Neuroscience 172 (2011) 164–176

EXCITATORY EFFECT OF THE A_{2A} ADENOSINE RECEPTOR AGONIST CGS-21680 ON SPONTANEOUS AND K^+-EVOKED ACETYLCHOLINE RELEASE AT THE MOUSE NEUROMUSCULAR JUNCTION

A. G. PALMA, S. MUCHNIK AND A. S. LOSAVIO*

Laboratorio de Neurofisiología, Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires, CONICET, Combatientes de Malvinas 3150, 1427—Buenos Aires, Argentina

Abstract—The mechanism of action of the A_{2A} adenosine receptor agonist 2-*p*-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680) in the facilitation of spontaneous (isotonic and hypertonic condition) and K^+-evoked acetylcholine (ACh) release was investigated in the mouse diaphragm muscles. At isotonic condition, the CGS-21680-induced excitatory effect on miniature end-plate potential (MEPP) frequency was not modified in the presence of $CdCl_2$ and in a medium free of Ca^{2+} ($0Ca^{2+}$-EGTA), but it was abolished after buffering the rise of intracellular Ca^{2+} with 1,2-bis-(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxy-methyl) (BAPTA-AM) and when the Ca^{2+}-ATPase inhibitor thapsigargin was used to deplete intracellular Ca^{2+} stores. CGS-21680 did not have a direct effect on the Ca^{2+}-independent neurotransmitter-releasing machinery, since the modulatory effect on the hypertonic response was also occluded by BAPTA-AM and thapsigargin. CGS-21680 facilitation on K^+-evoked ACh release was not altered by the P/Q-type voltage-dependent calcium channel (VDCC) blocker ω-Agatoxin IVA, but it was completely prevented by both, the L-type VDCC blocker nitrendipine (which is known to immobilize their gating charges), or thapsigargin, suggesting that the effects of CGS-21680 on L-type VDCC and thapsigargin-sensitive internal stores are associated. We found that the VDCC pore blocker Cd^{2+} (2 mM Ca^{2+} or $0Ca^{2+}$-EGTA) failed to affect the CGS-21680 effect in high K^+ whereas nitrendipine in $0Ca^{2+}$-EGTA+Cd^{2+} occluded its action. The blockade of Ca^{2+} release from endoplasmic reticulum with ryanodine antagonized the facilitating effect of CGS-21680 in control and high K^+ concentration. It is concluded that, at the mouse neuromuscular junction, activation of A_{2A} receptors facilitates spontaneous and K^+-evoked ACh release by an external Ca^{2+}-independent mechanism but that involves mobilization of Ca^{2+} from internal stores: during spontaneous ACh release stimulating directly the ryanodine-sensitive stores and, at high K^+, probably modulating the L-type VDCCs which may cause the opening of the ryanodine receptors that would be directly coupled to the channels. In both cases, Ca^{2+} released from the endoplasmic reticulum would be capable of activating the exocytotic machinery, thus producing facilitation of ACh release.

Key words: A_{2A} adenosine receptor, CGS-21680, intracellular Ca^{2+} stores, Ca^{2+} channels, ryanodine receptors, mouse neuromuscular junction.

Synaptic transmission is under the regulation of extracellular purines, which through an interaction between their own receptors and the steps implicated in the process of exocytosis modulate the neuronal activity. At motor nerve terminals, 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). Thus, the amount of synaptic adenosine depends mainly on nerve stimulation frequency (Silinsky, 1975; Cunha and Sebastião, 1993), although the nucleoside may also derive from activated muscles fibers (Smith, 1991). It has been shown that adenosine modulates ACh release through presynaptic inhibitory A_1 or facilitatory A_{2A} adenosine receptors, which seem to be co-localized at motor nerve terminals (Correia-de-Sá et al., 1991; Cunha et al., 1996). Activation of one or another receptor depends on the frequency and pattern of stimulation. So, whereas during low-frequency activity there is a predominant inhibition of ACh secretion mediated by A_1 receptors, facilitation of neuromuscular transmission is achieved during high-frequency long-lasting stimuli due to A_{2A} receptor activation (Correia-de-Sá et al., 1996; Oliveira et al., 2004).

At mammalian neuromuscular junctions, it was found that activation of A_1 adenosine receptors attenuated action potential-evoked ACh secretion by a mechanism that decreases P/Q-type Ca^{2+} currents (Hamilton and Smith, 1991; Silinsky, 2004) and, in a previous report, we have demonstrated that adenosine decreases spontaneous neurotransmitter release by affecting the nitrendipine-sensitive component of miniature end-plate potential (MEPP) frequency (De Lorenzo et al., 2004). However, it was recently found that a mechanism acting in a Ca^{2+}-independent step in the cascade of the exocitotic process appears also to be involved in the presynaptic inhibition induced by adenosine (Silinsky, 2005; Veggetti et al., 2008), raising to the possibility that an action of the nucleoside on strategic components of the secretory apparatus could modify the activation of calcium channels.

*Corresponding author. Tel: +54-11-4514-8701 (Int 160); fax: +54-11-4523-8947.
E-mail address: idimneurofisio@gmail.com or adrianalosavio@gmail.com (A. S. Losavio).

Abbreviations: ACh, acetylcholine; BAPTA-AM, 1,2-bis-(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxy-methyl); CGS-21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride; CICR, Ca^{2+}- induced Ca^{2+} release; DHP, dihydropyridine; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; MEPP, miniature end-plate potential; RyRs, ryanodyne receptors; SCH-58261, 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5,-c]pyrimidin-5-amine; VDCC, voltage-dependent calcium channel; VICaR, voltage-induced Ca^{2+} release; ω-Aga IVA, ω-Agatoxin IVA.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2010.10.015

On the other hand, in rat motor nerve terminals, Correia-de-Sá et al. (2000) have found that activation of A_{2A} adenosine receptors induced facilitation of evoked [^{3}H] ACh release by mobilizing Ca^{2+} from internal stores and/or stimulating Ca^{2+} influx *via* L-type and/or P-type voltage-dependent calcium channels (VDCCs) depending on the stimulation paradigm.

The aim of the present work was to identify the presynaptic mechanisms underlying the facilitatory action of the specific A_{2A} adenosine receptor agonist 2-*p*-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680), upon spontaneous and K^+-evoked ACh release. At mammalian neuromuscular junctions, it is known that tonic ACh secretion is related to Ca^{2+} influx through L-type and N-type VDCCs (Losavio and Muchnik, 1997), whereas phasic neurotransmitter release involves the P/Q-type VDCCs (Protti and Uchitel, 1993). In addition, there are evidences that two distinct vesicle pools (Koenig and Ikeda, 1999) and different release machineries may contribute to evoked and spontaneous secretion (Deitcher et al., 1998; Hua et al., 1998; see rev. Kidokoro, 2003). Thus, it is plausible that A_{2A} receptors may modulate each type of secretion by independent pathways. Our results reveal that CGS-21680 increases ACh secretion by an external calcium-independent mechanism but that involves Ca^{2+} mobilization from endoplasmic stores *via* ryanodine receptors. Interestingly, the mechanisms by which these endoplasmic Ca^{2+} channels are activated appear to be different for spontaneous and K^+-evoked ACh release.

EXPERIMENTAL PROCEDURES

Preparations and solutions

Experiments were carried out on phrenic nerve-diaphragm preparations taken from adult CF1 mice (30–40 g) of either sex. All animal procedures were performed under protocols approved by national guidelines, which are in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 80-23) revised 1996. Mice were anesthetized with sodium thiopental (Fada Pharma, Buenos Aires, Argentina, 50 mg kg^{-1}) i.p. and left hemidiaphragms were 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 each experimental group, the muscles were allowed to equilibrate in the respective solution for at least 20 min, except otherwise indicated (See Results). In some experiments, a saline solution containing 0 $CaCl_2$, 2 mM $MgCl_2$, and 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) (0Ca^{2+}-EGTA) was employed in order to eliminate the inward Ca^{2+} gradient. When KCl concentration of the Ringer Krebs solution was raised to 15 mM, an equal amount of NaCl was removed from the incubation medium to maintain the isotonicity. In experiments performed in 15 mM K^+-0Ca^{2+}-EGTA, 100 μM $CdCl_2$ was added to prevent Ca^{2+} outflow from depolarized nerve terminals when electrochemical Ca^{2+} gradient is reversed. In other group of experiments, nerve terminals were loaded with 1,2-bis-(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxy-methyl) (BAPTA-AM) to decrease $[Ca^{2+}]_i$: preparations were immersed in a Ca^{2+}-free Krebs solution containing BAPTA-AM (10 μM) for 2 h. After that, the diaphragms were washed during a 40-min period with Ca^{2+}-free Krebs solution and then recordings were performed. Hyperosmotic media were freshly prepared by adding 100 mM sucrose to Krebs solutions and their osmolarity were checked with a Fiske osmometer before each experiment. When using nitrendipine, experiments were performed with extreme care to minimize exposure of drug solutions to light. All recordings were carried out at room temperature (22–23 °C).

Electrophysiological recordings

MEPPs were recorded at the end-plate region of the muscle fibers using borosilicate glass microelectrodes (WP Instruments) 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. Before studying the time course of hyperosmotic response, 10 junctions were sampled in the isotonic solution and their values averaged. In each synapse, MEPP frequency was recorded during 100 s. Then, immediately after the change to the hyperosmotic solution, synapses were sampled repeatedly from the same small area of diaphragm over brief intervals during 30 min. An effort was made to keep the intervals between sampling as short as possible. In this case, MEPP frequency was recorded during 10 s in each synapse. Tetrodotoxin 10^{-6} M was added to hypertonic solutions to prevent the muscle from twitching violently, which otherwise occurred upon sudden exposure of preparations to hypertonic solutions. Frequency/amplitude data were acquired through an A/D converter (Digidata 1322A) controlled by computer and then analyzed using pClamp-8.2 (Axon Instruments, USA). MEPP amplitudes were normalized to a resting membrane potential of −75 mV, using the formula $V_c=[V_o\times(-75)]/E$, where V_c is the corrected MEPP amplitude, V_o is the observed MEPP amplitude, and E is the resting membrane potential.

