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RESEARCH ARTICLE

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Endogenous purines modulate K^+-evoked ACh secretion at the mouse neuromuscular junction

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Abstract

At the mouse neuromuscular junction, adenosine triphosphate (ATP) is co-released with the neurotransmitter acetylcholine (ACh), and once in the synaptic cleft, it is hydrolyzed to adenosine. Both ATP/adenosine diphosphate (ADP) and adenosine modulate ACh secretion by activating presynaptic $P2Y_{13}$ and A_1, A_{2A}, and A_3 receptors, respectively. To elucidate the action of endogenous purines on K^+-dependent ACh release, we studied the effect of purinergic receptor antagonists on miniature end-plate potential (MEPP) frequency in phrenic diaphragm preparations. At 10 mM K^+, the $P2Y_{13}$ antagonist N-[2-(methylthio)ethyl]-2-[3,3,3-trifluoropropyl]thio-5′-adenylic acid, monoanhydride with (dichloromethylene)bis[phosphonic acid], tetrasodium salt (AR-C69931MX) increased asynchronous ACh secretion while the A_1, A_3, and A_{2A} antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), (3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1, 4-(±)-dihydropyridine-3,5-, dicarboxylate (MRS-1191), and 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH-58261) did not modify neurosecretion. The inhibition of equilibrative adenosine transporters by S-(p-nitrobenzyl)-6-thioinosine provoked a reduction of 10 mM K^+-evoked ACh release, suggesting that the adenosine generated from ATP is being removed from the synaptic space by the transporters. At 15 and 20 mM K^+, endogenous ATP/ADP and adenosine bind to inhibitory $P2Y_{13}$ and A_1 and A_3 receptors since AR-C69931MX, DPCPX, and MRS-1191 increased MEPP frequency. Similar results were obtained when the generation of adenosine was prevented by using the ecto-5′-nucleotidase inhibitor α,β-methyleneadenosine 5′-diphosphate sodium salt. SCH-58261 only reduced neurosecretion at 20 mM K^+, suggesting that more adenosine is needed to activate excitatory A_{2A} receptors. At high K^+ concentration, the equilibrative transporters appear to be saturated allowing the accumulation of adenosine in the synaptic cleft. In conclusion, when motor nerve terminals are depolarized by increasing K^+ concentrations, the ATP/ADP and adenosine endogenously generated are able to modulate ACh secretion by sequential activation of different purinergic receptors.

KEYWORDS
adenosine, ATP/ADP, K^+ depolarization, purinergic receptors

Abbreviations: ACh, acetylcholine; AR-C69931MX, *N*-[2-(methylthio)ethyl]-2-[3,3,3-trifluoropropyl]thio-5′-adenylic acid, monoanhydride with (dichloromethylene) bis[phosphonic acid], tetrasodium salt; ADP, adenosine diphosphate; ANOVA, analysis of variance; ATP, adenosine triphosphate; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS-21680, 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl] amino] ethyl]benzenepropanoic acid hydrochloride; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EPP, end-plate potential; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; αβ-MeADP, α,β-methyleneadenosine 5′-diphosphate sodium salt; MEPP, miniature end-plate potential; 2-MeSADP, 2-methylthioadenosine 5′-diphosphate trisodium salt hydrate; MRS-1191, ; (3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1, 4-(±)-dihydropyridine-3,5-, dicarboxylate; NBTI, S-(p-nitrobenzyl)-6-thioinosine; SCH-58261, 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine; *SEM*, standard error of mean; VDCC, voltage-dependent calcium channel.

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Significance

At the mammalian neuromuscular junction, adenosine triphosphate (ATP) is co-released with the neurotransmitter acetylcholine (ACh), and once in the synaptic cleft, it is hydrolyzed to adenosine. We report that when motor nerve terminals are depolarized by increasing $[K^+]_o$, there is a sequence of activation of presynaptic purinergic receptors that are able to modulate neurosecretion. ATP/adenosine diphosphate binds $P2Y_{13}$ receptors at 10 to 20 mM K^+, reducing ACh secretion. Adenosine inhibits neurosecretion at 15 to 20 mM K^+ by activation of A_1 and A_3 receptors and facilitates it via A_{2A} receptors at 20 mM K^+. Equilibrative nucleoside transporters only achieve removal of adenosine from the synaptic space at 10 mM K^+.

1 | INTRODUCTION

Extracellular purines regulate synaptic transmission through their own receptors and the steps involved in the process of exocytosis. At the mammalian neuromuscular junction, adenosine triphosphate (ATP) is co-released with the neurotransmitter acetylcholine (ACh), and once in the synaptic cleft, it is hydrolyzed to adenosine via the ectonucleotidase cascade (Meriney & Grinnell, 1991; Redman & Silinsky, 1994; Ribeiro & Sebastião, 1987). Thus, the level of adenosine at the synaptic space is directly proportional to synaptic activity (Cunha & Sebastião, 1993; Silinsky & Redman, 1996), although it also depends on the action of equilibrative nucleoside transporters that carry nucleosides across cell membranes in either direction following their concentration gradient (reviewed in Kong, Engel, & Wang, 2004). On the other hand, purines may also be released from activated muscle fibers (Santos, Salgado, & Cunha, 2003; Smith, 1991) and from perisynaptic Schwann cells (Liu, Werry, & Bennett, 2005; discussed in Todd & Robitaille, 2006).

It was demonstrated that both purines, ATP and adenosine, modulate neurotransmitter release operating via presynaptic P2 and P1 receptors, respectively (De Lorenzo, Veggeti, Muchnik, & Losavio, 2004, 2006; Giniatullin & Sokolova, 1998; Sebastião & Ribeiro, 2000; Sokolova, Grishin, Shakirzyanova, Talantova, & Giniatullin, 2003). In previous reports performed in mouse neuromuscular junctions, we have found that at basal conditions (K^+ 5 mM), ATP and adenosine regulate ACh secretion by activating presynaptic P2Y receptors (De Lorenzo et al, 2006; Guarracino, Cinalli, Fernández, Roquel, & Losavio, 2016; Veggetti, Muchnik, & Losavio, 2008) and A_1 and A_{2A} receptors (De Lorenzo et al., 2004; Palma, Muchnik, & Losavio, 2011), respectively. Moreover, we have recently demonstrated, by pharmacological and immunohistochemical assays, that A_3 receptors are also present at the motor nerve terminals and that these receptors may be activated by adenosine and its metabolite inosine (Cinalli, Guarracino, Fernandez, Roquel, & Losavio, 2013). However, in preparations depolarized by 15 mM K^+, exogenous P2Y, A_1, and A_3 agonists failed to exert any modulatory effect on neurosecretion, while the activation of A_{2A} receptors induced the typical facilitatory action. One probable explanation for these findings is that the sustained depolarization of the presynaptic membrane induced by high K^+ provokes a greater secretion of ACh and ATP and, therefore, a major concentration of nucleotides and generation of endogenous nucleosides in the synaptic cleft. These purines might occupy P2Y, A_1, and A_3 receptors, impairing the action of the exogenous agonists, whereas it is possible that higher levels of synaptic adenosine would be necessary to occupy A_{2A} receptors and occlude the action of the A_{2A} agonist.

The purpose of this work was to summarize evidence on the above hypothesis and to analyze to what degree the metabolism of ATP and the action of the equilibrative nucleoside transporters contribute to the concentration of endogenous nucleosides in the synaptic cleft during asynchronous cholinergic secretion.

2 | METHODS

2.1 | Preparations and solutions

Electrophysiological recordings were performed on phrenic nerve diaphragm preparations taken from adult CF1 mice ($n = 133$; 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 publication 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) intraperitoneally, and left hemidiaphragms were excised and pinned in a 5-ml recording chamber superfused (3 ml/min) 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). When KCl concentration of the Krebs–Ringer solution was raised to 10–20 mM, an equal amount of NaCl was removed from the incubation medium to maintain the isotonicity. Experiments were carried out at room temperature (22 °C–23 °C).

2.2 | Electrophysiological recordings

Miniature end-plate potentials (MEPPs) were recorded at the end-plate region from muscle fibers using borosilicate glass microelectrodes (WP Instruments, Sarasota, FL) with a resistance of 5 to 10 MΩ filled with 3 M KCl. Muscle fibers with a resting membrane potential (Vm) less negative than −60 mV (control solution) or MEPPs with a rise time greater than 1 ms were rejected. We performed individual experiments for each drug (agonist/antagonist for $P2Y_{13}$, A_1, A_3, and A_{2A} receptors, nucleoside transporter blocker, or ecto-5′-nucleotidase inhibitor) and K^+ concentration (10, 15, or 20 mM K^+) used. Typically, in each experiment, MEPP frequency was measured at control solution (5 mM K^+, 10 fibers), high K^+ solution (10, 15, or 20 mM K^+, 10 fibers), control solution (washing for 20 min, 3–4 fibers, not shown in figures), control solution + drug (10 fibers), and high K^+ solution + drug (10 fibers). In each experimental group, before recordings were made, muscle fibers were equilibrated for at least 20 min in the drug solution and for 5 to 7 min in the high K^+ solution. In each fiber, MEPP frequency was recorded during 100 s. All signals were amplified with Axoclamp 2A (Molecular

Devices, Sunnyvale, CA) and digitized with Digidata 1322 (Molecular Devices). Responses were recorded and analyzed using pClamp 8.2 software (Molecular Devices).

