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PRESYNAPTIC INHIBITION OF SPONTANEOUS ACETYLCHOLINE RELEASE MEDIATED BY P2Y RECEPTORS AT THE MOUSE NEUROMUSCULAR JUNCTION

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Abstract—At the neuromuscular junction, ATP is co-released with the neurotransmitter acetylcholine (ACh) and once in the synaptic space, it is degraded to the presynaptically active metabolite adenosine. Intracellular recordings were performed on diaphragm fibers of CF1 mice to determine the action of extracellular ATP (100 μM) and the slowly hydrolysable ATP analog 5′-adenylylimidodiphosphate lithium ($\beta\gamma$-imido ATP) (30 μM) on miniature end-plate potential (MEPP) frequency. We found that application of ATP and $\beta\gamma$-imido ATP decreased spontaneous secretion by 45.3% and 55.9% respectively. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective A_1 adenosine receptor antagonist and α,β-methylene ADP sodium salt ($\alpha\beta$-MeADP), which is an inhibitor of ecto-5′-nucleotidase, did not prevent the inhibitory effect of ATP, demonstrating that the nucleotide is able to modulate spontaneous ACh release through a mechanism independent of the action of adenosine. Blockade of Ca^{2+} channels by both, Cd^{2+} or the combined application of nitrendipine and ω-conotoxin GVIA (ω-CgTx) (L-type and N-type Ca^{2+} channel antagonists, respectively) prevented the effect of $\beta\gamma$-imido ATP, indicating that the nucleotide modulates Ca^{2+} influx through the voltage-dependent Ca^{2+} channels related to spontaneous secretion. $\beta\gamma$-Imido ATP-induced modulation was antagonized by the non-specific P2 receptor antagonist suramin and the P2Y receptor antagonist 1-amino-4-[[4-[[4-chloro-6-[[3(or4)-sulfophenyl] amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl] amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid (reactive blue-2), but not by pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (PPADS), which has a preferential antagonist effect on P2X receptors. Pertussis toxin and *N*-ethylmaleimide (NEM), which are blockers of $G_{i/o}$ proteins, prevented the action of the nucleotide, suggesting that the effect is mediated by P2Y receptors coupled to $G_{i/o}$ proteins. The protein kinase C (PKC) antagonist chelerythrine and the calmodulin antagonist N-(6-aminohexil)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) occluded the effect of $\beta\gamma$-imido ATP, while the protein kinase A (PKA) antagonist KT-5720 and the inhibitor of the calcium/calmodulin-dependent protein kinase II (CAMKII) KN-62 failed to do so. $\beta\gamma$-Imido ATP did not affect 10, 15 and 20 mM K^+-evoked release and application of reactive blue-2 before incubation in high K^+ induced a higher asynchronous secretion. Thus, our results show that at mammalian neuromuscular junctions, ATP induces presynaptic inhibition of spontaneous ACh release due to the modulation of Ca^{2+} channels related to tonic secretion through the activation of P2Y receptors coupled to $G_{i/o}$ proteins. We also demonstrated that at increasing degrees of membrane depolarization evoked by K^+, endogenously released ATP induces presynaptic inhibition as a means of preventing excessive neurotransmitter secretion.

Key words: ATP, presynaptic inhibition, P2Y receptors, PKC, calmodulin, mammalian neuromuscular junction.

ATP is a neuromodulator and neurotransmitter (or co-transmitter) in the CNS (Edwards et al., 1992; Pankratov et al., 1998) and peripheral nervous system (Burnstock, 1990; Evans et al., 1992; Silinsky and Gerzanich, 1993). As a neuromodulator, ATP can induce either depression or facilitation of neurotransmitter secretion (Cunha and Ribeiro, 2000) by acting at the presynaptic membrane through their specific P2 receptors. These purine receptors can be mainly divided into two types: the P2X receptors, which are ligand-gated ion channels, and the P2Y receptors which are G protein-coupled receptors (Fredholm et al., 1994; Ralevic and Burnstock, 1998). At motor nerve endings, ATP is co-released with the neurotransmitter acetylcholine (ACh) and once in the synaptic space, it is degraded to adenosine via the ectonucleotidase cascade (Ribeiro and Sebastião, 1987; Meriney and Grinnell, 1991; Redman and Silinsky, 1994).In fact, it was classically accepted that ATP modulated ACh release via its metabolite adenosine by activating the presynaptic adenosine receptors (Ribeiro and Sebastião, 1987; Hamilton and Smith, 1991; Meriney and Grinnell, 1991; Redman and Silinsky, 1994). Recently, we have demonstrated that, unlike the

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Abbreviations: ACh, acetylcholine; AR-C69931MX, N-[2-(methyltthioethyl9]-2-[3,3,3-trifluoropropyl]thio-5′-adenylic acid, monoanhydride with dichloromethylenebiphosphonic acid, tetrasodium salt; CAMKII, calcium/calmodulin-dependent protein kinase II; CCPA, 2-chloro-N^6-cyclopentyl-adenosine; ω-CgTx, ω-conotoxin GVIA; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; $\beta\gamma$-imido ATP, 5′-adenylylimidodiphosphate lithium; $\alpha\beta$-MeADP, α,β-methylene ADP sodium salt; MEPP, miniature end-plate potential; NEM, *N*-ethylmaleimide; PC-PLC, phosphatidylcholine specific phospholipase C; PI-PLC, phosphatidylinositol specific phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt; PTX, pertussis toxin; reactive blue-2, 1-amino-4-[[4-[[4-chloro-6-[[3(or4)-sulfophenyl] amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl] amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid; suramin, 8,8′-[carbonylbis [imino-3,1-phenylenecarbonylimino (4-methyl-3,1-phenylene) carbonylimino]]bis-1,3,5-aphthalenetrisulfonic acid hexasodium salt; U73122, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien17-yl)amino)hexyl)-1H-pyrrole-2,5-dione; VDCC, voltage-dependent calcium channel; W-7, N-(6-aminohexil)-5-chloro-1-naphthalenesulfonamide hydrochloride; 2-MeSAMP, 2-methylthio-AMP triethylammonium salt.

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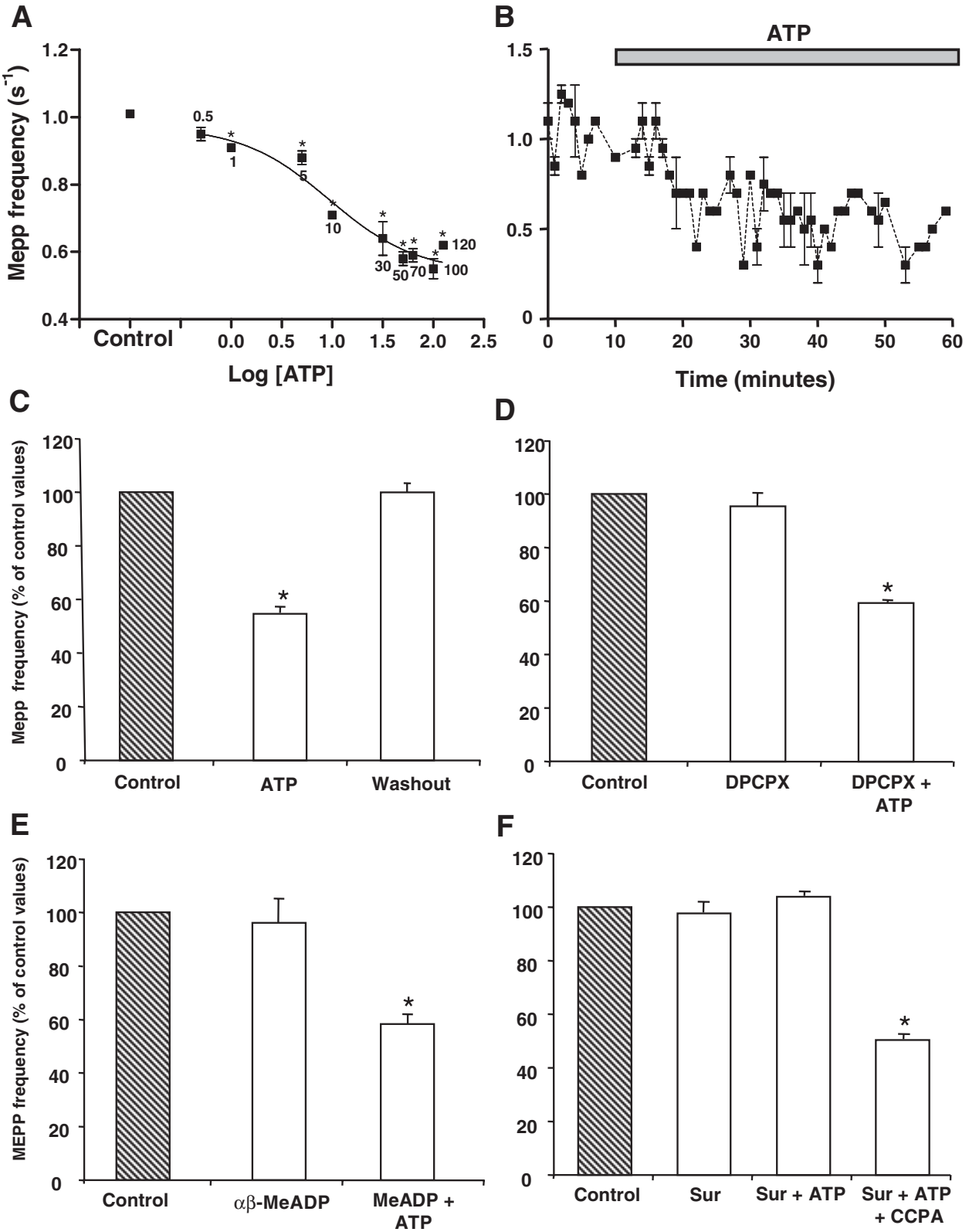