Data analysis

In all cases, data are reported as mean±SEM and *n* expresses number of animals (only left hemidiaphragm was used from each mouse for a given experiment). Areas under the hypertonic curves were calculated using Prism (version 3.02). Statistical comparison among three or more groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's post test. Two group comparisons were performed using paired or unpaired Student's *t*-test. Differences were considered to be significant when $P<0.05$.

Chemicals

ω-Agatoxin IVA (ω-Aga IVA), cadmium chloride ($CdCl_2$), CGS-21680, EGTA, nitrendipine, thapsigargin, and tetrodotoxin were from Sigma, St. Louis, MO, USA; BAPTA-AM was from Molecular Probes, Carlsbad, CA, USA, and ryanodine and SCH-58261 (2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5,-c]pyrimidin-5-amine) were from Tocris Bioscience, Ellisville, MO, USA. All other reagents were of the highest purity available. CGS-21680 and thapsigargin was made up as 1 mM stock solutions in dimethyl sulfoxide (DMSO). Nitrendipine was made up from a 10 mM stock solution in DMSO that was kept protected from the light to prevent photo-oxidation. Aqueous dilutions of these stock solutions were made daily, and appropriate solvent controls were done.

RESULTS

Facilitatory effect of CGS-21680 on spontaneous ACh release

Fig. 1 illustrates presynaptic effect of the specific A_{2A} receptor agonist CGS-21680 on spontaneous ACh release from mice phrenic nerve-hemidiaphragm preparations.

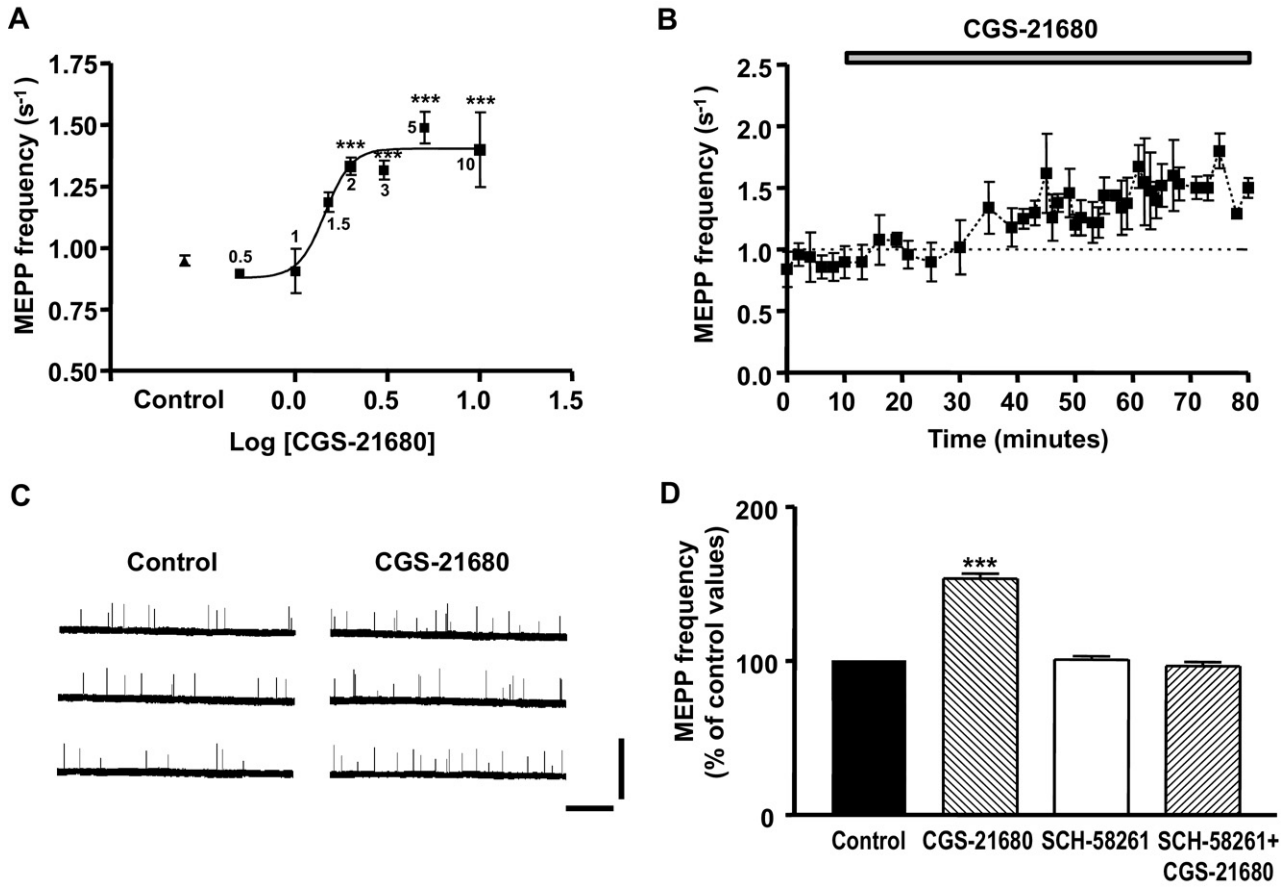

Fig. 1. Facilitatory effect of CGS-21680 upon spontaneous ACh release at the mouse neuromuscular junction. (A) Effect of the A_{2A} adenosine receptor agonist CGS-21680 on MEPP frequency (s^{-1}) as a function of its concentration. Each point represents mean±SEM (Control: 0.95±0.02, n=4; 0.5 nM: 0.9±0.01, n=4; 1 nM: 0.91±0.09, n=4; 1.5 nM: 1.19±0.04, n=3; 2 nM: 1.34±0.04, n=4; 3 nM: 1.32±0.04, n=4; 5 nM: 1.49±0.06, n=4; 10 nM: 1.40±0.15, n=4, *** P<0.001, ANOVA followed by Dunnett's test; EC_{50}: 1.46 nM). (B) Temporal course of the CGS-21680 facilitatory effect on MEPP frequency. CGS-21680 (5 nM) was applied as indicated by the bar. Symbols are mean±SEM values of MEPP frequency recorded from five muscles. (C) Representative MEPPs recorded from diaphragm muscle fibers bathed with control solution (Vm: −74.1 mV), and with 5 nM CGS-21680 (Vm: −74.8 mV). Calibration: 2.5 mV, 2 s. (D) Summary bar graph showing the presynaptic excitatory effect of 5 nM CGS-21680 on MEPP frequency (n=5) recorded after 45 min of incubation with the drug and its inhibition by the A_{2A} adenosine receptor antagonist SCH-58261 (50 nM, n=4). Data (mean±SEM) are expressed as percentage of control values (black bar). *** P<0.001, ANOVA followed by Tukey's test.

CGS-21680 (5 nM: see dose–response curve at Fig. 1A, EC_{50} 1.46 nM) increased MEPP frequency to 153.2±3.1% (n=5, P<0.001) of control values (Fig. 1C, D). In general, there was a lag period between 40 and 50 min before MEPP frequency reached its plateau after bath application of the A_{2A} agonist, and the effect persisted for more than 30 min (Fig. 1B). As it is shown in Fig. 1D, the action of CGS-21680 did not occur when preparations were previously incubated with 50 nM SCH-58261, selective A_{2A} receptor antagonist (SCH-58261 100.6±2.3% of control values, SCH-58261+CGS-21680 96.44±2.6%, n=4). We did not find any detectable change in MEPP amplitude (Control 1.10±0.09 mV, CGS-21680 1.05±0.07 mV, n=3), indicating that the sensitivity of endplates toward ACh remained unchanged. Thus, the facilitation of spontaneous ACh release induced by CGS-21680 was mainly the result of presynaptic modulation on transmitter secretion by activation of A_{2A} receptors.