2.3 | Data analysis

In all cases, data are reported as mean ± *SEM*, and *n* expresses the number of animals. The distribution of the data in each experiment was tested for normality using Shapiro–Wilk test. Statistical comparisons among 3 or more groups were performed using 1-way analysis of variance followed by Tukey (to compare all pairs of columns) or Dunnett posttest (to compare all other columns vs. control column). Differences were considered to be significant when $p < .05$.

2.4 | Chemicals

2-chloro-N^6-cyclopentyl-adenosine (CCPA, 500 nM), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1 μM), 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1, 4-(±)-dihydropyridine-3,5-, dicarboxylate (MRS-1191, 5 μM), inosine (100 μM), α,β-methyleneadenosine 5′-diphosphate sodium salt (αβ-MeADP, 100 μM), 2-methylthioadenosine 5′-diphosphate trisodium salt hydrate (2-MeSADP, 150 nM), and S-(p-nitrobenzyl)-6-thioinosine (NBTI, 30 μM) were purchased from Sigma-Aldrich Corp. (St. Louis, MO); 4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl] amino] ethyl]benzenepropanoic acid hydrochloride (CGS-21680, 5 nM) and 2-(2-furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH-58261, 50 nM) were obtained from Tocris Bioscience (Ellisville, MO); and *N*-[2-(methylthio)ethyl]-2-[3,3,3-trifluoropropyl]thio-5′-adenylic acid, monoanhydride with (dichloromethylene)bis[phosphonic acid], tetrasodium salt (AR-C69931MX, 1 μM) was kindly provided by the Medicines Company (Parsippany, NJ). All other reagents were of the highest purity available. CCPA, DPCPX, MRS-1191, NBTI, CGS-21680, and SCH-58261 were made up in dimethyl sulfoxide, and inosine, αβ-MeADP, 2-MeSADP, and AR-C69931MX were made up in pure water. All stock solutions were aliquoted and frozen at −20 °C. Aqueous dilutions of these stock solutions were made daily, and appropriate solvent controls were done.

3 | RESULTS

3.1 | Effect of increasing K^+ concentration on MEPP frequency

When phrenic diaphragm preparations are depolarized by exposing them to high K^+ concentrations, an increase is observed in ACh release (MEPP frequency) that depends on Ca^{2+} influx through P/Q-type voltage-dependent calcium channels (VDCCs; Losavio & Muchnik, 1997; Protti & Uchitel, 1993). Indeed, depolarizations by 10, 15, and 20 mM K^+ (Figure 1) result in a monoexponential elevation of MEPP frequency (MEPP/s: 5 mM K^+ [control solution] 1.06 ± 0.05, $n = 10$; 10 mM K^+ 3.41 ± 0.25, $n = 10$; 15 mM K^+ 10.59 ± 0.41, $n = 10$, $p < .001$; 20 mM K^+ 54.69 ± 2.88, $n = 10$, $p < .001$) that returns to control values after washout of the muscles.

3.2 | Activation of P2Y receptors by endogenous ATP/adenosine diphosphate when ACh secretion is evoked by high K^+ concentrations

In a previous report, we found that contrary to what happens in control Ringer solution (5 mM K^+), the slowly hydrolysable ATP analog βγ-imido ATP did not affect neurotransmitter secretion induced by 10, 15, and 20 mM K^+ (De Lorenzo et al., 2006). Since we have demonstrated that the P2Y receptors involved in cholinergic modulation are of the subtype $P2Y_{13}$ (Guarracino et al., 2016), we performed those experiments with the preferential agonist for these receptors, 2-MeSADP (150 nM). We found that, like βγ-imido ATP, 2-MeSADP did not exert any inhibitory effect on MEPP frequency when preparations were exposed to 10, 15, and 20 mM K^+ (Table 1, Figure 2a,b).

The lack of effect of 2-MeSADP at 10 to 20 mM K^+ may be due to the occupation of $P2Y_{13}$ receptors by endogenous ATP/adenosine diphosphate (ADP). To bring out the effect of such adenine nucleotides at high K^+ concentrations, we incubated the preparations with an antagonist for $P2Y_{13}$ receptors. Thus, we found that AR-C69931MX (1 μM; Fumagalli et al., 2004; Marteau et al., 2003; Takasaki et al., 2001) did not affect spontaneous secretion at control solution, but induced an increase in MEPP frequency at 10, 15, and 20 mM K^+ (10 mM K^+ 253.0% ± 17.0% of control values, 10 mM K^+ + AR-C69931MX 315.1% ± 18.8%, $n = 4$, $p < .05$; 15 mM K^+ 968.9% ± 52.0%, 15 mM K^+ + AR-C69931MX 1,428.0% ± 58.5%, $n = 4$, $p < .001$; 20 mM K^+ 4,399.0% ± 225.2%, 20 mM K^+ + AR-C69931MX 6,655.0% ± 525.1%, $n = 4$, $p < .001$, Figure 2c–f).

3.3 | Activation of P1 receptors by endogenous adenosine when ACh secretion is evoked by high K^+ concentrations

Adenosine is a key modulator of neuromuscular transmission, regulating ACh secretion by acting on inhibitory (A_1, A_3) and excitatory (A_{2A})

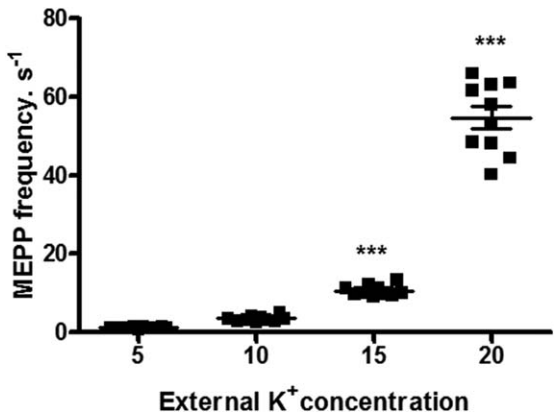

FIGURE 1 ACh secretion evoked by increasing K^+ concentrations. Data (mean ± *SEM*) express MEPP frequency per second recorded at control solution (5 mM K^+, $n = 10$) and at 10 ($n = 10$), 15 ($n = 10$), and 20 mM K^+ ($n = 10$). The increase in MEPP frequency follows a monoexponential curve ($R^2 = 0.999$). ***$p < .001$ (ANOVA followed by Dunnett test)