Fig. 1. Effect of ATP on spontaneous ACh release at the mouse skeletal muscle. (A) Effect of ATP on MEPP frequency (s^{-1}) as a function of concentration. Control: 1.01±0.01 (n=4); 0.5 μM: 0.95±0.02 (n=4); 1 μM: 0.91±0.01 (n=4); 5 μM: 0.88±0.02 (n=4); 10 μM: 0.71±0.01 (n=4); 30 μM: 0.64±0.05 (n=4); 50 μM: 0.58±0.02 (n=4); 70 μM: 0.59±0.02 (n=4); 100 μM: 0.55±0.03 (n=4); 120 μM: 0.62±0.01 (n=4); EC_{50}: 9.41 μM. (B) Temporal course of ATP inhibitory effect on MEPP frequency. ATP (100 μM) was applied as indicated by the bar. Symbols are means±S.E.M. values of individual cells from a representative muscle. (C) Changes of MEPP frequency after ATP application and after washout of the nucleotide. (D) DPCPX (0.1 μM) did not affect ATP-induced inhibition. (E) $\alpha\beta$-MeADP (100 μM) had no effect on the action of ATP. (F) Suramin (Sur, 100 μM) prevented the modulatory effect of ATP, but did not affect the action of CCPA. In C, D, E, and F data are expressed as percentage of control values (cross-hatched bar). Error bars indicate S.E.M. * $P<0.05$.

situation at frog neuromuscular synapse (Redman and Silinsky, 1994), adenosine and the specific adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyl-adenosine (CCPA) exert their modulatory role by decreasing exclusively the nitrendipine-sensitive component of miniature end-plate potential (MEPP) frequency through a mechanism related to the action of Ca^{2+}-calmodulin (De Lorenzo et al., 2004).

On the other hand, it was found that ATP, independently of being a source of adenosine, is able to modulate ACh release through its own presynaptic receptors. Several reports have demonstrated the presence of P2X and P2Y receptors in the neuromuscular synapses (Choi et al., 2001; Deuchars et al., 2001; Moores et al., 2005). However, the presynaptic role of ATP on neurosecretion is contradictory: a direct inhibitory effect of ATP on ACh release was described at frog neuromuscular junction (Giniatullin and Sokolova, 1998; Sokolova et al., 2003) whereas at mammalian motor nerve endings, ATP was found to potentate spontaneous secretion at developing and mature neuromuscular junctions (Fu and Poo, 1991; Hong and Chang, 1998) and to facilitate evoked [^{3}H]ACh release in adult rats (Salgado et al., 2000). Conversely, Galkin et al. (2001) reported that exogenous ATP reduces MEPP frequency at mouse diaphragm muscles. In the present work, we studied the modulatory effect of the slowly hydrolysable ATP analog, 5′-adenylylimidodiphosphate lithium ($\beta\gamma$-imido ATP) upon spontaneous quantal secretion at mature mammalian motor nerve endings. Our main goals were conducted (1) to clarify the type of response of ATP and $\beta\gamma$-imido ATP on MEPP frequency, i.e. facilitatory or inhibitory, (2) to identify the subtype of ATP receptor in the response, (3) to study the mechanism involved in P2 receptor activation, i.e. if ATP modulates Ca^{2+} influx through the Ca^{2+} channels related to spontaneous secretion (Losavio and Muchnik, 1997), (4) to identify the subtype/s of Ca^{2+} channel/s in the response, (5) to determine the intracellular mechanisms in the response, (6) to test the effect of $\beta\gamma$-imido ATP on quantal release triggered by KCl.

EXPERIMENTAL PROCEDURES

Preparations and solutions

CF1 mouse diaphragm muscles were used. Mice (30–40 g) were anesthetized with sodium thiopental (50 mg kg^{-1}) intraperitoneally and the left hemidiaphragm was excised and transferred to a 5 ml 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 some experiments the KCl concentration of the Ringer–Krebs was raised to 10, 15 or 20 mM. To maintain the isotonicity, an equal amount of NaCl was removed from the incubation medium. Pertussis toxin (PTX), 2 μM ml^{-1}, was applied to the muscle superfusate for 12–14 h. All recordings were made at room temperature (20–23 °C) using conventional intracellular recording techniques.

Electrophysiological recordings

MEPPs were recorded at the end-plate region of the muscle fibers 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 less negative than −60 mV or MEPPs with a rise time greater than 1 ms were rejected. In each experimental group, the muscles were allowed to equilibrate in the respective solution for at least 20 min after observing that MEPPs represented a period of stable spontaneous release. MEPP frequency in each solution was recorded during 100 s from at least 10 different neuromuscular junctions and their values were averaged.

Data analysis

In results, figures represent mean±S.E.M. and *n* expresses number of animals (only left hemidiaphragm was used from each mouse for a given experiment). To overcome the problem of variability associated with spontaneous release of different muscles, in each experiment MEPP frequency was expressed as percentage of that obtained in the control Ringer solution of that muscle. Data frequencies were measured by hand from the screen of the oscilloscope or as MEPP amplitudes, acquired through an A/D converter controlled by computer and analyzed using WCP software (Dagan Corporation, Minneapolis, MN, USA). MEPP amplitudes were normalized to a resting membrane potential of −75 mV.

Statistical significance of differences between means was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered to be significant when $P<0.05$ (*).

Chemicals

ATP (100 μM), $\beta\gamma$-imido ATP (30 μM), CCPA (100 μM), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1 μM), 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino (4-methyl-3,1-phenylene) carbonylimino]]bis-1,3,5-aphthalenetrisulfonic acid hexasodium salt (suramin, 100 μM), 1-amino-4-[[4-[[4-chloro-6-[[3(or4)-sulfophenyl] amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl] amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid (reactive blue-2, 5 μM), pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (PPADS, 10 μM), PTX (2 μM ml^{-1}); α,β-methylene ADP sodium salt ($\alpha\beta$-MeADP, 100 μM), 2-methylthio-ADP (2-MeSADP, 150 nM); 2-methylthio-AMP triethylammonium salt (2-MeSAMP, 30 μM), nitrendipine (5 μM, used in a darkened room to prevent photo oxidation of the compound), *N*-ethylmaleimide (NEM, 10 μM), and N-(6-aminohexil)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, 10 μM) were purchased from Sigma, St. Louis, MO, USA. ω-Conotoxin GVIA (ω-CgTx, 5 μM), KT5720 (500 nM), and chelerythrine (5 μM) were purchased from Alomone Laboratories, Jerusalem, Israel. KN-62 (10 μM), 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U73122, 10 μM), and tricyclodecan-9-yl xanthate potassium salt (D-609, 100 μM) were purchased from Biomol International, Plymouth Meeting, PA, USA. N-[2-(methylthioethyl9]-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, Waltham, MA, USA.

Table 1. Effect of ATP and $\beta\gamma$-imido ATP on spontaneous acetylcholine release

Characteristic	Control	100 μM ATP	30 μM $\beta\gamma$-Imido ATP
Frequency (s^{-1})	1.05±0.02 (n=18)	0.60±0.02* (n=7)	0.44±0.01* (n=11)
Amplitude (mV)	1.08±0.04 (n=6)	1.15±0.14 (n=3)	1.20±0.08 (n=3)

Values are means±SEM.
* $P<0.05$.