One of the possible mechanisms by which CGS-21680 can exert presynaptic facilitation on spontaneous secretion might be an enhancement of Ca^{2+} influx through the VDCCs present at the presynaptic membrane of mammalian neuromuscular junction (P/Q-type, L-type and N-type VDCC; Protti and Uchitel, 1993; Bowersox et al., 1995; Katz et al., 1997; Losavio and Muchnik, 1997). Therefore, we first investigated the effect of CGS-21680 in the presence of 100 μM $CdCl_2$, a universal VDCC blocker. As shown in Fig. 2A, Cd^{2+} reduced MEPP frequency to 50.6±3.5% of control values (P<0.01) but failed to prevent the CGS-21680 facilitatory effect on spontaneous ACh release (125.7±12.7% of control values, Cd^{2+} vs. Cd^{2+}+CGS-21680 P<0.001, n=6). It has been reported that Cd^{2+} itself can cause neurotransmitter release in addition to its blocking action, depending upon either concentration or time of exposure (Nilson and Volle, 1976; Cooper and Manalis, 1984; Nishimura et al., 1984; Braga and Rowan, 1994). So, we studied the effect of 100 μM Cd^{2+} on MEPP frequency during 75 min. We found that in the presence of the blocker, MEPP frequency (Control 0.93±0.05 s^{-1}) varied from 0.52±0.03 s^{-1} after 5 min of

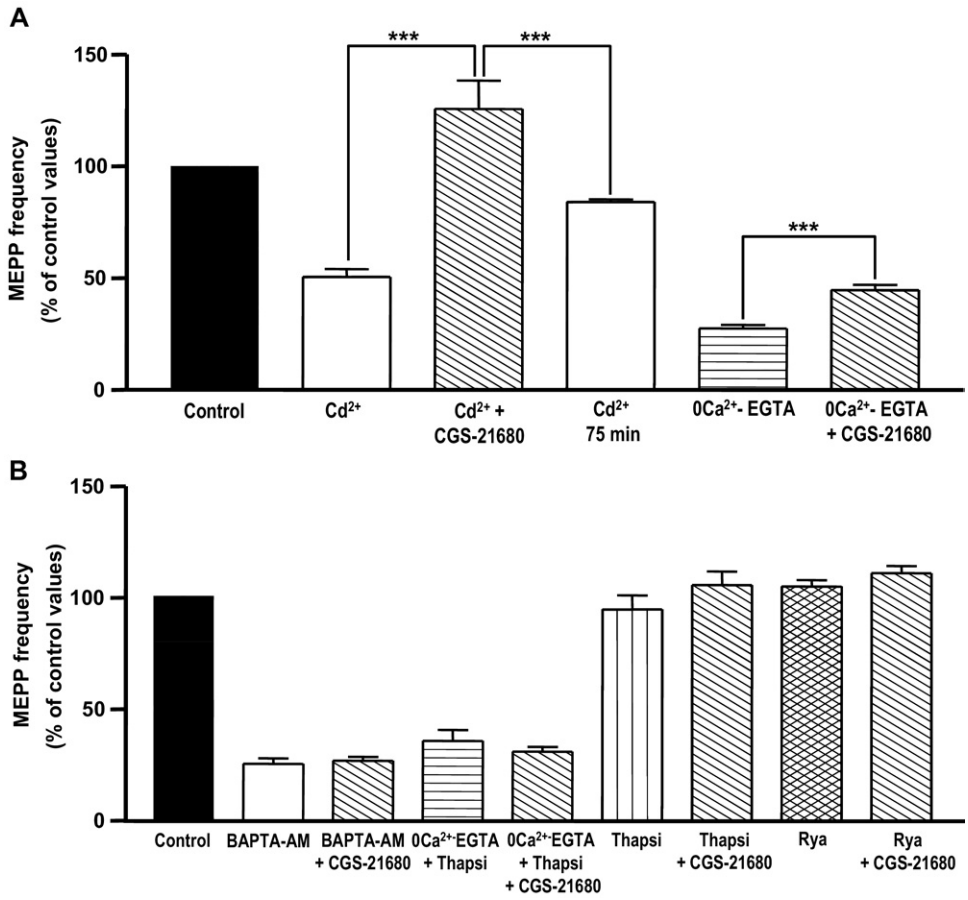

Fig. 2. Role of extracellular and intracellular calcium in the CGS-21680-induced increase of spontaneous ACh release. (A) The universal VDCC blocker, Cd^{2+} (100 μM, n=6) or the absence of extracellular Ca^{2+} ($0Ca^{2+}$-EGTA, n=5), did not prevent the effect of CGS-21680 on MEPP frequency. (B) The intracellular Ca^{2+} chelator BAPTA-AM (10 μM, n=5), the depletion of intracellular Ca^{2+} stores with thapsigargin (Thapsi, 2 μM) in $0Ca^{2+}$-EGTA (n=4) or in 2 mM Ca^{2+} (n=4), and the blockade of RyRs with ryanodine (Rya, 10 μM, n=4) abolished CGS-21680 action on spontaneous ACh secretion. Data (mean±SEM) are expressed as percentage of control values (black bars). *** $P<0.001$, ANOVA followed by Tukey's test.

exposure to 0.68±0.05 s^{-1} at about 75 min (n=3, $P>0.05$). When spontaneous release was recorded in solutions containing Cd^{2+}+CGS-21680, MEPP frequency raised to 1.28±0.10 s^{-1} (n=4) at time 75 min; this value is statistically different from those observed with the blocker alone at that time (Fig. 1, $P<0.01$). In Fig. 2A, fourth column, it is shown MEPP frequency values that are matching in time to data recorded in Cd^{2+}+CGS-21680 (Cd^{2+} vs. Cd^{2+}+CGS-21680 $P<0.001$). So, it is clear that the facilitatory effect corresponds to the action of the A_{2A} agonist. Moreover, treating the muscles with the A_{2A} agonist after 20 min incubation in $0Ca^{2+}$-EGTA solution still elicited a comparable increase in MEPP frequency ($0Ca^{2+}$-EGTA 27.6±1.5% of control values; $0Ca^{2+}$-EGTA+CGS-21680 44.7±2.3% of control values, $P<$ 0.001, n=5, Fig. 2A). Thus, Ca^{2+} influx from extracellular fluid is not the major cause of CGS-21680-induced facilitation.

Since it is well-known that the $[Ca^{2+}]_i$ level in the nerve terminal exerts a dominant effect on the rate of spontaneous transmitter release (Augustine et al., 1987; Rahamimoff and Alnaes, 1973; Blaustein et al., 1978; Emptage et al., 2001), we examined whether a rise in intracellular calcium is required for the excitatory effect of CGS-21680. In Fig. 2B it is depicted that the facilitation induced by CGS-21680 was completely blocked by buffering the $[Ca^{2+}]_i$ rise with the membrane-permeable Ca^{2+} chelator BAPTA-AM (10 μM) during 120 min (BAPTA-AM 25.5± 2.4% of control values, BAPTA-AM+CGS-21680 26.9± 1.7%, n=5), suggesting that the effect of CGS-21680 requires an elevation of Ca^{2+} concentration within motor nerve terminals. We next examined whether the Ca^{2+} released from intracellular stores is responsible for A_{2A} agonist-induced synaptic facilitation. To approach this question, the Ca^{2+}-ATPase inhibitor thapsigargin (2 μM) was used to deplete intracellular Ca^{2+} stores (Thastrup et al., 1990). Experiments were first performed in $0Ca^{2+}$-EGTA Krebs solution to exclude the possibility of internal Ca^{2+} store depletion-induced Ca^{2+} entry through store-operated channels in the plasma membrane (Kanzaki et al., 1999; Tempia et al., 2001). As it was observed by other authors (Chen and Grinnell, 1997; Liou et al., 2003), application of thapsigargin elicited an increase in MEPP frequency, which returned to control levels within 40–60 min.

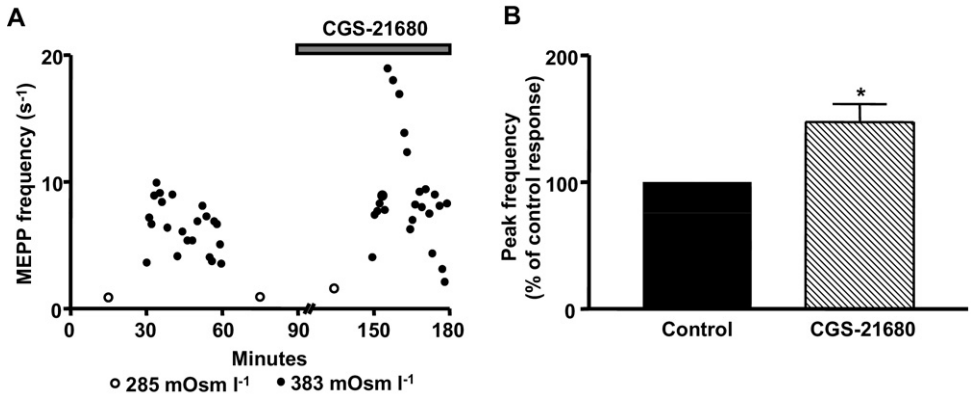

Fig. 3. Facilitatory effect of CGS-21680 upon hypertonic response. (A) Effect of CGS-21680 on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic condition. Open circles indicate mean values from 10 synapses obtained after exposing the preparations to isotonic conditions and closed circles represent the time course of hypertonic response (each point represents averaged value of MEPP frequency recorded during 10 s from a single synapse). (B) Summary bar graph shows the modulatory effect of CGS-21680 on the peak of the hypertonic response. Data (mean±SEM, $n=4$) are expressed as percentage of control values (black bars). * $P<0.02$, paired Student's *t*-test.

So, recordings were made after that period. Further exposure of preparations to CGS-21680 in the presence of thapsigargin no longer induced any changes in MEPP frequency ($0Ca^{2+}$-EGTA+thapsigargin 35.7±4.9% of control values, $0Ca^{2+}$-EGTA+thapsigargin+CGS-21680 30.9±2.1%, $n=4$, Fig. 2B). The same behavior was observed when this kind of experiments was performed in control saline (2 mM $CaCl_2$): thapsigargin 94.2±6.2% of control values, thapsigargin+CGS-21680 104.9±6.1%, $n=4$, Fig. 2B.

Calcium from internal stores can be released into the cytosol by activation of specific Ca^{2+} channels (ryanodine receptors, RyRs), a phenomenon antagonized by the alkaloid ryanodine (10 μM, Meissner, 1986). We found that application of ryanodine prevented the potentiating effect of CGS-21680 on MEPP frequency (ryanodine 104.3±2.9% of control values, ryanodine+CGS-21680 110.3±3.1%, $n=4$, Figs. 2B and 7D). Similar results were observed when 10 μM dantrolene was used as a blocker of RyRs (data not shown). These findings suggest that Ca^{2+} released from intracellular stores through RyRs is responsible for the spontaneous ACh release facilitation induced by the activation of A_{2A} adenosine receptors.