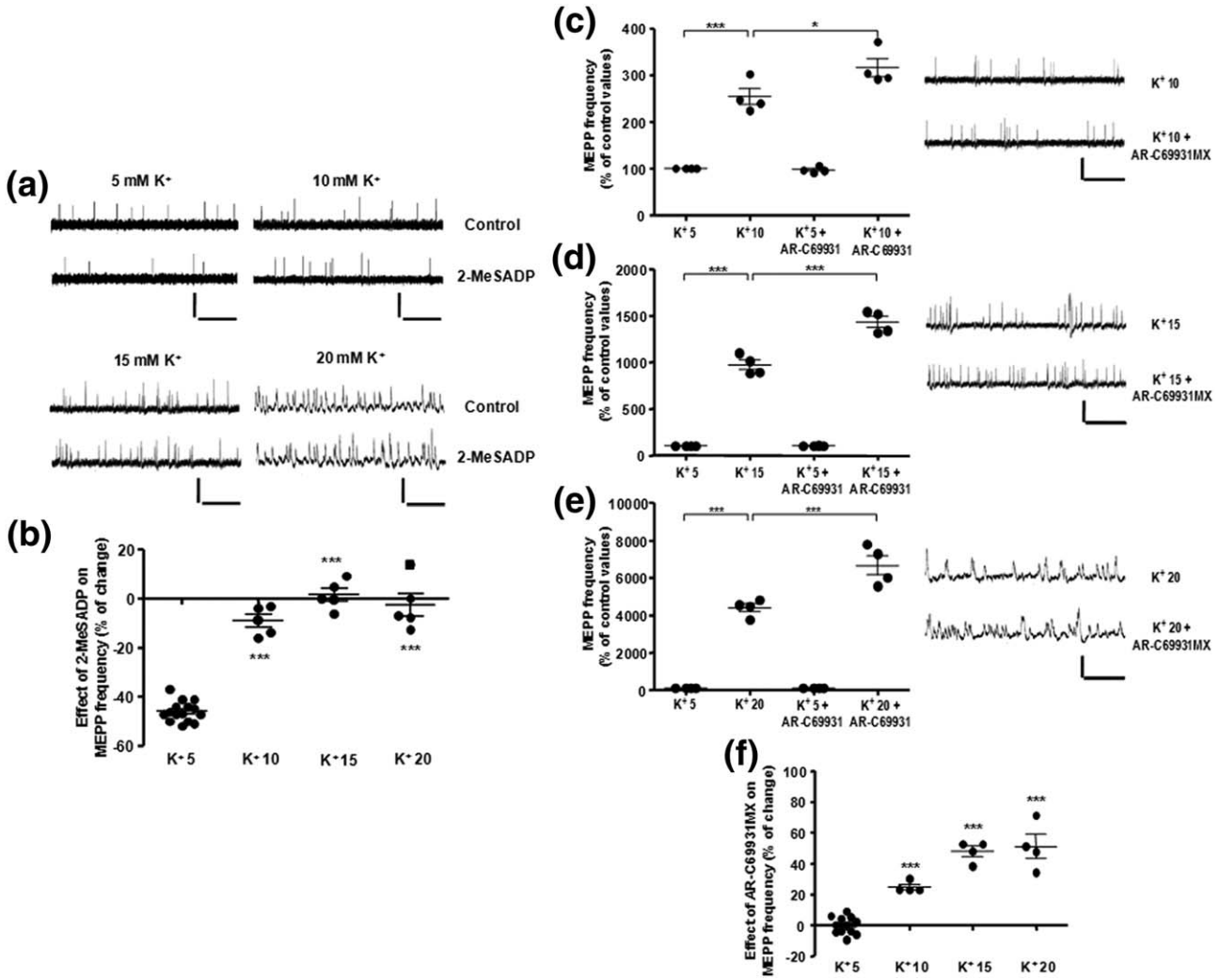

FIGURE 2 Effect of the $P2Y1_{13}$ agonist 2-MeSADP (150 nM) and the $P2Y1_{13}$ antagonist AR-C69931MX (1 μM) upon K^+-evoked ACh secretion. (a) Representative MEPPs recorded from diaphragm muscle fibers incubated in control solution (5 mM K^+, calibration 1 mV, 2,000 ms) and solutions containing 10 mM K^+ (calibration 1 mV, 1,000 ms), 15 mM K^+ (calibration 1 mV, 500 ms), and 20 mM K^+ (calibration 1 mV, 100 ms), in the absence or presence of 2-MeSADP. (b) Summary graph showing the action of 2-MeSADP upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of change induced by 2-MeSADP with respect to those observed at 5 (control solution), 10, 15, and 20 mM K^+ without the agonist. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. The inhibitory effect of 2-MeSADP was only observed at control solution ($n = 15$; 46.0% ± 1.1%), but not at 10 mM K^+ ($n = 5$), 15 mM K^+ ($n = 5$), and 20 mM K^+ ($n = 5$). ***$p < .001$ (ANOVA followed by Dunnett test to compare all other columns with the control column). (c–e) Effect of AR-C69931MX upon asynchronous secretion induced by 10 ($n = 4$), 15 ($n = 4$), and 20 mM K^+ ($n = 4$), respectively. Data (mean ± *SEM*) are expressed as percentage of control values (5 mM K^+). *$p < .05$, ***$p < .001$ (ANOVA followed by Tukey test). Symbols represent individual experiments. For each K^+ concentration (right panels), representative MEPPs are shown, recorded from diaphragm muscle fibers incubated in solutions containing 10 mM K^+ (calibration 1 mV, 1,000 ms), 15 mM K^+ (calibration 1 mV, 500 ms), and 20 mM K^+ (calibration 1 mV, 100 ms), in the absence or presence of AR-C69931MX. (f) Summary graph showing the action of AR-C69931MX upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of the change induced by AR-C69931MX with respect to those obtained at 5 (control solution), 10, 15, and 20 mM K^+ without the antagonist. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. AR-C69931MX significantly increased asynchronous release starting from 10 mM K^+. ***$p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline

adenosine receptors. Extracellular nucleoside concentration in the synaptic cleft depends on the extracellular conversion of adenine nucleotides into adenosine by ectonucleotidases and by the activity of bidirectional equilibrative nucleoside transporters (for review, see Latini & Pedata, 2001). To elucidate the relative contribution of these two pathways to the effect of endogenous adenosine at high K^+ concentration, we studied the action of the inhibitor of the ecto-5′-nucleotidase (last enzyme in the conversion of ATP into adenosine) αβ-MeADP

TABLE 1 Effect of $P2Y_{13}$, A_1, A_3, and A_{2A} receptor agonists on K^+-induced ACh release

Solutions	10 mM K^+ (% of control values)	15 mM K^+ (% of control values)	20 mM K^+ (% of control values)
Controlt 2-MeSADP	218.5 ± 7.6 199.0 ± 14.2 *n* = 5	976.5 ± 55.3 980.0 ± 42.1 *n* = 5	5290.0 ± 519.4 5096.0 ± 420.9 *n* = 5
Control CCPA	353.1 ± 23.9 175.4 ± 12.8*** *n* = 4	843.1 ± 76.2 869.4 ± 38.6 *n* = 7	4791.0 ± 582.3 4651.0 ± 456.1 *n* = 4
Control Inosine	198.7 ± 6.4 133.6 ± 12.0*** *n* = 4	844.3 ± 57.1 724.2 ± 36.0 *n* = 4	3203.0 ± 352.1 3230.0 ± 385.2 *n* = 4
Control CGS-21680	278.0 ± 13.7 390.4 ± 11.3*** *n* = 4	670.3 ± 94.3 1057.0 ± 117.6* *n* = 4	6928.0 ± 260.8 6746.0 ± 340.6 *n* = 4

Values are mean ± *SEM*. ***$p < .001$, *$p < .05$, ANOVA followed by Tukey test.

(100 mM; Naito & Lowenstein, 1985) or the adenosine transporter blocker NBTI (30 μM; Griffith & Jarvis, 1996; Kiss et al., 2000) on ACh secretion induced by 10, 15, and 20 mM K^+.

When analyzing asynchronous neurotransmitter release in the presence of αβ-MeADP to prevent extracellular adenosine formation from released ATP (Figure 3), we found that at 10 mM K^+ the inhibitor did not alter MEPP frequency (10 mM K^+ 395.3% ± 39.1% of control values, 10 mM K^+ + αβ-MeDP 354.1% ± 40.5%, $n = 4$). On the contrary, at 15 and 20 mM K^+, αβ-MeADP significantly increased ACh secretion (15 mM K^+ 996.5% ± 46.6%, 15 mM K^+ + αβ-MeADP 1,750.0% ± 72.1%, $n = 4$, $p < .001$; 20 mM K^+ 7,630.0% ± 917.0%, 20 mM K^+ + αβ-MeADP 10,718.0% ± 810.7%, $n = 4$, $p < .05$). These last results indicate that, without the inhibitor, enough endogenous adenosine is present at the synaptic space to activate adenosine receptors.

On the other hand, as illustrated in Figure 4a, at 10 mM K^+, the blockade of the adenosine transporters by NBTI significantly reduced asynchronous neurotransmitter secretion (10 mM K^+ 459.4% ± 42.6% of control values, 10 mM K^+ + NBTI 303.1% ± 34.5%, $n = 5$, $p < .01$), indicating that the inhibition of adenosine uptake into cells may effectively enhance its concentration in the synaptic cleft and exert its typical modulatory effect by activating inhibitory adenosine receptors. Addition of the specific A_1 adenosine receptor agonist CCPA (500 nM) to the solution containing NBTI could not induce further inhibition (10 mM K^+ + NBTI + CCPA 287.9% ± 28.5%), suggesting that A_1 receptors were occupied by the nontransported adenosine. Moreover, the action observed with NBTI was not observed when the preparations were previously incubated with the A_1 antagonist DPCPX (10 mM K^+ 344.3% ± 14.4% of control values, 10 mM K^+ + DPCPX + NBTI 314.6% ± 18.6%, $n = 4$, Figure 4b). Conversely, at 15 and 20 mM K^+, the inhibitor of the adenosine transporter failed to modify MEPP frequency (15 mM K^+ 967.8% ± 62.9%, 15 mM K^+ + NBTI 1,037.0% ± 69.4%, $n = 4$; 20 mM K^+ 4,471.0% ± 488.7%, 20 mM K^+ + NBTI 4,215.0% ± 252.4%, $n = 4$, Figure 4c–e), probably because, under such conditions, the adenosine that was not transported into the cells did not find available receptors (similar to what happened with CCPA and inosine at 15 and 20 mM K^+; see below).

Then, the next step was to analyze how the adenosine generated during 10, 15, and 20 mM K^+-evoked ACh secretion interacted with the action of agonists and antagonists of each adenosine receptor.