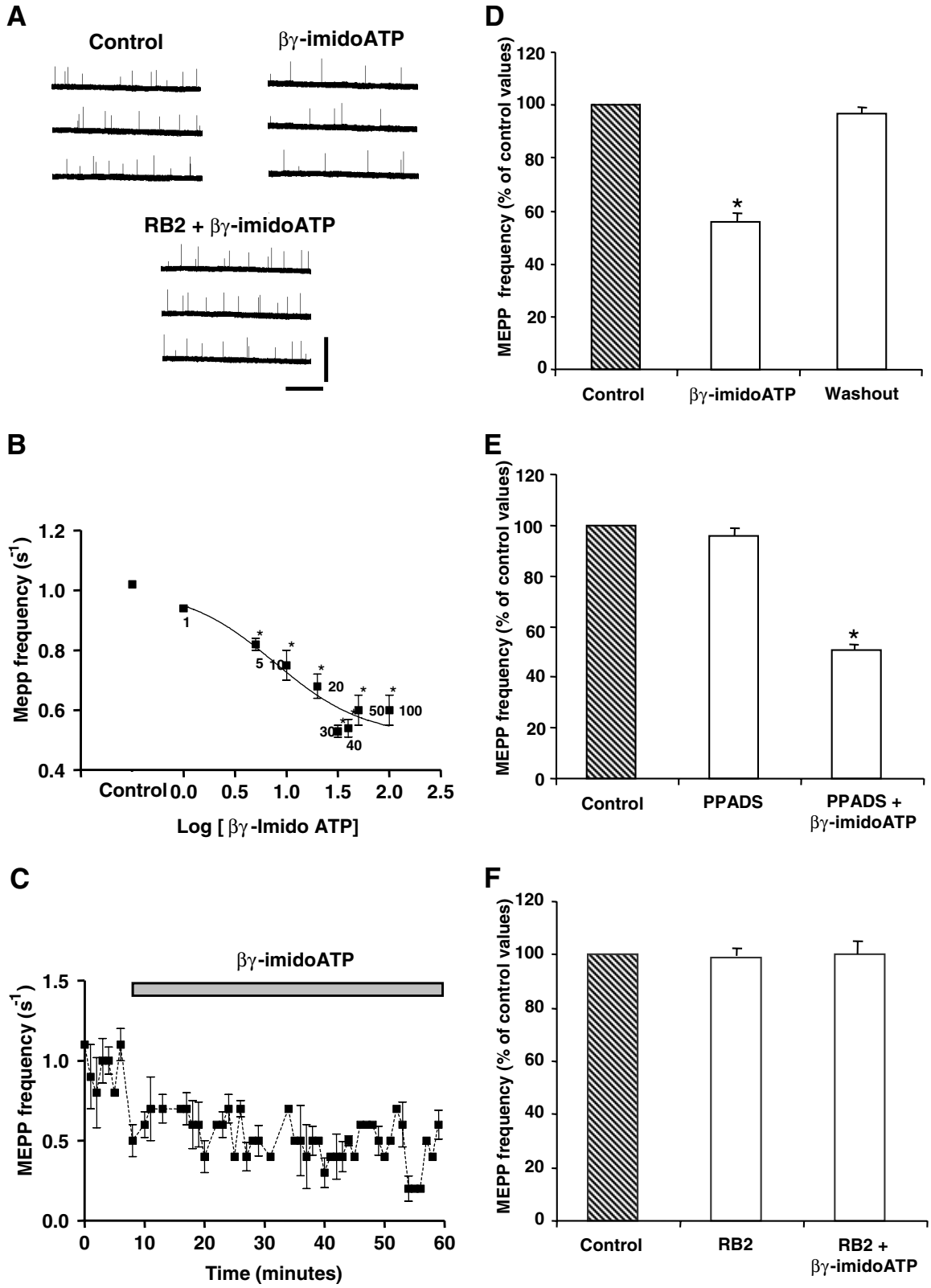

Fig. 2. Action of $\beta\gamma$-imido ATP on spontaneous ACh release. (A) MEPPs recorded from diaphragm muscle fibers bathed with control Ringer solution (Vm: −75.2 mV), with 30 μM $\beta\gamma$-imido ATP (Vm: −74.8 mV), and with 30 μM $\beta\gamma$-imido ATP in the presence of 5 μM reactive blue-2 (Vm: −74.1 mV). Calibration: 3 mV, 2.5 s. (B) Effect of $\beta\gamma$-imido ATP on MEPP frequency (s^{-1}) as a function of concentration. Control: 1.02±0.01 (n=6); 1 μM: 0.94±0.01 (n=6); 5 μM: 0.82±0.02 (n=6); 10 μM: 0.75±0.01 (n=6); 20 μM: 0.68±0.04 (n=6); 30 μM: 0.53±0.02 (n=6); 40 μM: 0.54±0.03 (n=6);

RESULTS

Effect of ATP on spontaneous acetylcholine release

Our first aim was to establish whether ATP exerts a modulatory effect via its own receptors (P2 receptors) on spontaneous ACh secretion at mice neuromuscular junction. ATP (100 μM: see dose-response curve at Fig. 1A, EC_{50} 9.41 μM) decreased MEPP frequency to 54.7±2.3% (n=7) of control values in a manner completely reversible on washout with ATP-free medium (Fig. 1C, Table 1), whereas MEPP amplitude was not affected by the nucleotide (Table 1). The time dependence of ATP-induced inhibition on MEPP frequency is shown in Fig. 1B. ATP reached its maximal effect approximately within 10–12 min after the application of the drug. In order to rule out an effect of ATP through its active metabolite adenosine, we studied the effect of ATP after the application of 0.1 μM DPCPX, a selective A_1 adenosine receptor antagonist. DPCPX did not modify MEPP frequency (98.6±5.0% of control values) and the addition of ATP to this solution induced the presynaptic effect (59.3±1.2%, n=7, Fig. 1D). In line with these experiments, we investigated the action of ATP in the presence of $\alpha\beta$-MeADP, which is an inhibitor of ecto-5′-nucleotidase, the enzyme that acts at the final step in the conversion of ATP to adenosine (Keller and Zimmermann, 1983; Redman and Silinsky, 1994; Magalhães-Cardoso et al., 2003). We found that $\alpha\beta$-MeADP (100 μM) did not affect the inhibitory action of ATP ($\alpha\beta$-MeADP 96.2±9.0%; $\alpha\beta$-MeADP+ATP 58.3±3.6%, n=4, Fig. 1E). To evaluate whether ATP induced presynaptic modulation via P2 receptors, the effect of ATP was investigated in the continuous presence of 100 μM suramin, a non-specific P2 receptor antagonist. This blocker abolished the presynaptic action of ATP, but did not interfere with the inhibitory effect of the specific adenosine A_1 receptor agonist CCPA (suramin 97.7±4.0% of control values; suramin+ATP 103.9±1.7%; suramin+ATP+CCPA 50.4±2.0%, n=4, Fig. 1F), confirming the antagonist selectivity. These results suggested that ATP has a direct effect on spontaneous ACh secretion by acting through its own receptors.

Then, we took advantage of an ATP analog, $\beta\gamma$-imido ATP, which is less susceptible to hydrolysis by ecto-ATPase (Cunha et al., 1998; Salgado et al., 2000). Fig. 2B shows the dose-response curve for the inhibitory effect of $\beta\gamma$-imido ATP: the maximal action was obtained at 30 μM (EC_{50} 7.71 μM). At this dose, $\beta\gamma$-imido ATP reversibly reduced MEPP frequency to 44.1±1.6% of control values (n=11, Fig. 2A and D and Table 1); this effect was evident since the beginning of the exposure to the drug (Fig. 2C). $\beta\gamma$-imido ATP did not exert significant alterations in MEPP amplitude (Table 1). To investigate the type of P2 receptor involved in the inhibition induced by $\beta\gamma$-imido ATP, various P2 antagonists were tested. We first analyzed the effect of PPADS, an antagonist with a preferential effect on P2X receptors, although it was also described its action on the $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{13}$ receptors (North and Barnard, 1997; North and Surprenant, 2000; Burnstock, 2001; Marteau et al., 2003; Kim et al., 2005). The presence of PPADS (10 μM) did not modify the $\beta\gamma$-imido ATP-induced inhibition of MEPP frequency (PPADS 95.9±2.7% of control values; PPADS+$\beta\gamma$-imido ATP 50.7±2.4%, n=4, Fig. 2E). Then, we tested the ability of the $P2Y_{4,6,11,12,13}$ receptor antagonist reactive blue-2 (5 μM, Ralevic and Burnstock, 1998; Burnstock, 2001; Marteau et al., 2003; Claes et al., 2004) to modify the effect of $\beta\gamma$-imido ATP. Reactive blue-2 did not change spontaneous secretion compared with control values (98.8±2.8%), but prevented the modulatory action of $\beta\gamma$-imido ATP (100.3±4.7%, n=8, Fig. 2A and F), suggesting that *in situ* ATP does not affect spontaneous ACh release and that the inhibition induced by $\beta\gamma$-imido ATP is mediated by P2Y receptors.