Modulatory effect of CGS-21680 on hypertonic response

In order to investigate whether the facilitatory action of CGS-21680 occurs concomitantly by stimulation of a step related to the neurotransmitter-releasing machinery downstream of Ca^{2+} influx, we studied the effect of the A_{2A} agonist on hypertonicity-induced enhancement of MEPP frequency, a situation that was probed to be independent of Ca^{2+} (Furshpan, 1956; Hubbard et al., 1968; Rosenmund and Stevens, 1996; Losavio and Muchnik, 1997; Kashani et al., 2001). When hypertonic solution was applied to diaphragm muscles, MEPP frequency increased from a frequency of 0.91±0.01 s^{-1} in isotonic condition to a peak of 9.25±1.56 s^{-1}, and declined gradually during continuous application of the hypertonic solution. The area under the curve was 130.5±24.3 ($n=4$). After complete washout of the preparation with isotonic solution MEPP frequency returned to control values. The addition of CGS-21680 to the preparations induced an enhancement of MEPP frequency in isotonic as well as in hypertonic condition (see Fig. 3A, B). So, the peak of the hypertonic response increased to 13.68±2.65 s^{-1} (147.9±14.3% of the control responses, $P<0.02$) and the area under the curve to 209.6±37.2 (160.1±8.1% of the control responses, $P<0.002$).

Afterward, we analyzed the role of extracellular and intracellular Ca^{2+} in the CGS-21680-induced changes upon hypertonic response. As expected, CGS-21680 continued to increase the hypertonic response when preparations were bathed in extracellular solution lacking Ca^{2+} ($0Ca^{2+}$-EGTA) (Fig. 4A–C). That is, the A_{2A} agonist significantly enhanced the peak of the response ($0Ca^{2+}$-EGTA 16.6±2.1% of the control response, $n=4$; $0Ca^{2+}$-EGTA+CGS-21680 42.4±6.2% of control values, $n=4$, $P<0.001$) and the area under the curve ($0Ca^{2+}$-EGTA 21.8±3.2% of the control response; $0Ca^{2+}$-EGTA+CGS-21680 36.9±3.9% of the control response, $P<0.01$). On the other hand, CGS-21680 did not significantly increased the hypertonic response when diaphragms were pretreated with the intracellular buffer BAPTA-AM (BAPTA-AM: peak 22.9±3.8% of control response, area 22.0±2.1% of control response, $n=4$; BAPTA-AM+CGS-21680: peak 22.7±4.0%, area 25.8±2.3%, $n=5$; see Fig. 4D–F), suggesting that the facilitatory effect of CGS-21680 on hypertonic response involve an increase of intracellular Ca^{2+} availability. We next examined whether Ca^{2+} released from intracellular stores was responsible for the increase of the hypertonic response observed when the A_{2A} receptors are activated. As it is shown in Fig. 5A, B, 2 μM thapsigargin alone did not modify the hypertonic curve (peak 95.6±4.9% of control response, area under the curve 87.8±5.2%), but prevent the excitatory action of CGS-21680 (peak 93.0±2.9% of control responses, area under the curve 92.2±10.3%, $n=4$), indicating that thap-

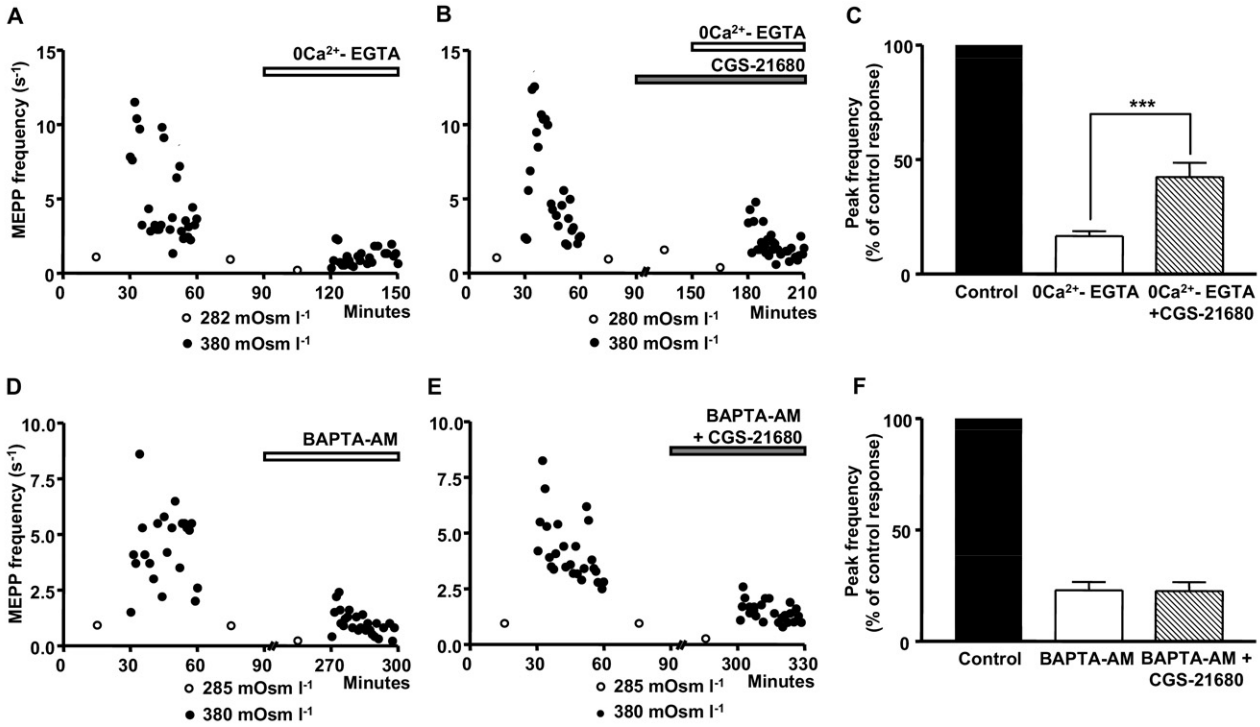

Fig. 4. Role of extracellular and intracellular calcium in the CGS-21680-mediated facilitation of the hypertonic response. (A) Effect of $0Ca^{2+}$-EGTA on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic condition. (B) Effect of CGS-21680 on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic condition in calcium free-solutions ($0Ca^{2+}$-EGTA). (C) Summary bar graph depicts the peak of the hypertonic response when preparations were incubated with $0Ca^{2+}$-EGTA(n=4) and with $0Ca^{2+}$-EGTA+CGS-21680 (n=4). (D) Effect of the intracellular Ca^{2+} chelator BAPTA-AM on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic condition. (E) Effect of CGS-21680 on ACh release when a diaphragm muscle was loaded with BAPTA-AM and then exposed to isotonic and hypertonic condition. (F) Summary bar graph shows the peak of the hypertonic response when preparations were incubated in BAPTA-AM (n=4) and in BAPTA-AM+CGS-21680 (n=5). In (A), (B), (D), and (E), open circles indicate mean values from 10 synapses obtained after exposing the preparations to isotonic conditions and closed circles represent the time course of hypertonic response (each point represents averaged value of MEPP frequency recorded during 10 s from a single synapse). In (C) and (F), data (mean±SEM) are expressed as percentage of control values (black bars), *** $P<0.001$, ANOVA followed by Tukey's test.

sigargin-sensitive Ca^{2+} stores within motor nerve endings are also involved in the action of the A_{2A} agonist in hypertonicity. Moreover, treatment of preparations with 10 μM ryanodine occluded the action of the A_{2A} agonist (ryanodine: peak 119.7±16.3% of control response, area 133.2±13.5% of control response; ryanodine+CGS-21680: peak 98.2±4.7%, area 99.7±19.6%, n=3; Fig. 5C, D).

Effect of CGS-21680 on K^+-evoked ACh release

To explore the action of CGS-21680 on ACh release triggered by depolarization, we studied its effect at high external K^+ concentration (15 mM). When preparations were expose to high K^+ solution, MEPP frequency increased to 591.9±74.8% of control values (n=3, $P<0.001$). After washout of muscles, the incubation with CGS-21680 in 15 mM K^+ induced facilitation of asynchronous ACh secretion to 947.7±62.3% of control values, a quantity significantly higher than those recorded in 15 mM K^+ without the A_{2A} adenosine receptor agonist ($P<0.01$, Fig. 6A, B). Then, in order to evaluate whether recruitment of Ca^{2+} from internal stores was also involved in the facilitatory action of CGS-21680 on K^+-evoked ACh release, we studied the effect of the A_{2A} agonist when muscles were bathed in thapsigargin. The results showed that thapsigargin did not modify the increase in MEPP frequency induced by high K^+ concentration (K^+ 15 mM 626.7±60.9% of control values; K^+ 15 mM+thapsigargin 577.8±14.6%, n=4). However, the Ca^{2+}-ATPase inhibitor was able to occlude the effect of CGS-21680 in 5 mM K^+ (see Fig. 7D) as well as in high K^+ concentration (K^+ 15 mM+thapsigargin+CGS-21680 523.2±13.5% of control values, Figs. 6C and 7E). In this case, 10 μM ryanodine also prevented the CGS-21680-induced facilitation of MEPP frequency in high K^+ (K^+ 15 mM 860.8±79.9% of control values; K^+ 15 mM+ryanodine 901.6±12.7%; K^+ 15 mM+ryanodine+CGS-21680 744.0±74.4%, n=4, Figs. 6D and 7E).