3.4 | A_1 adenosine receptors

As shown by De Lorenzo et al. (2004), the specific A_1 receptor agonist CCPA (500 nM) decreased MEPP frequency when muscles were bathed in 10 mM K^+, but at 15 and 20 mM K^+, asynchronous ACh release remained unchanged (Table 1, Figure 5a,b). As a counterpart, the selective antagonist for the A_1 adenosine receptor DPCPX (0.1 μM, Lohse et al., 1987) did not modify the asynchronous ACh secretion evoked by 10 mM K^+ (10 mM K^+ 353.1% ± 23.9% of control values, 10 mM K^+ + DPCPX 320.0% ± 29.9%, $n = 4$), but, at 15 and 20 mM K^+, the antagonist provoked a significant increase in MEPP frequency compared with values obtained in high K^+ without the antagonist (15 mM K^+ 843.1% ± 76.2%, 15 mM K^+ + DPCPX 1,357.0% ± 75.9%, $n = 4$, $p < .001$; 20 mM K^+ 4,791.0% ± 582.3%, 20 mM K^+ + DPCPX 7,117.0% ± 351.7%, $n = 5$, $p < .001$, Figure 5c–f).

3.5 | A_3 adenosine receptors

We have recently demonstrated, by pharmacological and immunohistochemical studies, that A_3 adenosine receptors are present at the motor nerve terminals and that inosine binds selectively to these receptors, but not to A_1 or A_{2A} receptors. At control Ringer solution (K^+ 5 mM), we found that 100 μM inosine is able to reduce MEPP frequency as well as EPP amplitude and its quantal content (Cinalli et al., 2013). Here, we show that inosine only reduced asynchronous neurotransmitter secretion in preparations incubated with 10 mM K^+; at 15 and 20 mM K^+ exogenous inosine was devoid of effect (Table 1, Figure 6a, b). In turn, the specific A_3 adenosine receptor antagonist MRS-1191 (5 μM; Jacobson et al., 1997; Jiang et al., 1996) did not modify MEPP frequency at 10 mM K^+, but increased 15 mM K^+ and 20 mM

K^+-evoked ACh release (10 mM K^+ 294.8% ± 31.4% of control values, 10 mM K^+ + MRS-1191 283.4% ± 11.0%, $n = 4$; 15 mM K^+ 793.5% ± 56.7%, 15 mM K^+ + MRS-1191 1,032.0% ± 92.0%, $n = 5$, $p < .05$; 20 mM K^+ 3,497.0% ± 156.9%, 20 mM K^+ + MRS-1191 4,525.0% ± 279.3%, $n = 4$, $p < .01$, Figure 6c–f).

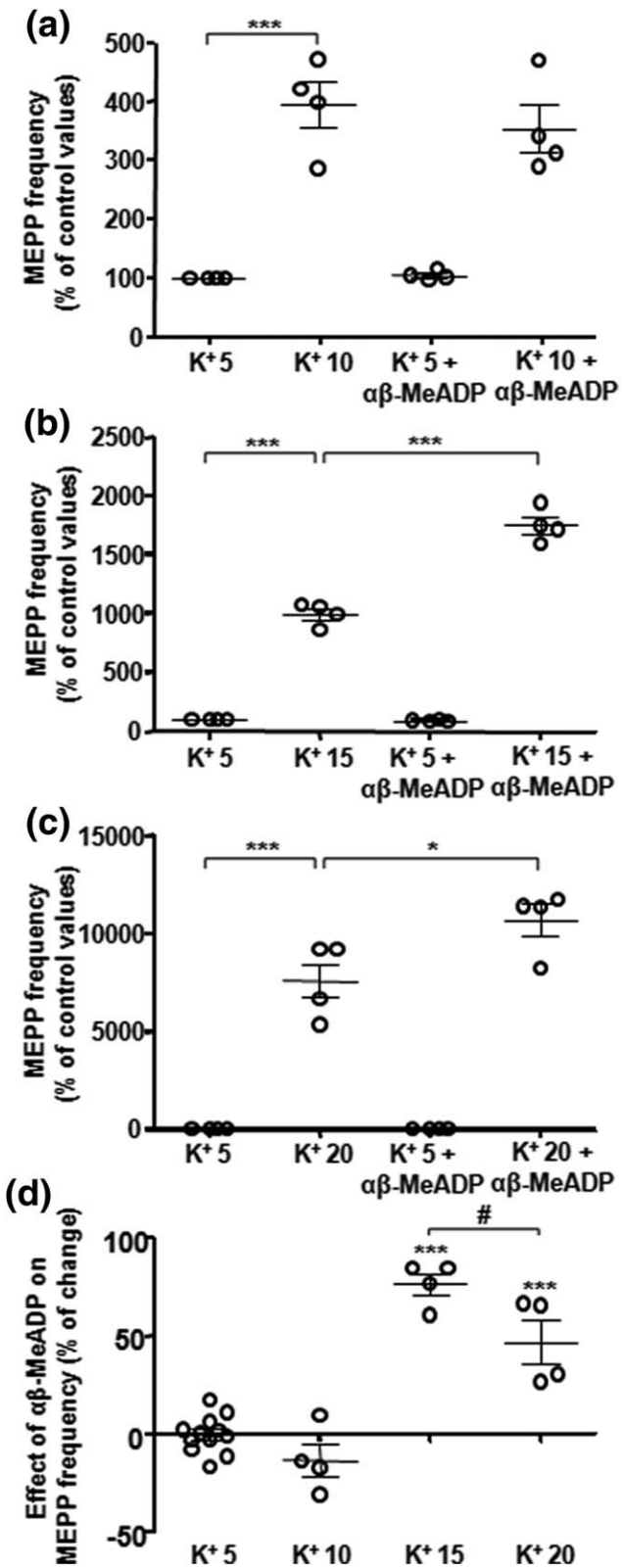

FIGURE 3.

3.6 | A_{2A} adenosine receptors

It is known that facilitation of ACh secretion due to activation of A_{2A} adenosine receptors becomes evident at high levels of endogenous adenosine, such as those generated during high-frequency, long-lasting stimuli (Oliveira, Timóteo, & Correia-de-Sá, 2004). In this investigation, application of the specific A_{2A} adenosine receptor agonist CGS-21680 (5 nM) to solutions containing 10 and 15 mM K^+ induced an increase in MEPP frequency, whereas at 20 mM K^+, facilitation was not observed (Table 1, Figure 7a,b). Opposite results were observed when the effect of the A_{2A} receptor antagonist SCH-58261 (50 nM; Zocchi et al., 1996) was evaluated at high K+ concentration. SCH-58261 did not alter 10–15 mM K^+-evoked ACh release, whereas at 20 mM K^+, the antagonist significantly reduced neurosecretion (10 mM K^+ 251.0% ± 34.6% of control values, 10 mM K^+ + SCH-58261 252.7% ± 19.3%, $n = 4$; 15 mM K^+ 851.8% ± 40.1%, 15 mM K^+ + SCH-58261 859.6% ± 84.1%, $n = 4$; 20 mM K^+ 6,623.0% ± 250.1%, 20 mM K^+ + SCH-58261 3,491.0% ± 300.0%, $n = 4$, $p < .001$, Figure 7c–f).

4 | DISCUSSION

In this study we analyzed the role of endogenous purines on K^+-evoked ACh secretion, in the range of 10 to 20 mM at the mammalian neuromuscular junction. Although the physiological relevance of K^+ stimulation remains to be elucidated, high extracellular K^+ concentration was detected during high-frequency neuronal discharge, and it was also implicated in pathological conditions, such as hypoxia and ischemia (Heinrich, Andó, Túri, Rózsa, & Sperlágh, 2012). This model also offers the possibility of analyzing purinergic modulation on transmitter release without the use of drugs needed to avoid the muscular

FIGURE 3 The inhibitor of the ecto-5′-nucleotidase αβ-MeADP induced an increase of MEPP frequency at 15 and 20 mM K^+-evoked ACh release. (a–c) Effect of αβ-MeADP (100 μM) upon asynchronous secretion evoked by 10 ($n = 4$), 15 ($n = 4$), and 20 mM K^+ ($n = 4$), respectively. Data (mean ± *SEM*) are expressed as percentage of control values (K^+ 5 mM). *$p < .05$, ***$p < .001$ (ANOVA followed by Tukey test). Symbols represent individual experiments. (d) Summary graph showing the action of αβ-MeADP upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of the change induced by αβ-MeADP with respect to those obtained at 5 (control solution), 10, 15, and 20 mM K^+ without the inhibitor. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. The inhibitor significantly increased asynchronous release at 15 and 20 mM K^+. ***$p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline. At 20 mM K^+, facilitation was significantly lower than at 15 mM K^+ because of the lack of effect of adenosine on A_{2A} receptors. #$p < .05$ (ANOVA followed by Tukey test)