Effect of $\beta\gamma$-imido ATP on voltage-dependent calcium channels

One of the possible mechanisms by which ATP can exert its presynaptic inhibition on spontaneous ACh release would be a reduction of the Ca^{2+} influx through the L-type and N-type voltage-dependent Ca^{2+} channels (VDCC), which regulate tonic secretion at mammalian neuromuscular junction (Losavio and Muchnik, 1997). Thus, we first investigated the effect of $\beta\gamma$-imido ATP in the presence of 100 μM Cd^{2+}, a universal VDCC blocker. As shown in Fig. 3A, Cd^{2+} reduced MEPP frequency to 45.9±5.9 (n=3) of control values and the addition of $\beta\gamma$-imido ATP to this solution failed to reduce spontaneous secretion (46.1±4.1%). We next studied the action of $\beta\gamma$-imido ATP on nerve terminals previously incubated with the specific channels blockers (Fig. 3B). The L-type VDCC blocker, nitrendipine (5 μM), inhibited spontaneous secretion to 50.3±1.8% (n=3) of the control values. The addition of the N-type VDCC blocker ω-CgTx (5 μM) induced a further decrease of MEPP frequency reaching to 38.4±3.3% of the control values. Introduction of $\beta\gamma$-imido ATP to the solution containing both blockers did not significantly further change MEPP frequency (42.0±0.6%), suggesting that Ca^{2+} channels play a key role in the mechanism of action of $\beta\gamma$-imido ATP. Afterward, we analyzed which type/s of the Ca^{2+} channel/s was modulated by $\beta\gamma$-imido ATP. Fig. 3C depicted that 5 μM nitrendipine inhibited spontaneous secretion to 56.5±1.6% (n=7) of the control values. Application of $\beta\gamma$-imido ATP to that solution showed a slight but significant (P<0.05) decrement of MEPP frequency (48.3±3.0%). Similar results were obtained when we used the no dihydropyridine L-type VDCC antagonist calciseptine (300 nM, Urbano et al., 2001; Piriz et al., 2003; Andreasen et

50 μM: 0.60±0.05 (n=6); 100 μM: 0.60±0.05 (n=6); EC_{50}: 7.71 μM. (C) Temporal course of $\beta\gamma$-imido ATP inhibitory effect on MEPP frequency. $\beta\gamma$-Imido ATP (30 μM) was applied as indicated by the bar. Symbols are means±S.E.M. values of individual cells from a representative muscle. (D) Changes of MEPP frequency after 30 μM $\beta\gamma$-imido ATP application and after washout of the ATP analog. (E) PPADS (10 μM) failed to affect $\beta\gamma$-imido ATP-induced inhibition. (F) Reactive blue-2 (RB2, 5 μM) prevented the action of $\beta\gamma$-imido ATP. In D, E, and F data are expressed as percentage of control values (cross-hatched bar). Error bars indicate S.E.M. * P<0.05.

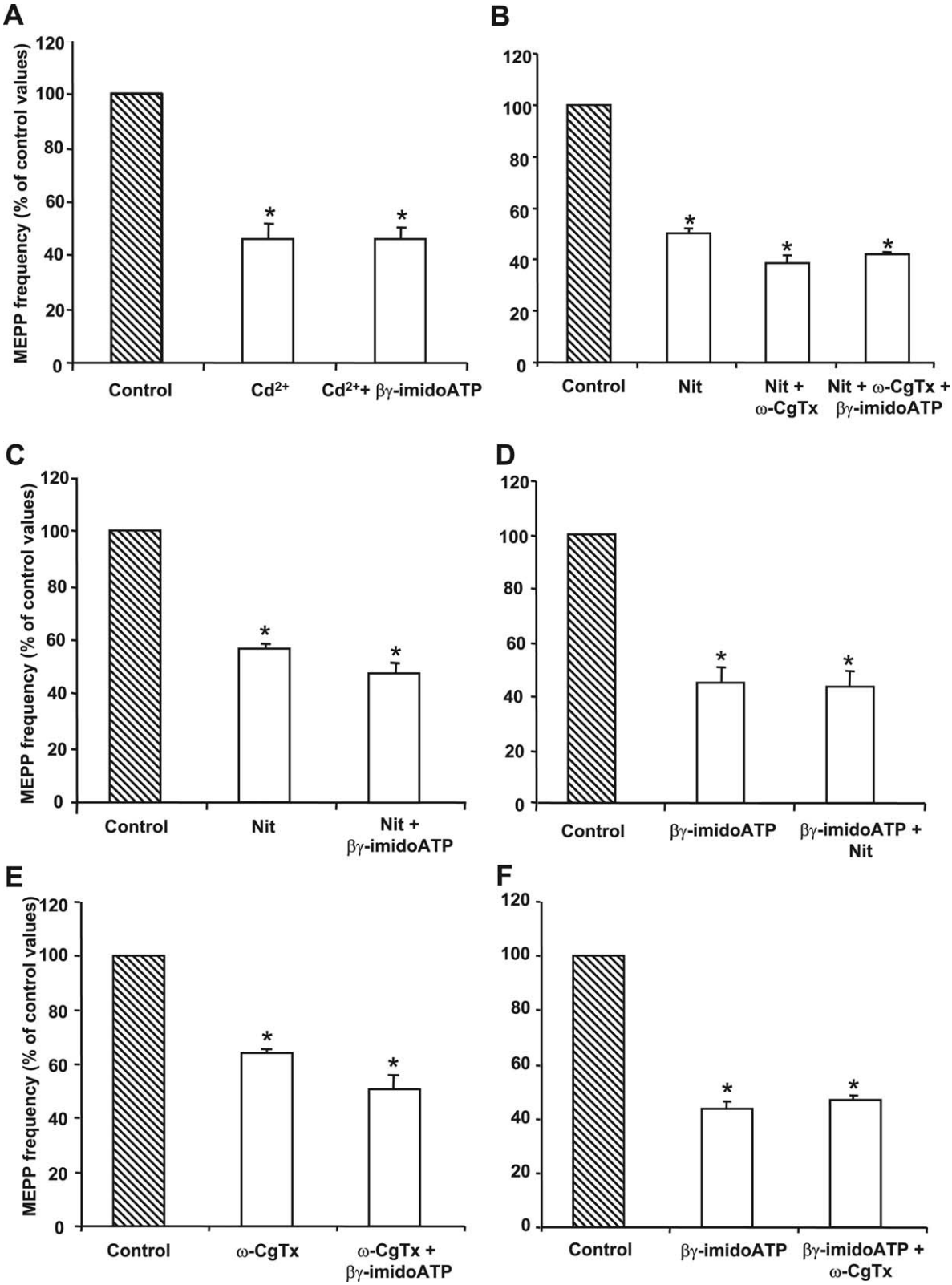

Fig. 3. Effect of $\beta\gamma$-imido ATP on VDCC related to spontaneous secretion. (A) The universal VDCC blocker Cd^{2+} (100 μM) diminished MEPP frequency and abolished the effect of $\beta\gamma$-imido ATP. (B) The combined application of 5 μM nitrendipine (Nit; L-type Ca^{2+} channel antagonist) and 5 μM ω-CgTx (N-type Ca^{2+} channel antagonist) also decreased MEPP frequency and prevented the inhibition induced by $\beta\gamma$-imido ATP. (C) Effect of $\beta\gamma$-imido ATP on spontaneous ACh secretion in the presence of 5 μM nitrendipine. $\beta\gamma$-Imido ATP induced a further reduction of MEPP frequency

al., 2006). This potent peptide inhibitor of L-type VDCC decreased MEPP frequency to 52.2±4.8% (n=4) of control values and application of βγ-imido ATP to the solution containing the antagonist, induced a further reduction of spontaneous secretion (39.8±4.8%, $P<0.05$). On reversing the order of administration, βγ-imido ATP induced a presynaptic inhibition to 44.4±5.5% (n=6) of control values, and the addition of nitrendipine did not exert any modification of these values (Fig. 3D). The same result was obtained when we used calciseptine after βγ-imido ATP (βγ-imido ATP 47.9±2.05% of control values, βγ-imido ATP+ calciseptine 52.1±1.1%, n=3). When preparations were bathed with ω-CgTx (5 μM), MEPP frequency decreased to 64.0±1.7% (n=4) of control values and the addition of βγ-imido ATP induced a further reduction ($P<0.05$) of spontaneous secretion (50.5±5.3%), implying that βγ-imido ATP was also modulating the L-type Ca^{2+} channels (Fig. 3E). The inversion of the sequence showed that βγ-imido ATP reduced MEPP frequency to 43.7±2.5% (n=4) and application of ω-CgTx did not alter these values (46.9±1.5%, Fig. 3F). Taken together, these results suggested that the activation of P2Y receptors by βγ-imido ATP led to a modulation of both Ca^{2+} channels related to spontaneous secretion.

Mechanism of action of βγ-imido ATP

Metabotropic P2Y receptors are coupled to second messenger pathways via either $G_{q/11}$ or $G_{i/o}$ proteins (Filippov et al., 1998; Ralevic and Burnstock, 1998; Torres et al., 2002). In order to establish whether the presynaptic inhibitory action of βγ-imido ATP in mice diaphragm muscles was due to the activation of P2Y receptors coupled to $G_{i/o}$ proteins, we investigated the effect of PTX (inactivates $G_{i/o}$ through ADP ribosylation, Gilman, 1984; Thomas and Hoffman, 1987; Watts, 2002) and NEM (uncouple G protein from several receptors, Wu et al., 1992; Shapiro et al., 1994; Yeon et al., 2004). Diaphragms incubated in PTX (2 μM ml^{-1}) for 12–14 h prevented the βγ-imido ATP-mediated reduction in MEPP frequency (12–14 h PTX 101.5±1.9% of control values; 12–14 h PTX+βγ-imido ATP 102.8±1.6%, n=4, Fig. 4A). In contrast, muscles bathed 12 h in normal solution without PTX, remained responsive to βγ-imido ATP (12 h Ringer–Krebs solution: 96.1±1.8% of control values, 12 h βγ-imido ATP 49.0±1.9% of control values, n=4, Fig. 4B). Like PTX, NEM (10 μM) also abolished the depressant effect of βγ-imido ATP (NEM 106.7+2.9% of control values; NEM+βγ-imido ATP 122.9±5.6%, n=4, Fig. 4C). These results indicate that the P2Y receptors involved in the inhibition of ACh release were $G_{i/o}$ linked.