As at rat phrenic nerve-hemidiaphragm preparations, Correia-de-Sá et al. (2000) found that A_{2A} receptor activation triggered facilitation of evoked [^{3}H]ACh release by mobilizing Ca^{2+} from internal (thapsigargin-sensitive) stores and/or by increasing Ca^{2+} influx *via* L-type or P-type VDCC, depending on the stimulation paradigm, we studied the effect of CGS-21680 upon asynchronous neurotransmitter secretion in the presence of P/Q-type or L-type VDCC blockers. It is known that, at mammalian neuromuscular junctions, the increase in MEPP frequency

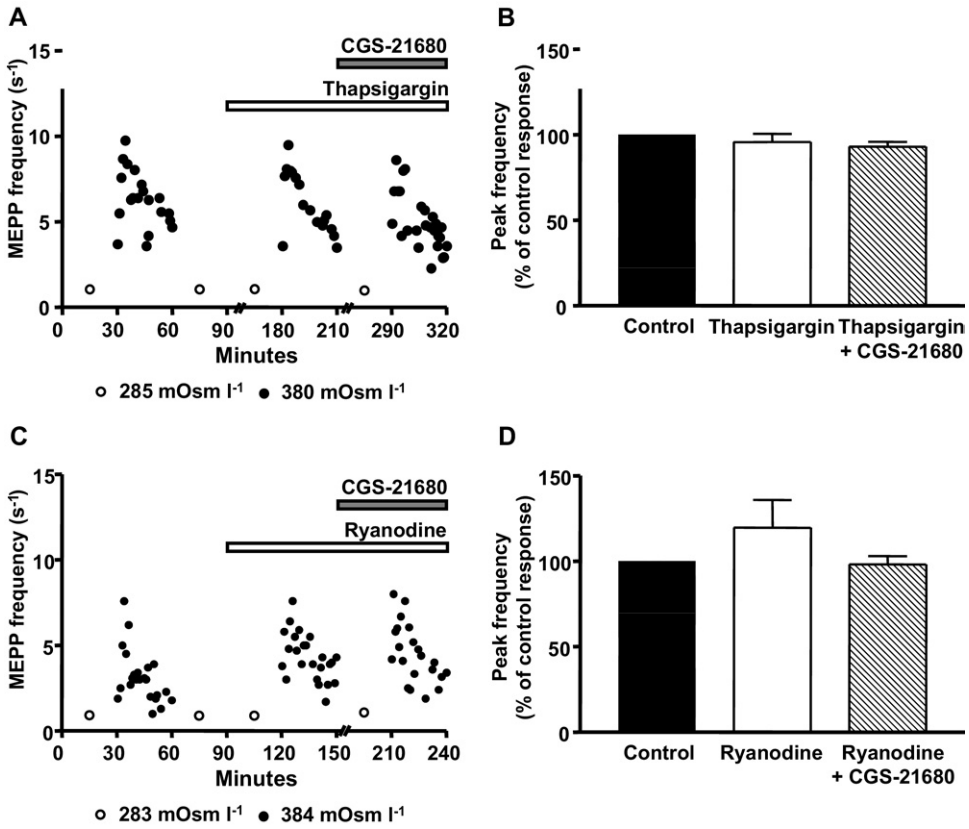

Fig. 5. The excitatory effect of CGS-21680 on hypertonic response depends on calcium mobilization from internal stores. (A) Effect of thapsigargin and thapsigargin+CGS-21680 on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic condition. (B) Summary bar graph shows the lack of effect of CGS-21680 on the peak frequency when internal stores were depleted with thapsigargin (n=4). (C) Effect of ryanodine and ryanodine+CGS-21680 on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic condition. (D) Summary bar graph shows the lack of effect of CGS-21680 on the peak frequency when Ca^{2+} release from endoplasmic reticulum was blocked by ryanodine (n=3). In (A) and (C), open circles indicate mean values from 10 synapses obtained after exposing the preparations to isotonic conditions and closed circles represent the time course of hypertonic response (each point represents averaged value of MEPP frequency recorded during 10 s from a single synapse). In (B) and (D), data (mean±SEM) are expressed as percentage of control values (black bars).

evoked by K^+ depolarization depends on Ca^{2+} influx through P/Q-type VDCC, since ω-Aga IVA (specific P/Q-type VDCC blocker) inhibits presynaptic Ca^{2+} current and ACh release (Protti and Uchitel, 1993; Losavio and Muchnik, 1997). Thus, ω-Aga IVA (100 nM) did not change MEPP frequency recorded in control solution, but significantly attenuated ACh release when preparations were incubated in high K^+ (K^+ 15 mM 850.2±80.8% of control values; K^+ 15 mM+ω-Aga IVA 368.0±42.7% of control values, n=4, P<0.001). In accordance with the experiments performed with Cd^{2+} or 0Ca^{2+}-EGTA (see Fig. 2A), further incubation of preparations with CGS-21680 in the presence of ω-Aga IVA (5 mM K^+) did not prevent the enhancement of MEPP frequency (167.8±10.0% of control values, ω-Aga IVA vs. ω-Aga IVA+CGS-21680 P<0.004; see also Fig. 7D). The same behavior was observed at high K^+ concentration (K^+ 15 mM+ω-Aga IVA+CGS-21680 555.6±52.2% of control values), a value significantly higher (P<0.05) than that observed in K^+ 15 mM+ω-Aga IVA without the A_{2A} agonist (Figs. 6E and 7E). These results demonstrate that the A_{2A} receptor-mediated facilitatory effect is hardly dependent on Ca^{2+} influx through P/Q-type VDCC. We then studied the involvement of dihydropyridine (DHP)-sensitive L-type VDCC in the facilitatory action of CGS-21680 on asynchronous ACh release. Fig. 6F shows that the specific L-type VDCC blocker nitrendipine (5 μM) reduced MEPP frequency in control Krebs solution to 58.3±2.7% of control values, but did not interfere with the Ca^{2+} entry associated with nerve terminal depolarization (K^+ 15 mM 510.1±18.1% of control values; K^+ 15 mM+nitrendipine 522.8±39.4%, n=4). After washout, incubation of preparations with nitrendipine+CGS-21680 in control saline did not alter the excitatory effect of the A_{2A} agonist (MEPP frequency 159.8±2.8% of control values, nitrendipine vs. nitrendipine+CGS-21680 P<0.003; see also Fig. 7D), whereas that, at high K^+ concentration, the CGS-21680 action was abolished (K^+ 15 mM+nitrendipine+CGS-21680 483.6±37.0% of control values, see Fig. 7E). These data suggest that L-type VDCCs are involved in the A_{2A} receptor-mediated facilitatory effect on K^+-evoked ACh release.

As it can be observed in Figs. 6C, F and 7E, thapsigargin as well as nitrendipine completely prevented CGS-21680 modulatory effect upon K^+-evoked ACh release, which led us to conjecture that the actions of the A_{2A} agonist on thapsigargin-sensitive internal stores and L-