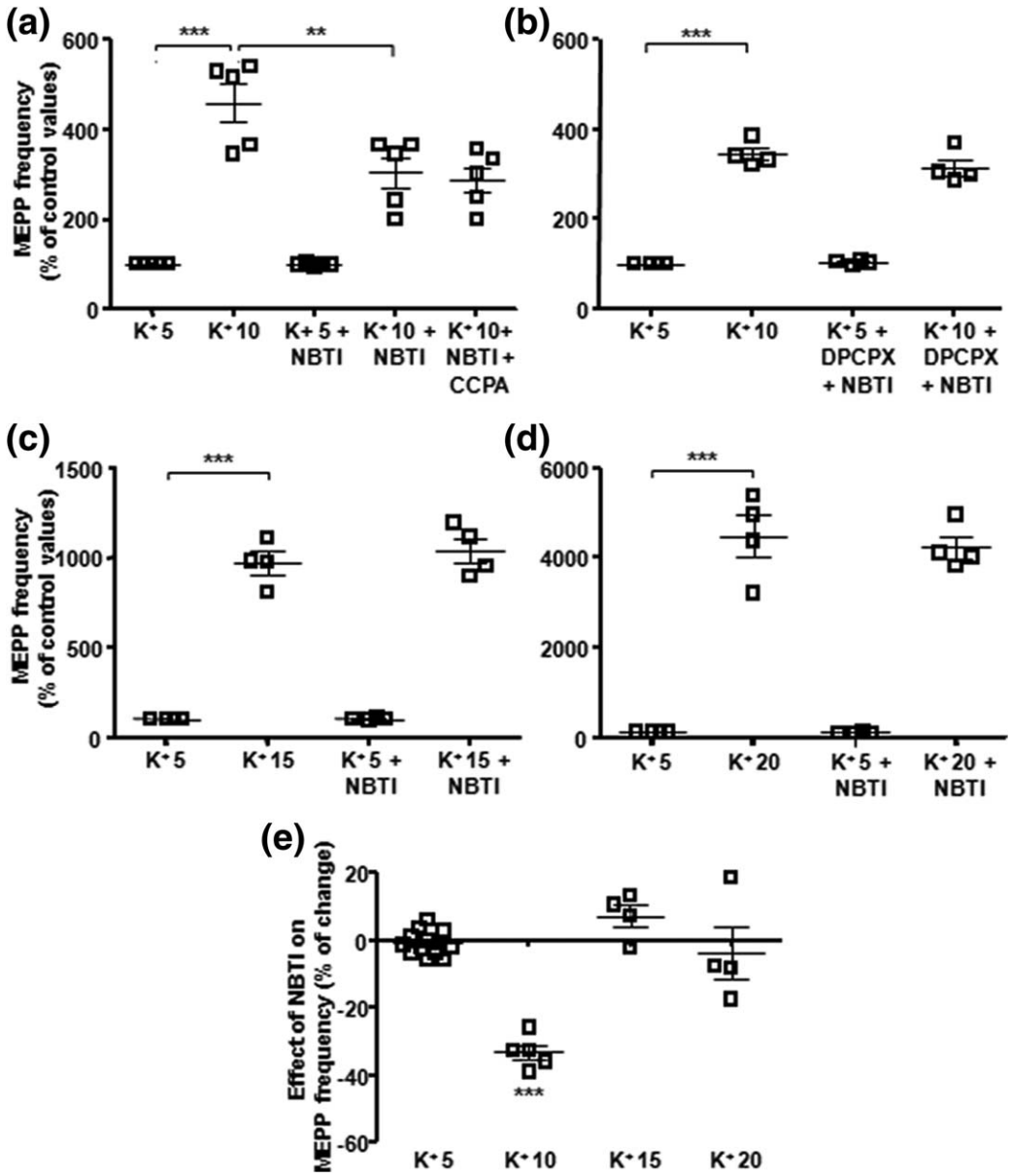

FIGURE 4 Effect of the equilibrative nucleoside transporter blocker NBTI (30 μM) upon 10–20 mM K^+-evoked ACh release. (a) At 10 mM K^+ ($n = 5$), NBTI reduced MEPP frequency, and addition of the A_1 adenosine receptor agonist CCPA (500 nM) was unable to induce any change. (b) At 10 mM K^+, the inhibitory effect on MEPP frequency induced by NBTI was prevented by the A_1 adenosine receptor antagonist DPCPX (0.1 μM, $n = 4$). (c,d) At 15 ($n = 4$) and 20 mM K^+ ($n = 4$), NBTI did not modify asynchronous ACh secretion. In panels a–d, data (mean ± *SEM*) are expressed as percentage of control values (K^+ 5 mM). $^{**}p < .01$, $^{***}p < .001$ (ANOVA followed by Tukey test). Symbols represent individual experiments. (e) Summary graph showing the action of NBTI upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of the change induced by NBTI with respect to those obtained at 5 (control solution), 10, 15, and 20 mM K^+ without the inhibitor. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. $^{***}p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline

contraction as it occurs when studying electrically evoked ACh secretion (e.g., high Mg^{2+}, D-tubocurarine or μ-conotoxin GIIIB).

Spontaneous secretion at rest depends, at least in part, on the stochastic activation of presynaptic VDCCs (Losavio & Muchnik, 1997, 1998). In the present investigation, incubation of the preparations with increasing K^+ solutions provokes depolarization of the membrane potential at values consistent with those predicted by the Goldman–Hodgkin–Katz equation. At the mammalian neuromuscular junction, K^+-induced depolarization of the presynaptic membrane causes an increase in MEPP frequency due to Ca^{2+} influx via P/Q-type VDCCs (Losavio & Muchnik, 1997; Protti & Uchitel, 1993). It is likely that the probability of P/Q-type VDCC openings increases as the membrane