In an attempt to determine which kinases are responsible for the modulation induced by βγ-imido ATP on MEPP frequency, we examined the effects of a number of pharmacological inhibitors in our system. To test the hypothesis that βγ-imido ATP decreases basal Ca^{2+} channels activity by inhibiting protein kinase A (PKA) pathway, via G_i protein, we examined if a specific PKA blocker such as KT5720 altered (i.e. mimicked or blocked) the effect of βγ-imido ATP on MEPP frequency. KT5720 (500 nM) did not modify MEPP frequency (101.9±3.0% of control values) and the application of βγ-imido ATP in the presence of the inhibitor still induced presynaptic inhibition (61.6±2.9% of control values, n=4, Fig. 5A). These results suggest that βγ-imido ATP-mediated modulation of Ca^{2+} channels does not occur by decreasing the basal activity of PKA.

Subsequently, we analyzed if activation of P2Y receptors coupled to a G_o protein could activate protein kinase C (PKC), leading in this case to a decrease of the activation of the VDCCs related to tonic secretion. As depicted in Fig. 5B, the specific PKC blocker chelerythrine (5 μM) completely prevented the inhibitory action of βγ-imido ATP (chelerythrine: 92.4±3.7% of control values; chelerythrine+βγ-imido ATP: 102.3±3.5%, n=5). Moreover, we obtained similar results inverting the sequence (βγ-imido ATP: 60.6±1.1% of control values; βγ-imido ATP+chelerythrine: 97.2±4.2%, n=5, Fig. 5C). These results suggested that PKC participates in the intracellular mechanisms activated by βγ-imido ATP.

We have recently found that in the mouse neuromuscular junction, the agonist of α1 receptors CCPA modulates MEPP frequency in a Ca^{2+}-calmodulin-dependent manner since presynaptic inhibition is eliminated by the calmodulin antagonist W-7 and EGTA-AM (De Lorenzo et al., 2004). To investigate the possibility that the above mechanism was also involved in the action of βγ-imido ATP, we examined the effect of the nucleotide in the presence of 50 μM W-7. As shown in Fig. 6A, W-7 did not modify MEPP frequency (96.2±2.4%, n=4), but occluded the effect of βγ-imido ATP (99.9±5.4%), indicating that calmodulin is also involved in P2Y signaling. Since calcium/calmodulin-dependent protein kinase II (CAMKII) may be associated with some ion channels (Hell et al., 1993; Dzhura et al., 2003), we analyzed whether KN-62, a specific inhibitor of the CAMKII, prevented the action of βγ-imido ATP. KN-62 (10 μM) neither modified significantly MEPP frequency when compared with control values (129.5±12.8%) nor occluded the effect of βγ-imido ATP (KN-62+βγ-imido ATP 64.6±1.6% of control values, n=3, Fig. 6B). These results suggested that the action of calmodulin is not associated to a phosphorylation induced by CAMKII.

Effect of βγ-imido ATP at different external K^+ concentrations

To explore the action of βγ-imido ATP on acetylcholine release triggered by depolarization, we studied its effect at

($P<0.05$) when applied after nitrendipine. (D) Lack of effect of nitrendipine on spontaneous ACh secretion in the presence of βγ-imido ATP. Nitrendipine effect was not observed when preparations were previously incubated with βγ-imido ATP. (E) Effect of βγ-imido ATP on spontaneous ACh secretion in the presence of 5 μM ω-CgTx. βγ-Imido ATP induced a further reduction of MEPP frequency ($P<0.05$) when applied after ω-CgTx. (F) ω-CgTx effect was not observed when preparations were previously incubated with βγ-imido ATP. Data are expressed as percentage of control values (cross-hatched bar). Error bars indicate S.E.M. * $P<0.05$.

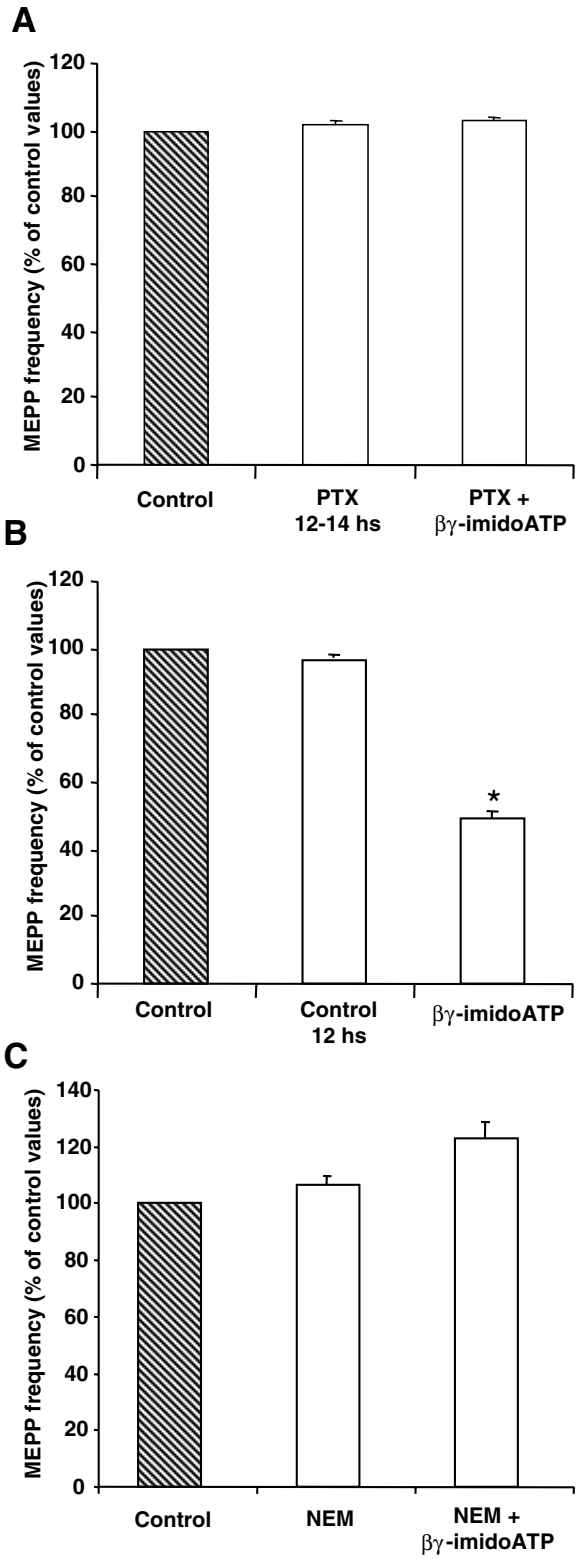

Fig. 4. Effect of PTX or NEM ($G_{i/o}$-protein blockers) on the depressant action induced by $\beta\gamma$-imido ATP. (A) The ATP analog did not inhibit MEPP frequency in muscles pre-treated for 12–14 h with 2 μM ml^{-1} PTX. (B) In corresponding control experiments, $\beta\gamma$-imido ATP reduced MEPP frequency after 12 h incubation in control saline not containing PTX. (C) NEM (10 μM) also prevented the action of $\beta\gamma$-imido ATP. Data are expressed as percentage of control values (cross-hatched bar). Error bars indicate S.E.M. * $P<0.05$.