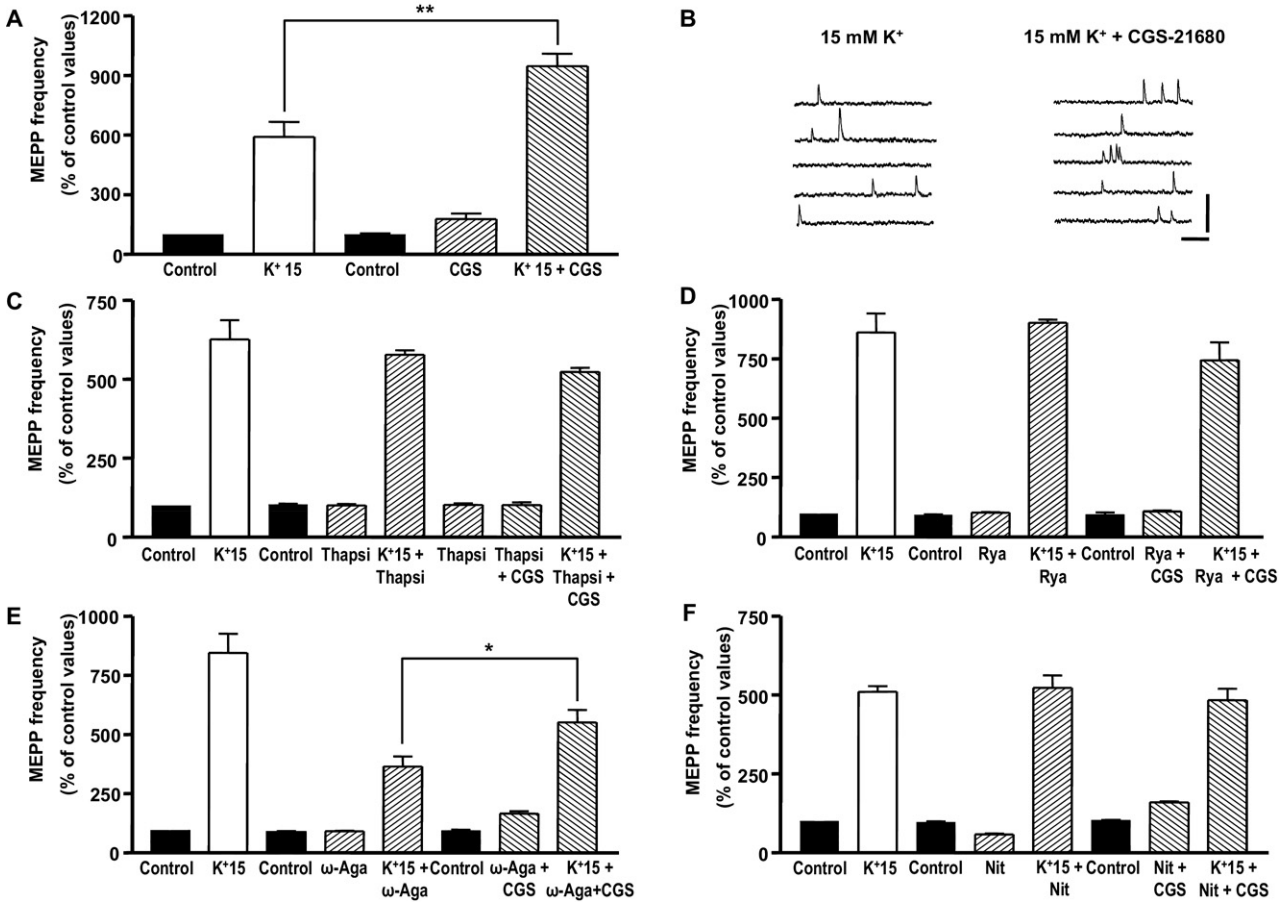

Fig. 6. Facilitatory effect of CGS-21680 upon K^+-evoked acetylcholine release. (A) CGS-21680 (CGS) increased ACh secretion when muscles were incubated in 15 mM K^+, $n=3$, ** $P<0.01$, ANOVA followed by Tukey's test. (B) Representative MEPPs recorded from diaphragm muscle fibers bathed in 15 mM K^+ (Vm: −58.4 mV), and in 15 mM K^++CGS-21680 (Vm: −57.7 mV). Calibration: 1 mV, 40 ms. (C) Depletion of intracellular calcium stores by thapsigargin (Thapsi) prevented CGS-21680 action in 15 mM K^+, $n=4$. (D) Blockade of RyRs with ryanodine (Rya) occluded the effect of CGS-21680 in high K^+, $n=4$. (E) The P/Q-type VDCC blocker, ω-Aga IVA (ω-Aga, 100 nM), decreased asynchronous ACh secretion in high K^+, but did not modify the facilitatory effect of CGS-21680, $n=4$, * $P<0.05$, ANOVA followed by Tukey's test. (F) The L-type VDCC antagonist, nitrendipine (Nit, 5 μM) reduced spontaneous ACh secretion (5 mM K^+) and occluded the CGS-21680-mediated facilitation in 15 mM K^+, $n=4$. Data (mean±SEM) are expressed as percentage of control values (black bars).

type VDCCs were associated. Moreover, similar experiments performed with both, thapsigargin and nitrendipine, in preparations depolarized by 15 mM K^+ did not reveal a summation of actions (data not shown). So, in order to approach this question, we investigated the possibility that, at high K^+ concentration, stimulation of A_{2A} receptors might increase Ca^{2+} influx through L-type VDCC which would provide the trigger for Ca^{2+} release from internal stores *via* the mechanism *Ca^{2+}-induced Ca^{2+} release* (CICR), increasing the exocytosis of synaptic vesicles. To evaluate this hypothesis we studied the effect of CGS-21680 upon K^+-evoked ACh release in the presence of $CdCl_2$ (100 μM). Fig. 7A shows that the universal VDCC blocker did not modify the modulatory effect of CGS-21680 in control saline (5 mM K^+, see Fig. 7D) and in high K^+ (15 mM K^++Cd^{2+} 149.6±10.9% of control values, 15 mM K^++Cd^{2+}+CGS-21680 217.0±21.3%, $n=4$, $P<0.001$, see Fig. 7E). Furthermore, similar results were observed in 5 mM K^+ (Fig. 7D) and 15 mM K^+ when extracellular Ca^{2+} was eliminated from the incubation medium (0Ca^{2+}-EGTA+$CdCl_2$, see methods): 15 mM K^++0Ca^{2+}-EGTA-Cd^{2+} 41.6±4.2% of control values, 15 mM K^++0Ca^{2+}-EGTA-Cd^{2+}+CGS-21680 67.9±9.1% of control values, $n=4$, $P<0.05$, Fig. 7B, E. These data suggest that the effect of the A_{2A} agonist in high K^+ is not associated to Ca^{2+} influx triggering release from stores *via* CICR. On the contrary, when experiments were performed in the presence of nitrendipine but in the absence of extracellular Ca^{2+}, the facilitation induced by CGS-21680 in control saline (5 mM K^+) persisted (Fig. 7D) while in high K^+ the effect was abolished (15 mM K^++nitrendipine+0Ca^{2+}-EGTA-Cd^{2+} 47.9±6.9% of control values, 15 mM K^++nitrendipine+0Ca^{2+}-EGTA-Cd^{2+}+CGS-21680 52.8±3.2% of control values, $n=4$, Fig. 7C, E), indicating that the action of nitrendipine cannot be a result of preventing Ca^{2+} entry.

DISCUSSION

The present results demonstrate that, at the mouse neuromuscular junction, the activation of A_{2A} adenosine re-

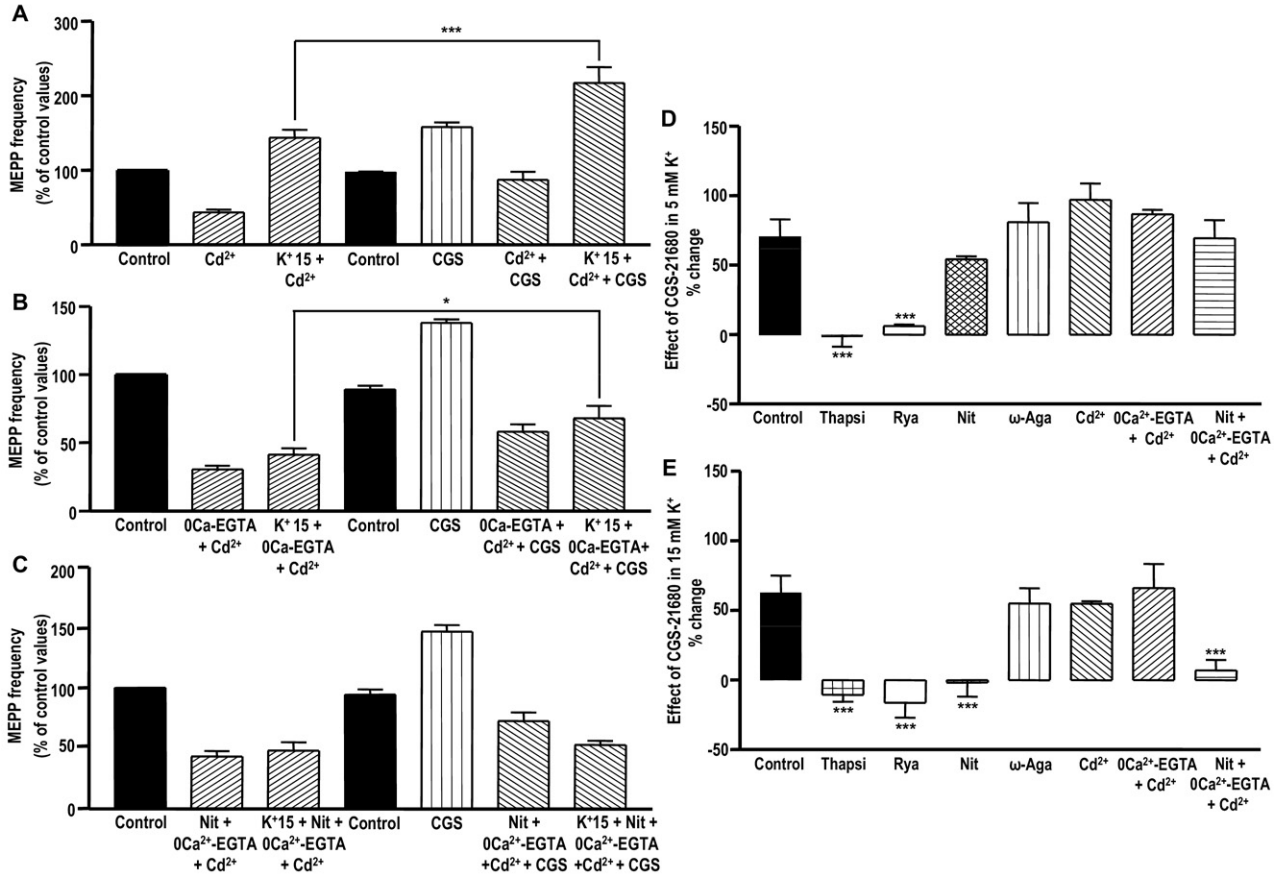

Fig. 7. CGS-21680-mediated modulation on asynchronous ACh secretion is not associated to Ca^{2+} influx through L-type VDCC. (A) and (B) The effect of CGS-21680 (CGS) upon K^+-evoked acetylcholine release was not altered in solutions containing Cd^{2+} (n=4) or $0Ca^{2+}$-EGTA-Cd^{2+} (n=4), respectively. (C) Nitrendipine (Nit) in the absence of extracellular calcium ($0Ca^{2+}$-EGTA+Cd^{2+}) prevented the action of CGS-21680 in high K^+ (n=4). (D) and (E) Summary bar graph showing the effect of CGS-21680 on spontaneous (5 mM K^+) and asynchronous ACh release (15 mM K^+) respectively, when preparations were incubated in control saline (n=5), or solutions containing thapsigargin (Thapsi, n=4), ryanodine (Rya, n=4), nitrendipine (Nit, n=4), ω-Aga IVA (ω-Aga, n=4), Cd^{2+} (n=4), $0Ca^{2+}$-EGTA-Cd^{2+}(n=4), and Nit+$0Ca^{2+}$-EGTA-Cd^{2+} (n=4). In (A–C), data (mean±SEM) are expressed as percentage of control values (black bars). * $P<0.05$, *** $P<0.001$, ANOVA followed by Tukey's test. In (D) and (E), each column (mean±SEM) depicts the effect of CGS-21680 on MEPP frequency, expressed as the percentage of change, *** $P<0.001$ (ANOVA followed by Dunnett's test).