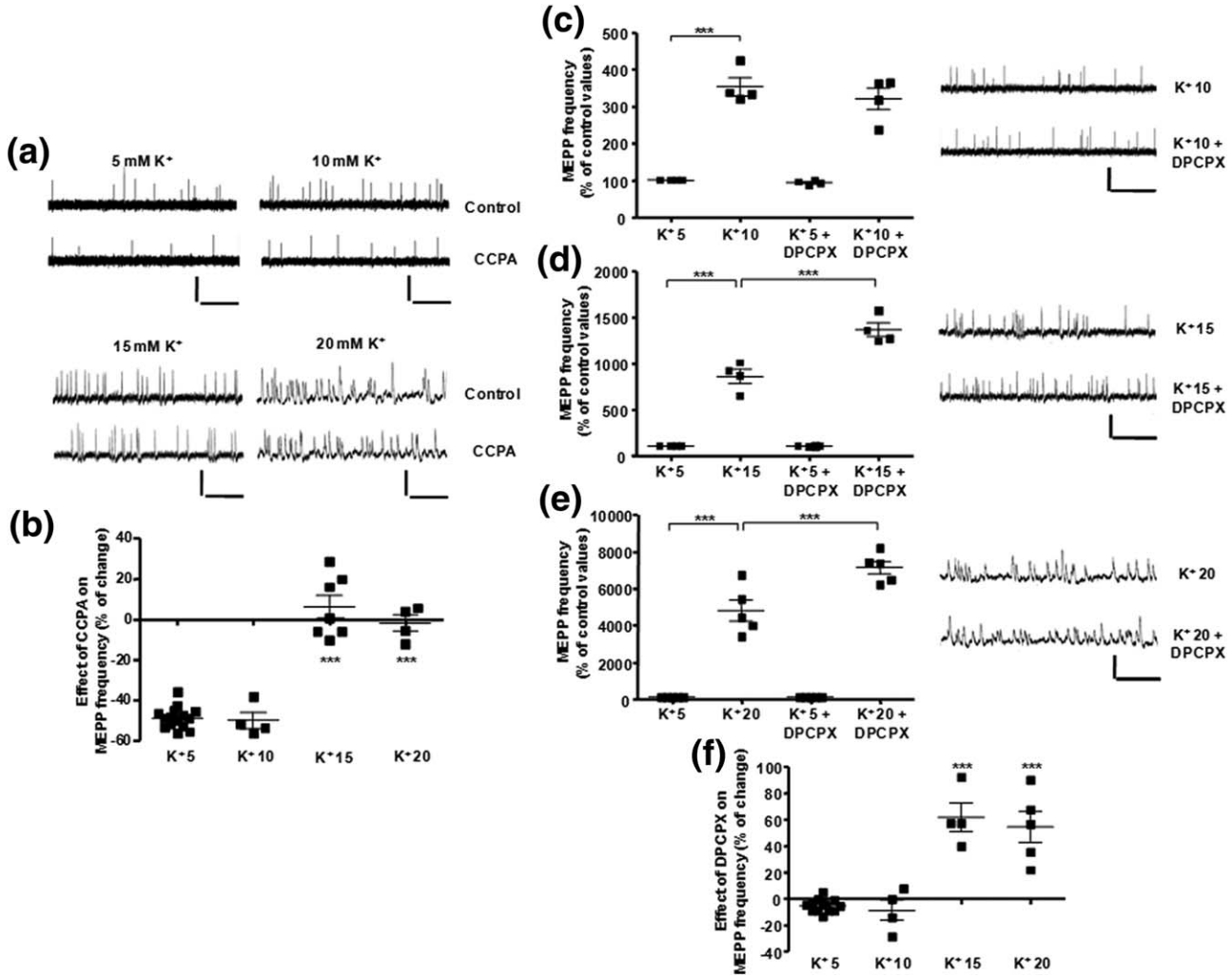

FIGURE 5 Effect of the A_1 adenosine receptor agonist CCPA (500 nM) and the A_1 adenosine receptor antagonist DPCPX (0.1 μM) upon K^+-evoked ACh secretion. (a) Representative MEPPs recorded from diaphragm muscle fibers incubated in control solution (5 mM K^+, calibration 1 mV, 2,000 ms) and solutions containing 10 mM K^+ (calibration 1 mV, 1,000 ms), 15 mM K^+ (calibration 1 mV, 500 ms), and 20 mM K^+ (calibration 1 mV, 100 ms), in the absence or presence of CCPA. (b) Summary graph showing the action of CCPA upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of the change induced by CCPA with respect to those observed at 5 (control solution), 10, 15, and 20 mM K^+ without the agonist. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. The inhibitory effect of CCPA was observed at 5 mM K^+ ($n = 15$; 48.7% ± 1.4%) and at 10 mM K^+ ($n = 4$; 50.0% ± 4.1%), but was devoid of effect at 15 mM K^+ ($n = 7$) and 20 mM K^+ ($n = 4$). ***$p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline. (c–e) Effect of DPCPX on 10 ($n = 4$), 15 ($n = 4$), and 20 mM ($n = 5$) K^+-evoked ACh release, respectively. Data (mean ± *SEM*) are expressed as percentage of control values (5 mM K^+). ***$p < .001$ (ANOVA followed by Tukey test). Symbols represent individual experiments. For each K^+ concentration (right panels), representative MEPPs are depicted, recorded from diaphragm muscle fibers incubated in solutions containing 10 mM K^+ (calibration 1 mV, 1,000 ms), 15 mM K^+ (calibration 1 mV, 500 ms), and 20 mM K^+ (calibration 1 mV, 100 ms), in the absence or presence of DPCPX. (f) Summary graph showing the action of DPCPX upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of change induced by DPCPX with respect to those obtained at 5 (control solution), 10, 15, and 20 mM K^+ without the antagonist. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. DPCPX significantly increased asynchronous release at 15 and 20 mM K^+. ***$p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline

potential get closer the potential of activation of them. Modeling studies indicate that a 10-mV depolarization in membrane potential would significantly increase the likelihood of stochastic activation of VDCC causing an increase in spontaneous release (Ermolyuk et al., 2013). So, as at high K^+ concentration, secretion of ACh and ATP from nerve terminals is increased (Jonzon & Fredholm, 1985), it is expected that more nucleotides of adenine and nucleosides were present in the synaptic space that were able to activate presynaptic purinergic receptors. On the other hand, it cannot be ruled out that K^+ depolarization also provokes secretion of purines from other cells (i.e., muscle cells,

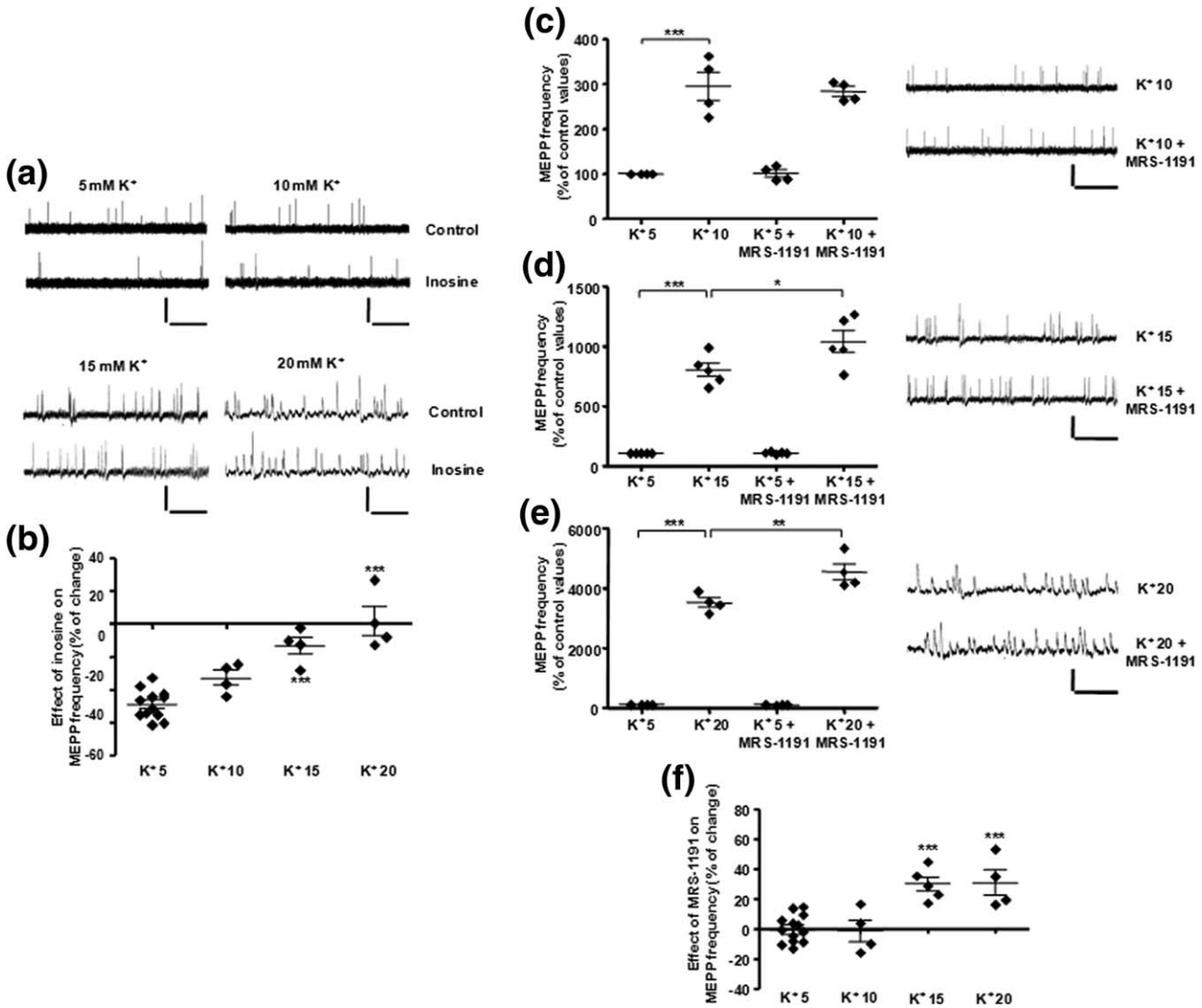

FIGURE 6 Effect of 100 μM inosine and 5 μM MRS-1191 (A_3 adenosine receptor agonist and antagonist, respectively) upon K^+-evoked ACh secretion. (a) Representative MEPPs recorded from diaphragm muscle fibers incubated in control solution (5 mM K^+, calibration 1 mV, 2,000 ms) and solutions containing 10 mM K^+ (calibration 1 mV, 1,000 ms), 15 mM K^+ (calibration 1 mV, 500 ms), and 20 mM K^+ (calibration 1 mV, 100 ms), in the absence or presence of inosine. (b) Summary graph showing the action of inosine upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of change induced by inosine with respect to those observed at 5 (control solution), 10, 15, and 20 mM K^+ without the agonist. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. Inosine by activating A_3 adenosine receptors reduced ACh secretion at 5 mM K^+ ($n = 12$; 48.9% ± 2.5%) and at 10 mM K^+ ($n = 4$; 33.0% ± 4.5%), whereas it did not alter MEPP frequency at 15 mM K^+ ($n = 4$) and 20 mM K^+ ($n = 4$). ***$p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline. (c–e) Effect of MRS-1191 on MEPP frequency evoked by 10 ($n = 4$), 15 ($n = 5$), and 20 mM ($n = 4$) K^+. Data (mean ± *SEM*) are expressed as percentage of control values (5 mM K^+). *$p < .05$, **$p < .01$, ***$p < .001$ (ANOVA followed by Tukey test). Symbols represent individual experiments. For each K^+ concentration (right panels), representative MEPPs are depicted, recorded from diaphragm muscle fibers incubated in solutions containing 10 mM K^+ (calibration 1 mV, 1,000 ms), 15 mM K^+ (calibration 1 mV, 500 ms), and 20 mM K^+ (calibration 1 mV, 100 ms), in the absence or presence of MRS-1191. (f) Summary graph showing the action of MRS-1191 upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of the change induced by MRS-1191 with respect to those obtained at 5 (control solution), 10, 15, and 20 mM K^+ without the antagonist. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. MRS-1191 significantly increased asynchronous release at 15 and 20 mM K^+. ***$p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline