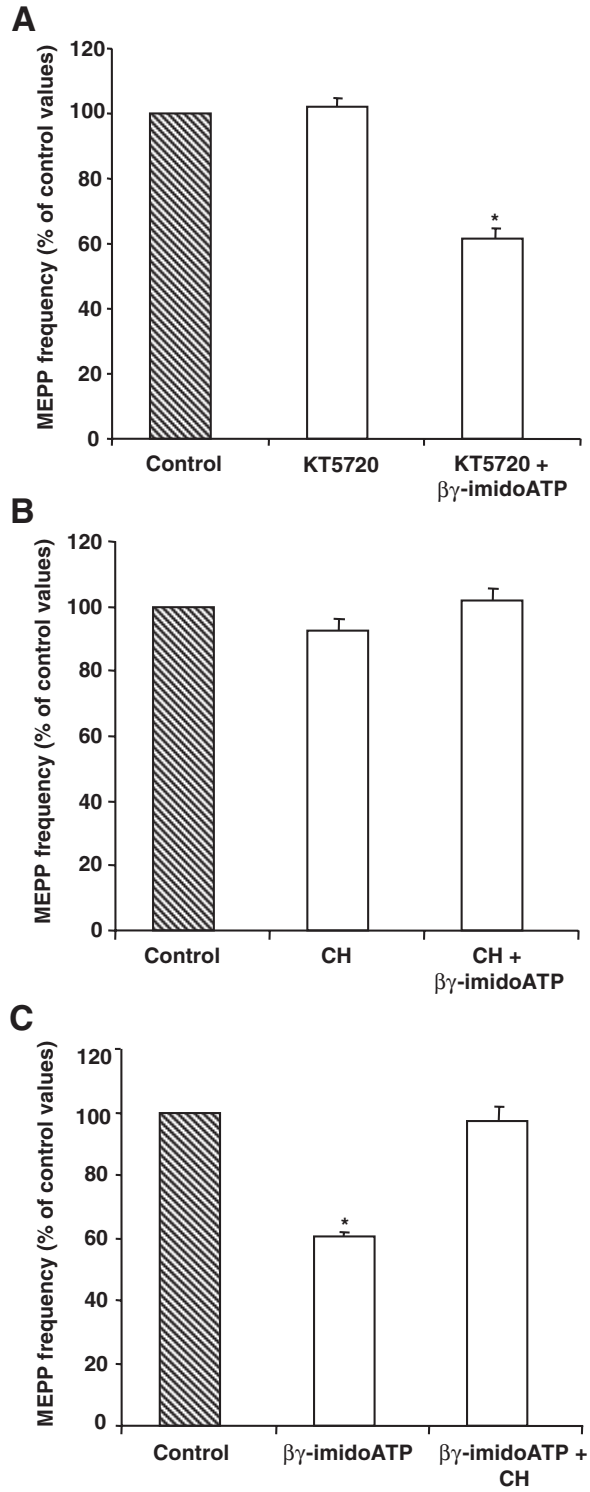

Fig. 5. Second messenger pathways related to PKC but not to PKA are involved in the $\beta\gamma$-imido ATP-induced presynaptic inhibition. (A) The specific PKA inhibitor KT5720 (500 nM) neither mimicked nor occluded the effect of $\beta\gamma$-imido ATP. Application of the specific PKC blocker chelerythrine (CH, 5 μM) before (B) or after (C) $\beta\gamma$-imido ATP, prevented the action of the nucleotide. Data are expressed as percentage of control values (cross-hatched bar). Error bars indicate S.E.M. * $P<0.05$.

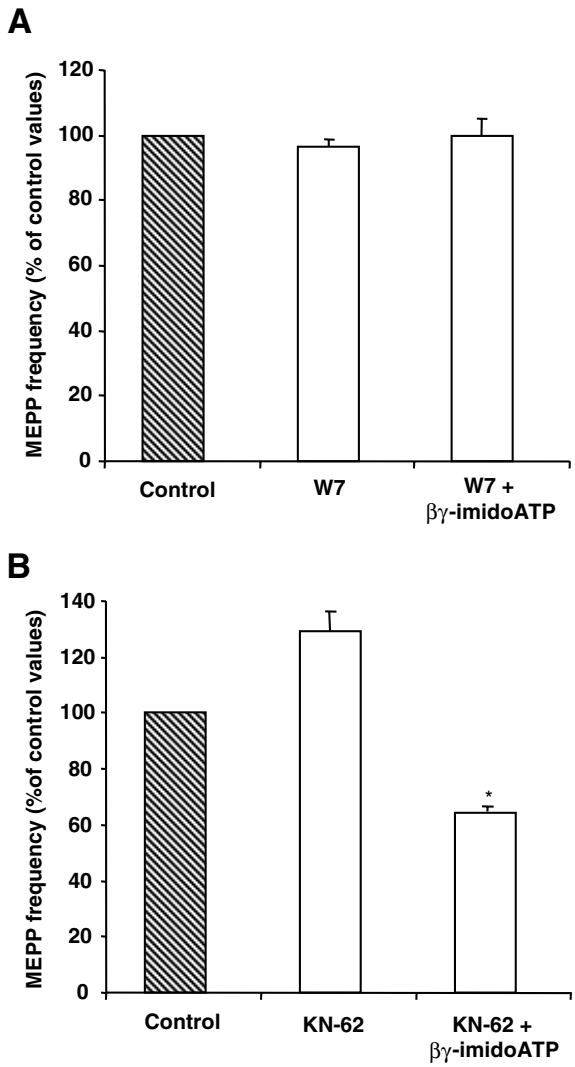

Fig. 6. Calmodulin is involved in the presynaptic inhibition induced by $\beta\gamma$-imido ATP. (A) W-7 (50 μM), an antagonist of calmodulin, did not alter MEPP frequency and prevented the effect of $\beta\gamma$-imido ATP on spontaneous ACh release. (B) Previous application of CaMKII inhibitor KN62 (10 μM) did not modify the presynaptic modulation induced by $\beta\gamma$-imido ATP. Data are expressed as percentage of control values (cross-hatched bar). Error bars indicate S.E.M. * $P<0.05$.

different external K^+ concentrations (10, 15 and 20 mM). Fig. 7A, B, C and D shows the increase of asynchronous ACh release when muscle fibers were exposed to 10, 15 and 20 mM K^+ (10 mM K^+ 365.5±11.2% of control values, $n=5$; 15 mM K^+ 976.7±67.7%, $n=6$; 20 mM K^+ 5108.9± 414.5%, $n=4$). Interestingly, the addition of 30 μM $\beta\gamma$-imido ATP to the solutions with high K^+ did not exert any modulation (10 mM K^++$\beta\gamma$-imido ATP 357.6±18.5% of control values; 15 mM K^++$\beta\gamma$-imido ATP 979.0±39.2%; 20 mM K^++$\beta\gamma$-imido ATP 5462.3±144.6%, Fig. 7A, B, C and D). In another group of experiments, to evaluate the possibility that the lack of effect of $\beta\gamma$-imido ATP upon asynchronous ACh secretion was due to the fact that presynaptic P2Y receptors were previously occupied by endogenous ATP, we applied 5 μM reactive blue-2 to the Ringer solution before the incubation in high K^+ concentration. Then we recorded MEPP frequency in 10, 15, and 20 mM K^+ and observed an increase to 408.9±25.5% ($n=4$), 2098.2±108.3% ($n=4$) and 7198.9±210.5% ($n=4$) of control values, respectively. These values were significantly higher than those recorded previously in 10 mM, 15 mM and 20 mM K^+ without the P2Y receptor antagonist (10 mM K^+ 305.6±29.7% of control values; 15 mM K^+ 1242.2±154.1%; 20 mM K^+ 4585.5±104.2%, $P<0.05$ in all cases, Fig. 8A and C), confirming the hypothesis that in high K^+ concentration, endogenous ATP modulates asynchronous neurotransmitter secretion by occupying P2Y receptors. In our previous paper, we demonstrated that, at 15 and 20 mM K^+, endogenous adenosine appears to occupy A_1 receptors, impairing the action of CCPA. Incubation with the A_1 receptor antagonist DPCPX and adenosine deaminase, which degrades adenosine into the inactive metabolite inosine, increased MEPP frequency compared with that obtained in 15 and 20 mM K^+ in the absence of the drugs. This behavior was not observed at moderate levels of K^+ (10 mM), where CCPA could exert its modulatory action (De Lorenzo et al., 2004). Thus, it resulted interesting to investigate the relative significance of P2Y and A_1 receptors activation on MEPP frequency, by exposing the preparations to both antagonist receptors. Fig. 8B shows the increase of asynchronous ACh release when muscles were incubated in 10, 15 and 20 mM K^+ in the presence of 0.1 μM DPCPX and 5 μM reactive blue-2 (10 mM K^+ 238.9±19.2% of control values, 10 mM K^++reactive blue-2+DPCPX 380.6±22.2%, $n=3$; 15 mM K^+ 1115.0±74.1%, 15 mM K^++reactive blue-2+DPCPX 2532.0±113.4%, $n=4$; 20 mM K^+ 4698.0±134.6%, 20 mM K^++reactive blue-2+DPCPX 9305.0±173.4%, $n=4$). MEPP frequency recorded with both antagonists was higher than that observed with the P2Y antagonist alone, except in 10 mM K^+ (see Fig. 8C). These results suggest that at high K^+ concentration, endogenous ATP as well as its metabolite adenosine modulates neurosecretion.

DISCUSSION

In this study, we demonstrated that, at the mouse neuromuscular junction, extracellular ATP induced presynaptic inhibition of spontaneous acetylcholine release via its own P2Y receptors, independently of the action of the metabolite adenosine. We found that ATP and the slowly hydrolysable ATP analog $\beta\gamma$-imido ATP decreased MEPP frequency by 45.3% and 55.9% respectively. Presynaptic modulation induced by ATP might be mediated by ionotropic P2X receptors or metabotropic P2Y receptors. The present results suggest that P2X receptors are not involved in the action of $\beta\gamma$-imido ATP, as P2X receptor blockade by PPADS failed to alter the inhibitory effect of the nucleotide. In contrast, P2Y receptors appear to mediate the effect of $\beta\gamma$-imido ATP since reactive blue-2 ($P2Y_{4,6,11,12,13}$, receptor antagonist) prevented the decrease of spontaneous secretion. Currently, eight different types of P2Y receptors have been identified ($P2Y_{1,2,4,6,11,12,13,}$ and $_{14}$; Ralevic and Burnstock, 1998;

