TK (1998) Agonist action of adenosine triphosphates at the human P2Y1 receptor. Mol Pharmacol 54:1118–1123.
Queiroz G, Talaia C, Gonçalves J (2003) ATP modulates noradrenaline release by activation of inhibitory P2Y receptors and facilitatory P2X receptors in the rat vas deferens. J Pharmacol Exp Ther 307:809–815.
Quintas C, Fraga S, Gonçalves J, Queiroz G (2009) The P2Y1 and P2Y12 receptors mediate autoinhibition of transmitter release in sympathetic innervated tissues. Neurochem Int 55:505–513.
Quintas C, Fraga S, Gonçalves J, Queiroz G (2011) Opposite modulation of astroglial proliferation by adenosine 5′-O-(2-thio)-diphosphate and 2-methylthioadenosine-5′-diphosphate: mechanisms involved. Neuroscience 182:32–42.
Ravelic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492.
Redman RS, Silinsky EM (1994) ATP released together with acetylcholine as the mediator of neuromuscular depression at frog motor nerve endings. J Physiol 447:127–177.
Ribeiro JA, Sebastião AM (1987) On the role, inactivation, and origin of endogenous adenosine at the frog neuromuscular junction. J Physiol 384:571–585.
Ribeiro JA, Walker J (1975) The effects of adenosine triphosphate and adenosine diphosphate on transmission at the rat and frog neuromuscular junctions. Br J Pharmacol 54:213–218.
Salgado AI, Cunha RA, Ribeiro JA (2000) Facilitation by P_2 receptor activation of acetylcholine release from rat motor nerve terminals: interaction with presynaptic nicotinic receptors. Brain Res 877:245–250.
Santos DA, Salgado AI, Cunha RA (2003) ATP is released from nerve terminals and from activated muscle fibres on stimulation of the rat phrenic nerve. Neurosci Lett 338:225–228.
Sebastião AM, Ribeiro JA (2000) Fine-tuning neuromodulation by adenosine. Trends Pharmacol Sci 21:341–346.
Shirasaki H, Kanaizumi E, Seki N, Kikuchi M, Himi T (2013) Expression and localization of purinergic P2Y(12) receptor in human nasal mucosa. Allergol Int 62:239–244.
Sokolova E, Grishin S, Shakirzyanova A, Talantova M, Giniatullin R (2003) Distinct receptors and different transduction mechanisms for ATP and adenosine at the frog motor nerve endings. Eur J Neurosci 18:1254–1264.
Smith DO (1991) Sources of adenosine released during neuromuscular transmission in the rat. J Physiol 432:343–354.
Sttaford MR, Bartlett PF, Adams DJ (2007) Purinergic receptor activation inhibits mitogen-stimulated proliferation in primary neurospheres from the adult mouse subventricular zone. Mol Cell Neurosci 35:535–548.
Takasaki J, Kamohara M, Saito T, Matsumoto M, Matsumoto S, Ohishi T, Soga T, Matsushime H, Furuichi K (2001) Molecular cloning of the platelet P2T(AC) ADP receptor: pharmacological

comparison with another ADP receptor, the P2Y1 receptor. Mol Pharmacol 60:432–439.
Todd KJ, Robitaille R (2006) Purinergic modulation of synaptic signaling at the neuromuscular junction. Pflugers Arch 452:608–614.
Unterberger U, Moskvina E, Scholze T, Freissmuth M, Boehm S (2002) Inhibition of adenylyl cyclase by neuronal P2Y receptors. Br J Pharmacol 135:673–684.
Veggetti M, Muchnik S, Losavio A (2008) Effect of purines on calcium-independent acetylcholine release at the mouse neuromuscular junction. Neuroscience 154:1324–1336.
Voss AA (2009) Extracellular ATP inhibits chloride channels in mature mammalian skeletal muscle by activating $P2Y_1$ receptors. J Physiol 587:5739–5752.
Waldo GL, Harden TK (2004) Agonist binding and Gq-stimulating activities of the purified human P2Y1 receptor. Mol Pharmacol 65:426–436.
Wang L, Olivecrona G, Götberg M, Olsson ML, Winzell MS, Erlinge D (2005) ADP acting on $P2Y_{13}$ receptors is a negative feedback pathway for ATP release from human red blood cells. Circ Res 96:189–196.
White PJ, Webb TE, Boarder MR (2003) Characterization of a Ca^{2+} response to both UTP and ATP at human $P2Y_{11}$ receptors: evidence for agonist-specific signaling. Mol Pharmacol 63: 1356–1363.
Winlow W, Usherwood PN (1975) Ultrastructural studies of normal and degenerating mouse neuromuscular junctions. J Neurocytol 4:377–394.
Wittenberger T, Schaller HC, Hellebrand S (2001) An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. J Mol Biol 307:799–813.
Wurm A, Lipp S, Pannicke T, Linnertz R, Krugel U, Schulz A, Färber K, Zahn D, Grosse J, Wiedemann P, Chen J, Schöneberg T, Illes P, Reichenbach A, Bringmann A (2010) Endogenous purinergic signaling is required for osmotic volume regulation of retinal glial cells. J Neurochem 112:1261–1272.
Xu B, Stephens A, Kirschenheuter G, Greslin AF, Cheng X, Sennelo J, Cattaneo M, Zighetti ML, Chen A, Kim SA, Kim HS, Bischofberger N, Cook G, Jacobson KA (2002) Acyclic analogues of adenosine bisphosphates as P2Y receptor antagonists: phosphate substitution leads to multiple pathways of inhibition of platelet aggregation. J Med Chem 45:5694–5709.
Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, Qiao X, Liu YH, Chen G, Pramanik B, Laz TM, Palmer K, Bayne M, Monsma Jr FJ (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. J Biol Chem 276:8608–8615.
Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, Yang S, Laz TM, Bayne M, Monsma Jr F (2002) $P2Y_{13}$: identification and characterization of a novel Gαi-coupled ADP receptor from human and mouse. J Pharmacol Exp Ther 301:705–713.
Zizzo MG, Mulè F, Serio R (2007) Inhibitory purinergic transmission in mouse caecum: role for $P2Y_1$ receptors as prejunctional modulators of ATP release. Neuroscience 150:658–664.

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