Schwann cells), contributing to the concentration of purines in the extracellular space. In this regard, pannexin 1 was recently proposed as a possible pathway for ATP release from skeletal muscle (Arias-Calderón et al., 2016; Buvinic et al., 2009; Cea, Riquelme, Vargas, Urrutia, & Sáez, 2013; Riquelme et al., 2013), and among other stimuli, high extracellular K^+ increases the activity of pannexin 1 channels (D'Hondt

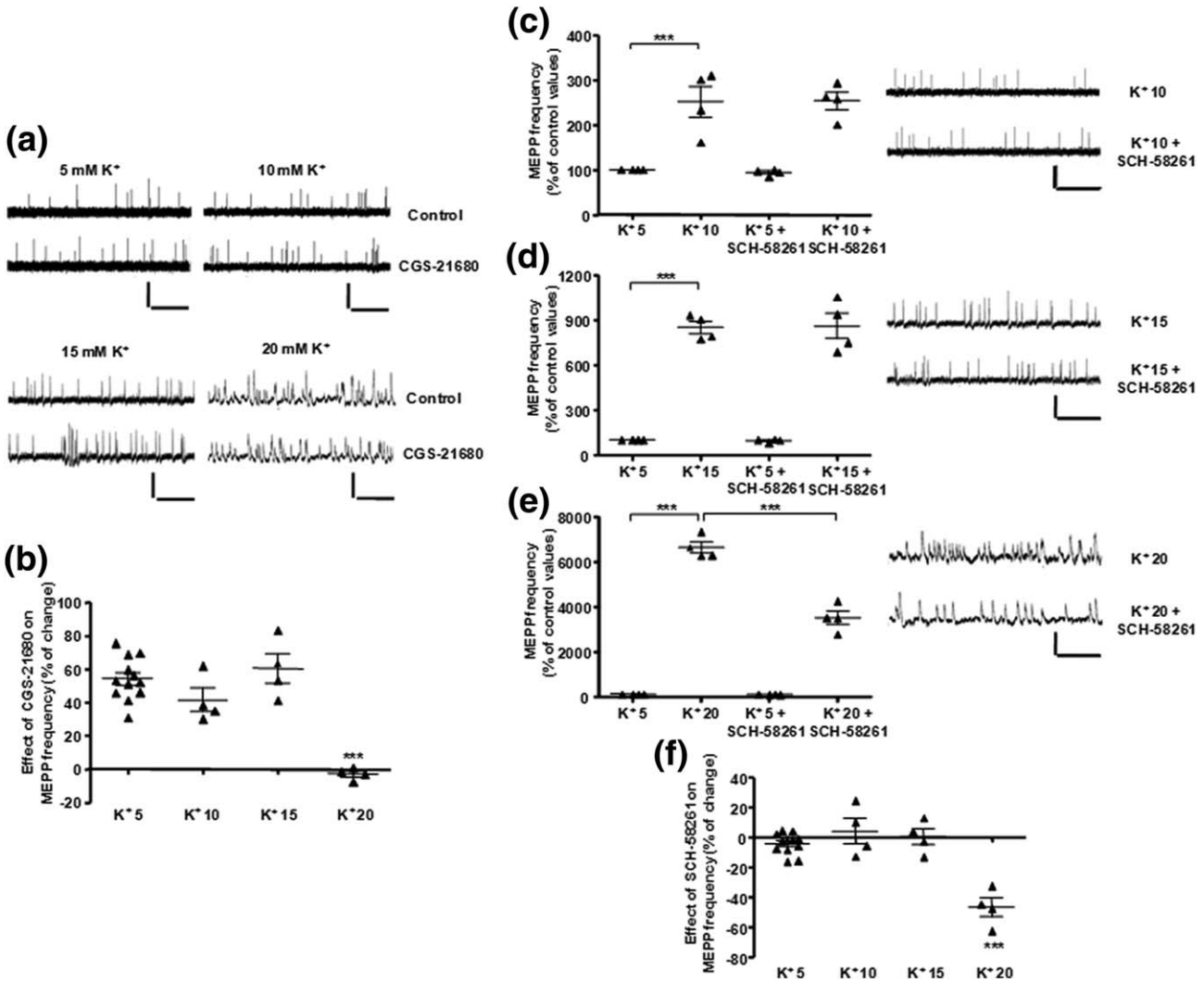

FIGURE 7 Effect of 5 nM CGS-21680 and 50 nM SCH-58261 (A_{2A} adenosine receptor agonist and antagonist, respectively) upon K^+-evoked ACh secretion. (a) Representative MEPPs recorded from diaphragm muscle fibers incubated in control solution (5 mM K^+, calibration 1 mV, 2,000 ms) and solutions containing 10 mM K^+ (calibration 1 mV, 1,000 ms), 15 mM K^+ (calibration 1 mV, 500 ms), and 20 mM K^+ (calibration 1 mV, 100 ms), in the absence or presence of CGS-21680. (b) Summary graph showing the action of CGS-21680 upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of the change induced by CGS-21680 with respect to those observed at 5 (control solution), 10, 15, and 20 mM K^+ without the agonist. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. CGS-21680 facilitated neurosecretion at 5 mM K^+ ($n = 12$; 51.1% ± 3.7%), 10 mM K^+ ($n = 4$; 41.30% ± 7.1%), and 15 mM K + ($n = 4$; 60.3% ± 8.9%) but not at 20 mM K^+ ($n = 4$). ***$p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline. (c–e) Effect of SCH-58261 on MEPP frequency evoked by 10 ($n = 4$), 15 ($n = 4$), and 20 mM ($n = 4$) K^+. Data (mean ± *SEM*) are expressed as percentage of control values (5 mM K^+). ***$p < .001$ (ANOVA followed by Tukey test). Symbols represent individual experiments. For each K^+ concentration (right panels), representative MEPPs are depicted, recorded from diaphragm muscle fibers incubated in solutions containing 10 mM K^+ (calibration 1 mV, 1,000 ms), 15 mM K^+ (calibration 1 mV, 500 ms), and 20 mM K^+ (calibration 1 mV, 100 ms), in the absence or presence of SCH-58261. (f) Summary graph showing the action of SCH-58261 upon ACh secretion when the preparations were incubated in control solution or increasing K^+ concentrations. Data (mean ± *SEM*) are expressed as percentage of change induced by SCH-58261 with respect to those obtained at 5 (control solution), 10, 15, and 20 mM K^+ without the antagonist. Symbols represent individual experiments. In K^+ 5 were included the values of percentage of change obtained in all high K^+ experiments. SCH-58261 was able to significantly decrease asynchronous release only at 20 mM K^+. ***$p < .001$ (ANOVA followed by Dunnett test). Asterisks indicate significance with respect to the results obtained in control saline

et al., 2013). However, pannexin 1 is localized in the sarcolemma of the T-tubules (Cea et al., 2012; Cea, Riquelme, Vargas, Urrutia, & Sáez, 2014; Jorquera et al., 2013; Riquelme et al., 2013) far from the synaptic area. More investigation is needed to elucidate this point.

As we have observed in our previous papers (Cinalli et al., 2013; De Lorenzo et al., 2004, 2006; Guarracino et al., 2016; Palma et al., 2011; Veggetti et al., 2008), our experiments do not reveal any involvement of endogenous purines at basal conditions (K^+ 5 mM), since

incubation of the diaphragms with the antagonists for the $P2Y_{13}$, A_1, A_3, or A_{2A} receptors (AR-C69931MX, DPCPX, MRS-1191, or SCH-58261, respectively) did not affect spontaneous neurotransmitter secretion. On the contrary, the application of exogenous agonists for $P2Y_{13}$, A_1, or A_3 receptors (2-MeSADP, CCPA, or inosine, respectively) decreases MEPP frequency, whereas the A_{2A} receptor agonist CGS-21680 increases it. In this sense, Sokolova et al. (2003) have demonstrated that exogenous ATP and adenosine reduce MEPP frequency in basal conditions at the frog neuromuscular junction. However, some of our results differ from those obtained by Garcia et al. (2013) in mouse levator auris longus muscles; they found that the blockade of A_1 receptors by DPCPX increased MEPP frequency, while the activation of A_1 and A_{2A} receptors by CCPA or CGS-21680, respectively, did not change spontaneous secretion, supporting the idea that endogenous adenosine contributes to limiting the spontaneous quantal leak of ACh. In addition to the fact that the muscle, mouse strain, and experimental conditions (their experiments were performed in the presence of μ-conotoxin GIIIB) were different from ours, it is not expected an increase of adenosine in the synaptic space at basal conditions, since any change in its concentration would be dissipated by the equilibrative adenosine transporters. Our experiments in control solution in the presence of NBTI or αβ-MeADP, to block the action of the transporters or to inhibit the conversion of adenine nucleotides into adenosine, respectively, did not modify MEPP frequency, indicating no endogenous adenosine in such conditions.