ceptors by its specific agonist CGS-21680 facilitates spontaneous ACh secretion as result of an increase in cytosolic nerve terminal Ca^{2+} concentration due to release of this ion from thapsigargin-sensitive internal stores. Conversely, several lines of evidence indicate that recruitment of calcium from external pool does not contribute to the excitatory effect of CGS-21680 on MEPP frequency. So, CGS-21680-induced rise in MEPP frequency remained unaffected by the blockade of presynaptic VDCC with Cd^{2+} or in the absence of extracellular Ca^{2+} ($0Ca^{2+}$-EGTA). Additionally, at Figs. 6E, F and 7D, it is also observed that ω-Aga IVA and nitrendipine (specific P/Q-type and L-type VDCC blockers, respectively) did not affect the facilitatory action of CGS-21680 in physiological saline. These findings suggest that the A_{2A} agonist facilitates the secretory process at a step downstream from Ca^{2+} influx. Similar external calcium-independent mechanisms of presynaptic facilitation have been observed in the globus pallidus (Shindou et al., 2002). On the other hand, buffering the internal Ca^{2+} concentration with the membrane-permeable Ca^{2+} chelator BAPTA-AM effectively prevents the CGS-21680-induced MEPP frequency facilitation, suggesting that the effect of the A_{2A} agonist on spontaneous ACh release requires an increase in Ca^{2+} concentration within presynaptic neurons. In order to find out what the source of this Ca^{2+} was, we depleted the intracellular Ca^{2+} stores by incubating the muscles with the Ca^{2+}-ATPase inhibitor thapsigargin. In this condition, CGS-21680 (in $0Ca^{2+}$-EGTA or 2 mM Ca^{2+} solution) failed to elicit presynaptic facilitation of spontaneous secretion. Moreover, we found that the blockade of RyRs with ryanodine also occlude the action of the A2A agonist. Taken together, these results suggest that at resting conditions, the activation of A_{2A} receptors with CGS-21680 stimulates directly the thapsigargin/ryanodine-sensitive Ca^{2+} stores leading to an intracellular Ca^{2+} rise that in turn increases spontaneous ACh release.

Independently of the effect induced by CGS-21680 on internal Ca^{2+} stores and taking into account that this action is not related to external calcium, the possibility that

the A_{2A} agonist might additionally exert a direct modulation of the release machinery could not be rule out. Transmitter release can be induced by raising the tonicity of a superfusing solution (Fatt and Katz, 1952; Hubbard et al., 1968), a situation known to be independent of $[Ca^{2+}]_o$ (Furshpan, 1956; Hubbard et al., 1968; Rosenmund and Stevens, 1996; Losavio and Muchnik, 1997; Mochida et al., 1998; Kashani et al., 2001) but that seems to share the major elements of the basic Ca^{2+} triggered vesicle fusion (e.g. the soluble N-ethylmaleimide-sensitive factor attachment receptor [SNARE] proteins, Dreyer et al., 1987; Gansel et al., 1987; Aravamudan et al., 1999). Thus, hypertonicity is a useful tool for analyzing release mechanisms between the Ca^{2+}-triggering step and vesicle fusion (Grinnell et al., 2003). Our results demonstrated that CGS-21680 enhanced the peak and the area under the curve of the hypertonic response, even when the responses were studied in a calcium free-solution ($0Ca^{2+}$-EGTA). On the contrary, the action of CGS-21680 in hypertonicity was not observed when $[Ca^{2+}]_i$ was decreased by loading nerve terminals with BAPTA-AM, suggesting that an increase of the intracellular Ca^{2+} availability rather than a direct action on the release machinery is involved in the action of the A_{2A} agonist. We found that depletion of Ca^{2+} stores with thapsigargin or the blockade of RyRs with ryanodine, did not modify the hypertonic response *per se*, but precluded the action of CGS-21680 in hypertonicity. These results indicate that, as it was observed by other authors (Rosenmund and Stevens, 1996), hypertonicity-induced increase in MEPP frequency does not depend on Ca^{2+} release from intracellular stores and that the A_{2A} receptor-mediated facilitatory effect depends on calcium mobilization from thapsigargin/ryanodine-sensitive endoplasmic stores.

When studying the effect of A_{2A} receptor activation upon ACh release induced by K^+ depolarization, our results demonstrated that CGS-21680 increased neurotransmitter secretion by ~63% with respect to the ACh released in high K^+ without the A_{2A} agonist (see Fig. 7E). At rat phrenic nerve-diaphragm preparations, Correia-de-Sá et al. (2000) found that, with brief stimulation pulses (5 Hz, 0.04 ms), both thapsigargin and nifedipine attenuated the CGS-21680 facilitatory effect on [^{3}H]ACh release, whereas with long stimulation pulses (5 Hz, 1 ms), the modulatory action of the A_{2A} agonist was reduced by pretreatment with the P-type and L-type VDCC blockers. Our results showed that depletion of intracellular Ca^{2+} stores with thapsigargin completely abolished the CGS-21680-induced modulation in high K^+ concentration. Furthermore, the same result was observed when Ca^{2+} release from endoplasmic reticulum through RyRs was blocked by ryanodine. On the other hand, the blockade of presynaptic P/Q-type VDCCs with ω-Aga IVA significantly reduced ACh release induced by K^+ depolarization, but did not affect the facilitatory action of CGS-21680 on this type of secretion, producing an increase in MEPP frequency of about 55% (Fig. 7E).

L-type VDCCs seem to be located away from the active zones (Tsien et al., 1988; Robitaille et al., 1990) and have been shown to be involved in spontaneous ACh release from mammalian motor nerve terminals in the presence of physiological concentration of extracellular KCl (Losavio and Muchnik, 1997). Although L-type VDCCs do not primarily participate in the ACh secretion induced by electrical or K^+ depolarization, they appear to be recruited when nerve terminals have been treated chronically with immunoglobulin from patients with Lambert–Eaton syndrome (Flink and Atchison, 2002) or amyotrophic lateral sclerosis (Frantantoni et al., 2000), at reinnervating motor end-plates (Katz et al., 1996) or during functional recovery from neuromuscular paralysis produced by botulinum toxin type A intoxication (Santafé et al., 2000). Our findings indicated that nitrendipine did not affect ACh release induced by K^+, but prevented completely the facilitatory action of CGS-21680 on this type of secretion. This suggests that DHP-sensitive L-type VDCCs are involved in the mechanism action of the A_{2A} agonist upon nerve terminals depolarized by high K^+. The results obtained with thapsigargin and nitrendipine in the K^+ experiments resemble those found by Correia-de-Sá et al. (2000) when they evaluated [^{3}H]ACh release with brief stimulation pulses. However, in our case, both thapsigargin and nitrendipine completely, and not partially as those authors described, occluded the CGS-21680 modulatory effect on K^+ evoked release, which supports the idea that the effects of the A_{2A} agonist on thapsigargin-sensitive internal stores and L-type VDCC are associated and that they are not individual targets. This discrepancy could be due mainly to differences in the experimental protocols (ACh release evoked by electrical stimulation vs. induced by high K^+) or it may be due to differences between species.

The mechanisms for mobilizing Ca^{2+} from internal stores are not known in all types of synapses, and it is often assumed that activation of CICR may occur in response to Ca^{2+} influx through VDCC. Interestingly, our data evidenced that the facilitatory effect of CGS-21680 in high K^+ remained unaffected when preparation were incubated with $CdCl_2$ in presence or absence of extracellular Ca^{2+} ($0Ca^{2+}$-EGTA), suggesting that a mechanism related to CICR was not involved in the modulatory action of CGS-21680 on ACh release induced by K^+. In contrast, when activation of A_{2A} receptors was studied in the presence of nitrendipine in a $0Ca^{2+}$-EGTA-Cd^{2+} solution the excitatory effect was not evident (Fig. 7E). How to explain that nitrendipine abolished the effect of CGS-21680 in high K^+ while Cd^{2+}/$0Ca^{2+}$-EGTA failed to do that? The main difference is that nitrendipine, as DHP, acts on L-type VDCCs by immobilizing the gating charges movements (Schneider and Chandler, 1973; Chameau et al., 1995), whereas Cd^{2+} blocks the ionic pore without affecting the gating mechanism (Nachshen, 1984; Lansman et al., 1986). In hypothalamic magnocellular neurons, De Crescenzo et al. (2006) found that depolarization of nerve terminals in the absence of extracellular Ca^{2+} elicited Ca^{2+} release from intraterminal stores *via* a process they designed as *voltage-induced Ca^{2+} release* (VICaR), which was blocked by nifedipine but not by Cd^{2+}. Calcium released from endoplasmic reticulum by this mechanism is called syntilla and it occurs through type 1 ryanodine receptors (De Crescenzo et al., 2006; see rev. Collin et al.,

2005; Berridge, 2006). Co-immunoprecipitation studies would support the idea of a direct physical interaction between type 1 ryanodine receptors and DHP receptors (Anderson, 1998; Mouton et al., 2001; Ouardouz et al., 2003; De Crescenzo et al., 2006), similar to that found in skeletal muscle (Coronado et al., 2004). At neuromuscular junction a mechanism related to VICaR was not yet described; however, it is possible that this system may be involved in the facilitatory action of the A_{2A} agonist on ACh release induced by K^+: CGS-21680 may increase L-type VDCC activation, not in its role as channel to allow Ca^{2+} entry and trigger release from internal stores *via* CICR, instead by increasing their gating charge movements and providing the mechanical displacement to interact with the RyRs of the endoplasmic reticulum resulting in the opening of these receptors and Ca^{2+} release to the cytosol. The increment of Ca^{2+} concentration within the nerve terminal would be sensed by the release machinery, thus producing an increase of ACh secretion.