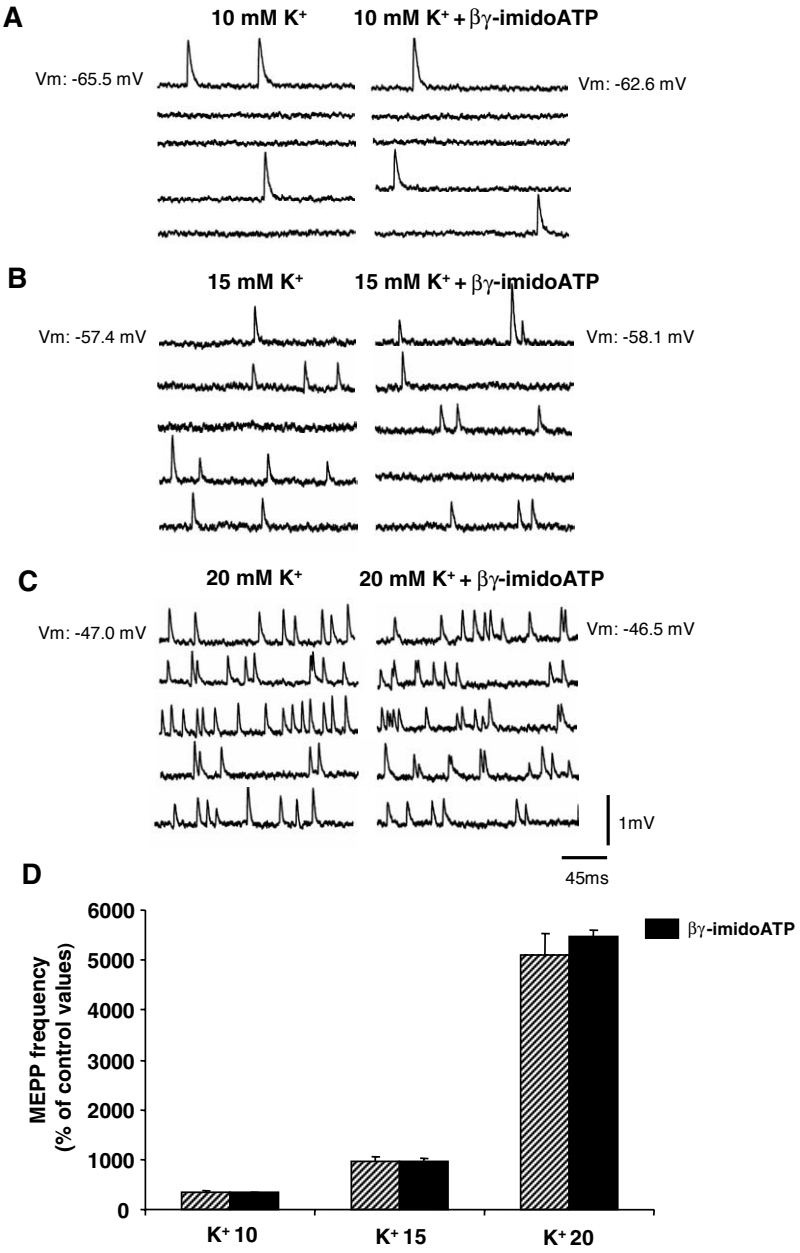

Fig. 7. $\beta\gamma$-Imido ATP failed to reduce asynchronous acetylcholine release induced by high K^+ concentration. (A–C) Representative MEPP traces recorded from diaphragm muscle fibers bathed with solutions containing 10, 15 and 20 mM K^+ respectively, in the absence and the presence of $\beta\gamma$-imido ATP. (D) Addition of $\beta\gamma$-imido ATP to high K^+ solutions did not affect K^+-evoked increase of MEPP frequency. Data are expressed as percentage of control values. Error bars indicate S.E.M.

Communi et al., 2001; Hollopeter et al., 2001; Zhang et al., 2001; Abbracchio et al., 2003). Among them, $P2Y_{1,2,4,6,11}$ couple to phospholipase C and mediate increase in inositol triphosphate via the $G_{q/11}$ proteins (Ralevic and Burnstock, 1998; Communi et al., 2001), except $P2Y_{11}$, which is also coupled to the cAMP pathway. On the other hand, $P2Y_{12,13,14}$ couple to $G_{i/o}$ proteins and mediate an inhibition of adenylyl cyclase (Communi et al., 2001; Hollopeter et al., 2001). Our results suggest that, as occurs in frog motor terminals (Sokolova et al., 2003), the P2Y receptors involved in the $\beta\gamma$-imido ATP-induced inhibition are coupled to $G_{i/o}$ proteins, since incubation with PTX or NEM, prevented the effect of the nucleotide. Therefore, one could speculate that the inhibitory effect may be mediated by $P2Y_{12}$ or $P2Y_{13}$ receptors, which act via $G_{i/o}$ protein (Simon et al., 2002; Fumagalli et al., 2004). In fact, in recent experiments, we found that 30 μM 2-MeSAMP and 1 μM AR-C69931MX, selective antagonists for $P2Y_{12}$ or $P2Y_{13}$ receptors (Takasaki et al., 2001; Marteau et al., 2003), abolished the action of 30 μM $\beta\gamma$-imido ATP and 150 nM 2-MeSADP, a preferential agonist for these types of receptors (S. De Lorenzo and A. Losavio, unpublished observations).

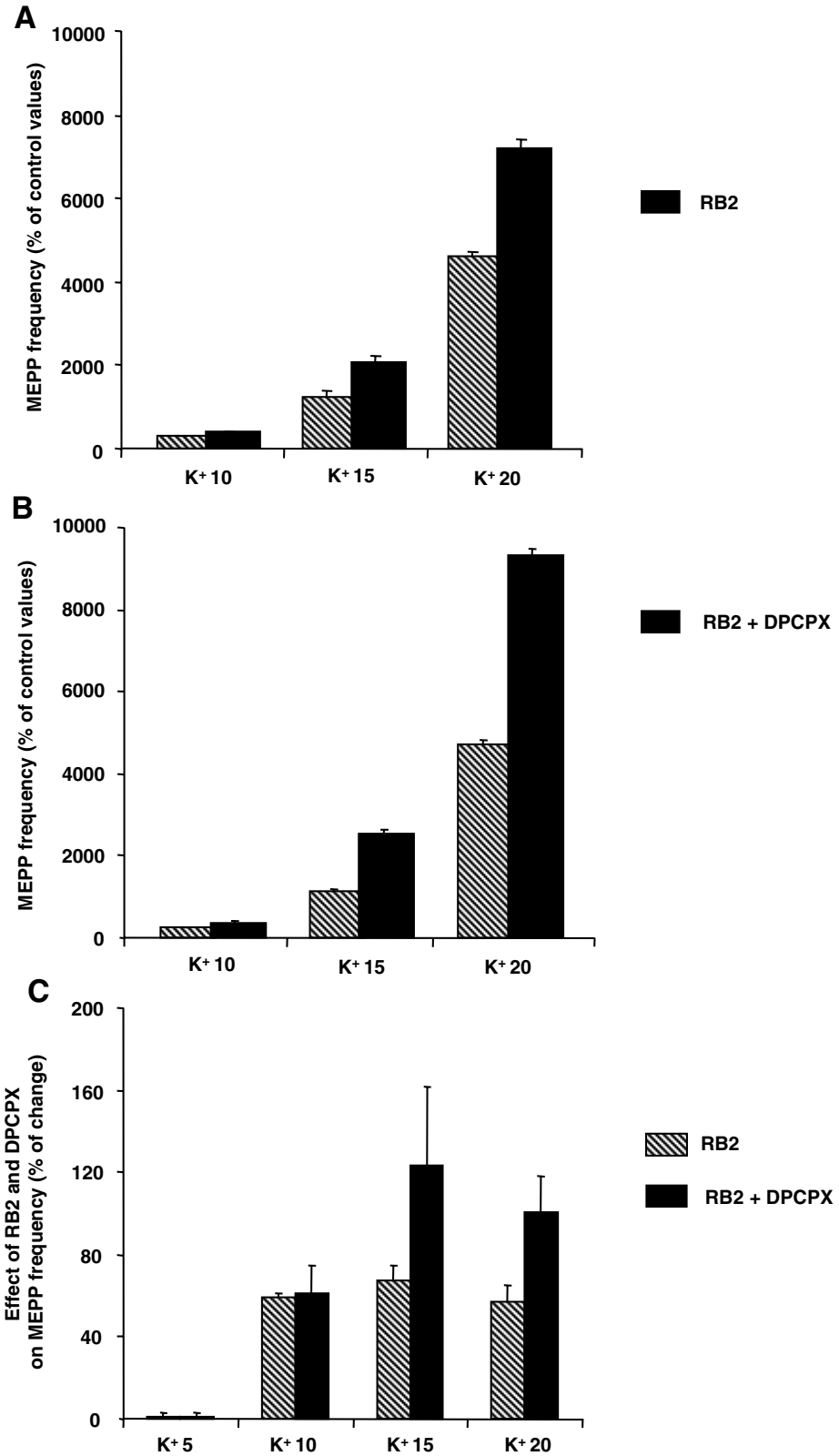

Fig. 8. (A) Effect of reactive blue-2 (RB2) on MEPP frequency induced by 10, 15 and 20 mM K^+. The blockade of P2Y receptors by RB2 (5 μM) caused an increase of MEPP frequency compared with that obtained before the application of RB2 in the same muscles. Data are expressed as percentage of MEPP frequency recorded in control Ringer solution (K^+ 5 mM). (B) Effect of RB2 (5 μM) and DPCPX on MEPP frequency induced by 10, 15 and 20 mM K^+. The blockade of P2Y and A_1 receptors induced a greater increase of MEPP frequency in 15 and 20 mM K^+. Data are expressed as percentage of MEPP frequency recorded in control Ringer solution (K^+ 5 mM). (C) Effect of RB2 and DPCPX on MEPP frequency at 5, 10, 15 and 20 mM K^+ expressed as percentage of change with respect to MEPP frequency obtained in 5, 10, 15 and 20 mM K^+ without the antagonist receptors. Error bars indicate S.E.M.