When the preparations were exposed to 10 mM K^+, the concentration of endogenous nucleotides of adenine (ATP and ADP) in the synaptic cleft seems to be enough to activate $P2Y_{13}$ receptors and decrease asynchronous ACh secretion. The blockade of the $P2Y_{13}$ receptors by AR-C69931MX significantly increased MEPP frequency. This finding is in accordance with the lack of effect of the $P2Y_{13}$ agonist 2-MeSADP, probably due to the occupancy of the receptors by the endogenous nucleotides. At the same time, part of ATP is being metabolized to adenosine, but as its concentration begins to rise in the synaptic cleft, the nucleoside is transported into the cells by equilibrative nucleoside transporters. The action of nucleoside transporters in removing extracellular endogenous adenosine was already described at rat neuromuscular junctions (Correia-de-Sá & Ribeiro, 1996; Sebastião & Ribeiro, 1988). This might explain why we did not obtain any change in ACh secretion when the muscles were analyzed in the presence of the A_1, A_{2A}, or A_3 antagonists, suggesting that at 10 mM K^+, adenosine receptors are not tonically activated by endogenous adenosine, thus allowing the modulatory action of the exogenous agonists CCPA, CGS-21680, or inosine, respectively. Moreover, we demonstrated that the blockade of the nucleoside transporters with NBTI provoked an increase in the concentration of endogenous adenosine in the synaptic cleft that was able to reduce 10 mM K^+-evoked ACh release by activation of the A_1 adenosine receptors. Additionally, incubation of the preparations with the A_1 antagonist DPCPX in the presence of NBTI prevented the inhibitory action of the nucleoside.

At 15 mM K^+, the $P2Y_{13}$ antagonist AR-C69931MX significantly increased ACh secretion, suggesting that endogenous ATP/ADP were activating $P2Y_{13}$ receptors. Similar to what happens at 10 mM K^+, incubation of the preparations with the $P2Y_{13}$ agonist 2-MeSADP did not induce any response upon asynchronous secretion. Besides, adenosine derived from adenine nucleotides was able to activate A_1 and A_3 inhibitory receptors and exert a tonic inhibitory action since incubation with the antagonists DPCPX and MRS-1191, respectively, increased 15 mM K^+-evoked ACh release. Similar results were obtained when the generation of adenosine from ATP was prevented by impairing the action of ecto-5′-nucleotidase by αβ-MeADP. These findings suggest that, at 15 mM K^+, enough endogenous adenosine can effectively reduce the ACh secretion via its inhibitory receptors. Furthermore, the specific A_1 and A_3 agonists CCPA and inosine also failed in modulating ACh release, suggesting that A_1 and A_3 receptors were occupied by the endogenous nucleoside. In this case, it is likely that the action of the equilibrative transporters was insufficient to reduce the concentration of the nucleosides in the synaptic cleft since the excess adenosine induced by NBTI did not find free receptors. On the other hand, at

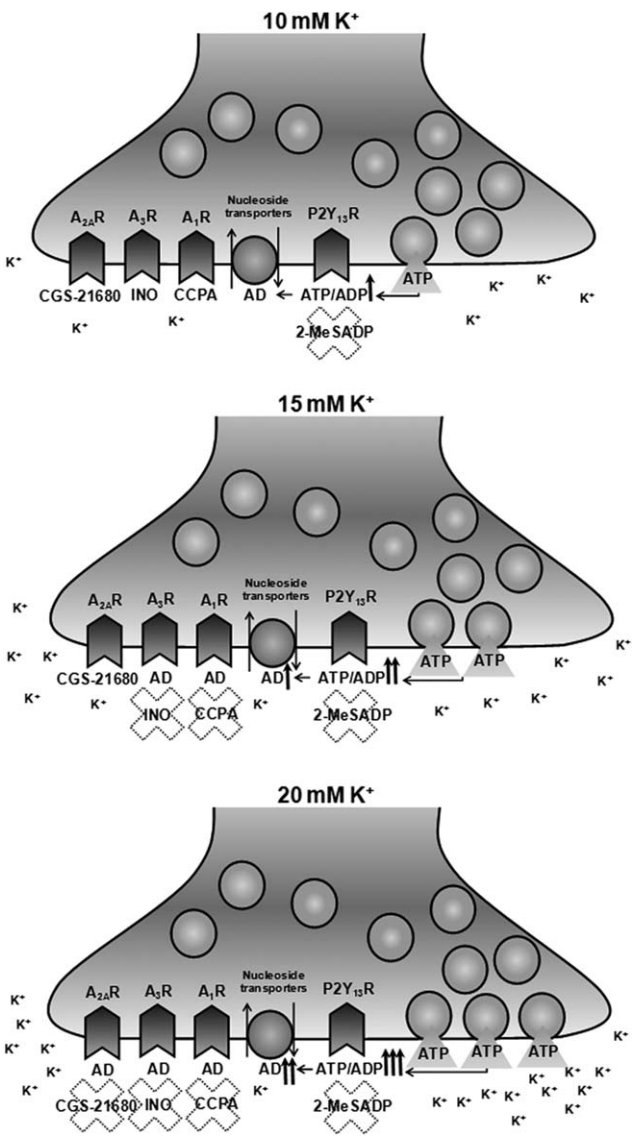

FIGURE 8 Effect of endogenous purines upon ACh secretion induced by increasing K^+ concentrations. See explanation in the text

15 mM K^+, the A_{2A} antagonist SCH-58261 did not change MEPP frequency, and, as expected, the specific agonist CGS-21680 facilitated ACh secretion by activation of free A_{2A} receptors, suggesting that higher adenosine concentration was required to activate A_{2A} receptors, probably because they have less affinity for the nucleoside (Correia-de-Sa et al., 1996; Daly, Butts-Lamb, & Padgett, 1983; de Lera Ruiz, Lim, & Zheng, 2014). Indeed, data on the potency of adenosine in Chinese hamster ovary cells transfected with human A_1, A_{2A}, A_{2B}, and A_3 receptors demonstrated that the highest potency was observed at the A_1 (EC_{50} 0.31 μM) and A_3 receptors (EC_{50} 0.29 μM), followed by the A_{2A} receptor (EC_{50} 0.73 μM) and much less at the A_{2B} receptor (EC_{50} 23.5 μM) (Fredholm, Irenius, Kull, & Schulte, 2001).

When K^+ concentration was raised to 20 mM, the amount of adenine nucleotides and adenosine in the synaptic cleft was high enough to activate all purinergic receptors, even the excitatory A_{2A} receptors. The blockade of the inhibitory $P2Y_{13}$, A_1, or A_3 receptors by AR-C69931MX, DPCPX, or MRS-1191, respectively, induced an increase in asynchronous ACh release, while the A_{2A} antagonist SCH-58261 reduced it. Conversely, when the effects of the agonists 2-MeSADP, CCPA, inosine, and CGS-21680 were analyzed, it was found that they did not exert any change in neurosecretion. Interestingly, in the experiments where the generation of adenosine was prevented by αβ-MeADP, an increase was observed in 20 mM K^+-evoked ACh release. However, the percentage of enhanced MEPP frequency in this K^+ concentration was significantly lower than that observed in 15 mM K^+ ($p < .05$; see Figure 3d), revealing the lack of effect of endogenous adenosine on facilitatory A_{2A} receptors at 15 mM K^+.

In conclusion, when motor nerve terminals are depolarized by increasing K^+ concentrations, there is a sequence of activation of purinergic receptors by the ATP/ADP and adenosine endogenously generated that are able to fine-tune neurosecretion (see Figure 8). So, at 10 mM K^+, released ATP and/or generated ADP would bind to $P2Y_{13}$ receptors, provoking inhibition of ACh secretion and impairing the action of 2-MeSADP. On the other hand, adenosine levels in the synaptic cleft appear not to be enough to activate A_1, A_{2A}, and A_3 receptors, possibly because the nucleoside is taken by equilibrative transporters to the intracellular space. At 15 and 20 mM K^+, ATP concentration is such that the nucleotides not only occupy the receptors but the adenosine formed from it, in an amount that could bind to A_1 and A_3 inhibitory receptors. In this situation, the inhibitory action of CCPA and inosine could not be observed. Excitatory A_{2A} receptors are only activated at 20 mM K^+ because of the lower affinity of the receptors to the nucleoside. It is likely that, at high K^+ concentration, equilibrative nucleoside transporters become saturated, allowing the accumulation of adenosine in the synaptic cleft.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

All authors had full access to the data in the study and take responsibility for the integrity of the data analysis. Conceptualization A.S.L., Methodology J.F.G., A.R.C., M.I.V. and A.S.L., Investigation J.F.G., A.R.C., M.I.V. and A.S.L., Formal analysis J.F.G., A.R.C., M.I.V. and A.S.L., Resources A.S.L., Writing - Original draft A.S.L., Writing - Review & Editing J.F.G., A.R.C., M.I.V. and A.S.L., Visualization A.S.L., Supervision A.S.L., Project administration A.S.L. and Funding Acquisition A.S.L.

DATA ACCESSIBILITY

All data supporting this paper are stored in the Laboratory of Neurophysiology, Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires (IDIM-CONICET) and are available upon request (idimneurofisio@gmail.com).

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