ATP-induced inhibition may be due to a reduced entry of calcium through the calcium channels related to spontaneous secretion and/or to an inhibition of the releasing machinery downstream of Ca^{2+} influx to the nerve terminal. Our results provide evidence that the action of ATP is mediated by modulation of Ca^{2+} channels, since Cd^{2+} or the combined application of nitrendipine and ω-CgTx, occluded completely the effect of the nucleotide. Both components of MEPP frequency, nitrendipine-sensitive and ω-CgTx-sensitive seem to be involved in the $\beta\gamma$-imido ATP-mediated inhibition (see Fig. 3). A concomitant effect of ATP on the neurotransmitter-releasing machinery could not be ruled out. At the frog neuromuscular junction, Grishin et al. (2005) demonstrated that the presynaptic depressant action of ATP is mediated by inhibition of Ca^{2+} currents and by mechanism acting downstream of Ca^{2+} entry.

Two major types of signaling pathways have been identified in the G protein-dependent modulation of Ca^{2+} channels: pathways involving the synthesis of diffusible second messengers via α subunits of G protein, and a membrane-delimited pathway based on a direct interaction between G protein $\beta\gamma$-subunits and VDCCs. Activation of G_i proteins by ATP might cause an inhibition of adenylate cyclase–cAMP signal–transduction pathway, leading to an inactivation of the Ca^{2+} channels. However, in our experiments this mechanism seems not to be involved since the specific PKA blocker KT5720, neither mimicked nor occluded the action of $\beta\gamma$-imido ATP. Afterward, we tested the possibility that activation of P2Y receptors, via G_o proteins, might activate PKC, leading in this case to a decrease of Ca^{2+} current, as it was observed at frog neuromuscular junction (Sokolova et al., 2003). The application of chelerytrine, a specific PKC inhibitor, completely eliminated the effect of $\beta\gamma$-imido ATP, suggesting that PKC participates in the intracellular mechanisms activated by G_o protein coupled to P2Y receptors. In an attempt to elucidate some pathways that could activate PKC, we studied whether phosphatidylinositol specific phospholipase C (PI-PLC) and/or phosphatidylcholine specific phospholipase C (PC-PLC) were involved in $\beta\gamma$-imido ATP-mediated modulation of Ca^{2+} channels. The application of U73122 (10 μM), a specific inhibitor of PI-PLC (Davletov et al., 1998; Tsang et al., 2000), was not effective in reducing the ability of $\beta\gamma$-imido ATP to induce presynaptic inhibition of MEPP frequency (data not shown), indicating that activation of PKC is not mediated by DAG generated by PI-PLC. Unfortunately, we could not reach to any conclusion regarding the participation of PC-PLC, since studies with the specific inhibitor of PC-PLC, D-609 100 μM (Neary et al., 1999; Sokolova et al., 2003), depicted a drastic increase of MEPP frequency due to depolarization of membrane potential (muscle membrane potential $-18/-20$ mV), making very difficult the analysis of the effect of $\beta\gamma$-imido ATP in the presence of the drug.

Another alternative is that Ca^{2+} channels may be inactivated through G protein $\beta\gamma$ subunit via a membrane-delimited pathway independently of soluble intracellular messenger (Hille, 1994; Herlitze et al., 1996; Ikeda, 1996; Dolphin, 1998). Ivanina et al. (2000), have demonstrated that at basal cellular levels of Ca^{2+}, G protein $\beta\gamma$ subunits have an inhibitory effect on L-type Ca^{2+} channels in a voltage-independent but calmodulin-dependent manner. In our previous paper, we showed that CCPA inhibits spontaneous ACh secretion by a mechanism that involves Ca^{2+}-calmodulin. Here, we suggest that this mechanism is also involved in the $\beta\gamma$-imido ATP-mediated modulation, since treatment of preparations with the calmodulin antagonist W-7 prevented the effect of the nucleotide. In addition to binding directly to Ca^{2+} channels, calmodulin can interact with CaMKII. However, evidence presented here indicates that application of CaMKII inhibitor KN62 did not modify the presynaptic modulation induced by $\beta\gamma$-imido ATP. Further investigations are needed to clarify the types of signaling pathways involved in the activation of P2Y receptors and their relationship with the inactivation of L-type and N-type Ca^{2+} channels.

In order to study the effect of $\beta\gamma$-imido ATP on different degrees of depolarization of nerve terminals, we investigated its action at different external K^+ concentrations (10, 15 and 20 mM). We found that at moderate levels of K^+ (10 mM K^+) as well as at high levels of K^+ (15 and 20 mM), 30 μM $\beta\gamma$-imido ATP failed to exert any inhibitory effect. At the same muscles, Hong and Chang (1998) showed that application of puffed ATP or α,β-methylene ATP at high concentrations (3 μl, 100 mM) elicited a transient reduction of MEPP frequency in preparation exposed to 17 mM K^+. These apparently contradictory results may be attributed to different concentrations and experimental protocols. The lack of effect of the ATP analog at high K^+ concentration may be due to the extracellular accumulation of ATP in the synaptic cleft as result of the excessive neurotransmitter release induced by K^+ (endogenous ATP). This ATP would occupy the presynaptic P2Y receptors, impairing the effect of $\beta\gamma$-imido ATP. Functional consequences of such a mechanism were evaluated by investigating the effect of reactive blue-2, the blocker of P2Y receptors, on asynchronous neurotransmitter release induced by K^+. Reactive blue-2 applied to high K^+ solutions induced an increase of MEPP frequency of 59%, 67% and 57% when compared with values obtained at 10, 15 and 20 mM K^+ without the antagonist, respectively, confirming the assumption that at high K^+ concentration, endogenous ATP exerts an inhibitory tonus via the presynaptic P2Y receptors. Besides its antagonizing effect on P2Y receptors, reactive blue-2, at higher concentrations, inhibits ecto-ATPase and ecto-ATP diphosphohydrolase degradation of P2-receptor agonists (Chen et al., 1996; Heine et al., 1999; Grobben et al., 2000). In our experiments, the action on the ecto-enzymes appears to be ruled out, since it would result in a potentiation of the ATP-mediated inhibition rather than an increase of the asynchronous neurotransmitter release. In our previous paper, we demonstrated that endogenous AD, coming mainly from the hydrolysis of released ATP, appears to occupy all A_1 receptors when preparations are bathed with high levels of K^+ (15 and 20 mM). This behavior was not observed at moderate levels of K^+ (10 mM), since in this case CCPA was able to attenuate asynchronous ACh secretion by modulating P/Q- type

VDCC (De Lorenzo et al., 2004). The fact that endogenous ATP occluded the effect of $\beta\gamma$-imido ATP since 10 mM K^+ might suggest that most of the ATP released during the exposure at 10 mM K^+ bound to P2Y receptors and that the AD coming from ATP was not sufficient to occupy all A_1 receptors and/or it was taken from the synaptic space by the adenosine transporters (Correia-de-Sá et al., 1996). On the contrary, at 15 and 20 mM K^+ the amount of ATP released is such that the nucleotide not only occupied its own receptors but it also broke down to AD occupying all A_1 receptors. This hypothesis was challenge by exposing the preparations to P2Y and A_1 antagonist receptors. At 10 mM K^+ the increase of MEPP frequency observed with both antagonists was not different from that observed with the P2Y antagonist alone. This result was coherent, taking into account that at this K^+ concentration, A_1 receptors appear not to be occupied by endogenous adenosine. On the contrary, at 15 and 20 mM K^+, the increase of asynchronous secretion was higher with both antagonists, suggesting that both, P2Y and A_1 receptors are being activated by the endogenous ATP and its metabolite adenosine, respectively (see Fig. 8C).

CONCLUSION

In summary, our results are consistent with the hypothesis that, at mammalian neuromuscular junctions, ATP induces presynaptic inhibition of spontaneous ACh release due to the modulation of the nitrendipine- and ω-CgTx-sensitive component of MEPP frequency by activation of P2Y receptors and independently of the inhibitory effect of its active metabolite adenosine. The modulatory effect of ATP upon Ca^{2+} channels depends on the relative contribution of many pathways (we found that PKC and calmodulin, but not PKA and CaMKII are involved), but certainly this point needs further investigation. On the other hand, we demonstrated that when spontaneous secretion is increased by exposing nerve terminals to high K^+ concentrations, endogenously released ATP induced presynaptic inhibition through the activation of P2Y receptors as a means of preventing excessive neurotransmitter secretion, confirming the modulatory role of the nucleotide.

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