CONCLUSION

Taken collectively, all the data obtained in this paper, we can conclude that, at the mouse neuromuscular junction, the activation of A_{2A} adenosine receptors facilitates spontaneous and asynchronous ACh secretion as result of an increase in cytosolic nerve terminal Ca^{2+} concentration due to release of this ion from thapsigargin/ryanodine-sensitive internal stores. According to our experiments, Ca^{2+} coming from endoplasmic reticulum does not contribute for triggering ACh secretion at basal conditions, during the hypertonic response and when nerve terminal are depolarized by high K^+, suggesting that silent systems may become evident under the modulation exerted by the activation of A_{2A} receptors. On the other hand, the mechanism by which this internal Ca^{2+} source is activated is different depending on the type of secretion. Thus, during spontaneous ACh release, activation of the A_{2A} receptors seems to stimulate directly the thapsigargin-sensitive Ca^{2+} stores acting on ryanodine receptors. The same system would be used by the A_{2A} agonist to modulate hypertonic response. Conversely, upon K^+ depolarization, activation of A_{2A} receptors might increase conformational changes in L-type VDCCs that would generate intramembrane charge movements which could cause the opening of RyRs (type 1?), allowing Ca^{2+} release to the cytosol and exocytosis facilitation.

Acknowledgments—We thank Mrs. María Fernanda Rodriguez and Mrs. Raquel Almirón for technical assistance. The research was supported by grants from CONICET (PIP 5919) and UBACYT (M041).

REFERENCES

Anderson NG (1998) Co-immunoprecipitation. Identification of interacting proteins. Methods Mol Biol 88:35–45.

Aravamudan B, Fergestad T, Davis WS, Rodesch CK, Broadie K (1999) *Drosophila* Unc-13 is essential for synaptic transmission. Nat Neurosci 2:965–971.

Augustine GJ, Charlton MP, Smith SJ (1987) Calcium action in synaptic transmitter release. Annu Rev Neurosci 10:633–693.

Berridge MJ (2006) Calcium microdomains: organization and function. Cell Calcium 40:405–412.

Blaustein MP, Ratzlaff RW, Kendrick NK (1978) The regulation of intracellular calcium in presynaptic nerve terminals. Ann N Y Acad Sci 307:195–212.

Bowersox SS, Miljanich GP, Sugiura Y, Li C, Nadasdi L, Hoffman BB, Ramachandran J, Ko CP (1995) Differential blockade of voltage-sensitive calcium channels at the mouse neuromuscular junction by novel omega-conopeptides and omega-agatoxin-IVA. J Pharmacol Exp Ther 273:248–256.

Braga MF, Rowan EG (1994) The pharmacological effects of cadmium on skeletal neuromuscular transmission. Gen Pharmacol 25: 1729–1739.

Chameau P, Bournaud R, Shimahara T (1995) Asymmetric intramembrane charge movement in mouse hippocampal pyramidal cells. Neurosci Lett 201:159–162.

Chen B-M, Grinnell AD (1997) Kinetics, Ca^{2+} dependence, and biophysical properties of integrin-mediated mechanical modulation of transmitter release from frog motor nerve terminals. J Neurosci 17:904–916.

Collin T, Marty A, Llano I (2005) Presynaptic calcium stores and synaptic transmission. Curr Opin Neurobiol 15:275–281.

Cooper GP, Manalis RS (1984) Cadmium: effects on transmitter release at the frog neuromuscular junction. Eur J Pharmacol 99:251–256.

Coronado R, Ahern CA, Sheridan DC, Cheng W, Carbonneau L, Bhattacharya D (2004) Functional equivalence of dihydropyridine receptor alpha1S and beta1a subunits in triggering excitation-contraction coupling in skeletal muscle. Biol Res 37:565–575.

Correia-de-Sá P, Sebastião AM, Ribeiro JA (1991) Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve endings of the rat. Br J Pharmacol 103:1614–1620.

Correia-de-Sá P, Timóteo MA, Ribeiro JA (1996) Presynaptic A_1 inhibitory/A_{2A} facilitatory adenosine receptor activation balance depends on motor nerve stimulation paradigm at the rat hemidiaphragm. J Neurophysiol 76:3910–3919.

Correia-de-Sá P, Timóteo MA, Ribeiro JA (2000) A_{2A} adenosine receptor facilitation of neuromuscular transmission: influence of stimulus paradigm on calcium mobilization. J Neurochem 74:2462–2469.

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

Cunha RA, Sebastião AM (1993) Adenosine and adenine nucleotides are independently released from both the nerve terminals and the muscle fibres upon electrical stimulation of the innervated skeletal muscle of the frog. Pflügers Arch 424:503–510.

De Crescenzo V, Fogarty KE, ZhuGe R, Tuft RA, Lifshitz LM, Carmichael J, Bellvé KD, Baker SP, Zissimopoulos S, Lai FA, Lemos JR, Walsh JV Jr (2006) Dihydropyridine receptors and type 1 ryanodine receptors constitute the molecular machinery for voltage-induced Ca^{2+} release in nerve terminals. J Neurosci 26: 7565–7574.

Deitcher DL, Ueda A, Stewart BA, Burgess RW, Kidokoro Y, Schwarz TL (1998) Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the Drosophila gene neuronal-synaptobrevin. J Neurosci 18:2028–2039.

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

Dreyer F, Rosenberg F, Becker C, Bigalke H, Penner R (1987) Differential effects of various secretagogues on quantal transmitter release from mouse motor nerve terminals treated with botulinum A

and tetanus toxin. Naunyn Schmiedebergs Arch Pharmacol 335:1–7.
Emptage NJ, Reid CA, Fine A (2001) Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca^{2+} entry, and spontaneous transmitter release. Neuron 29: 197–208.
Fatt P, Katz B (1952) Spontaneous subthreshold activity at the motor nerve endings. J Physiol 117:109–128.
Flink MT, Atchison WD (2002) Passive transfer of Lambert-Eaton syndrome to mice induces dihydropyridine sensitivity of neuromuscular transmission. J Physiol 543:567–576.
Frantantoni SA, Weisz G, Pardal AM, Reisin RC, Uchitel OD (2000) Amyotrophic lateral sclerosis IgG-treated neuromuscular junctions develop sensitivity to L-type calcium channel blocker. Muscle Nerve 23:543–550.
Furshpan EJ (1956) The effects of osmotic pressure changes on the spontaneous activity at motor nerve endings. J Physiol 134: 689–697.
Gansel M, Penner R, Dreyer F (1987) Distinct sites of action of clostridial neurotoxins revealed by double-poisoning of mouse motor nerve terminals. Pflügers Arch 409:533–539.
Grinnell AD, Chen BM, Kashani A, Lin J, Suzuki K, Kidokoro Y (2003) The role of integrins in the modulation of neurotransmitter release from motor nerve terminals by stretch and hypertonicity. J Neurocytol 32:489–503.
Hamilton BR, Smith DO (1991) Autoreceptor-mediated purinergic and cholinergic inhibition of motor nerve terminal calcium currents in the rat. J Physiol 432:327–341.
Hua S-Y, Raciborska DA, Trimble WS, Charlton MP (1998) Different VAMP/synaptobrevin complexes for spontaneous and evoked transmitter release at the crayfish neuromuscular junction. J Neurophysiol 80:3233–3246.
Hubbard JI, Jones SF, Landau EM (1968) An examination of the effects of osmotic pressure changes upon transmitter release from mammalian motor nerve terminals. J Physiol 197:639–657.
Kanzaki M, Zhang YQ, Mashima H, Li L, Shibata H, Kojima I (1999) Translocation of a calcium-permeable cation channel induced by insulin-like growth factor-I. Nat Cell Biol 1:165–170.
Kashani AH, Chen B-M, Grinnell AD (2001) Hypertonic enhancement of transmitter release from frog motor nerve terminals: Ca^{2+} independence and role of integrins. J Physiol 530:243–252.
Katz E, Ferro PA, Weisz G, Uchitel OD (1996) Calcium channels involved in synaptic transmission at the mature and regenerating mouse neuromuscular junction. J Physiol 497:687–697.
Katz E, Protti DA, Ferro PA, Rosato Siri MD, Uchitel OD (1997) Effects of Ca^{2+} channel blocker neurotoxins on transmitter release and presynaptic currents at the mouse neuromuscular junction. Br J Pharmacol 121:1531–1540.
Kidokoro Y (2003) Roles of SNARE proteins and synaptotagmin I in synaptic transmission: studies at the Drosophila neuromuscular synapse. Neurosignals 12:13–30.
Koenig JH, Ikeda K (1999) Contribution of active zone subpopulation of vesicles to evoked and spontaneous release. J Neurophysiol 81:1495–1505.
Lansman JB, Hess P, Tsien RW (1986) Blockade of current through single calcium channels by Cd^{2+}, Mg^{2+}, and Ca^{2+}. Voltage and concentration dependence of calcium entry into the pore. J Gen Physiol 88:321–347.
Liou J-C, Tsai F-Z, Ho S-Y (2003) Potentiation of quantal secretion by insulin-like growth factor-1 at developing motoneurons in Xenopus cell culture. J Physiol 553:719–728.
Losavio A, Muchnik S (1997) Spontaneous acetylcholine release in mammalian neuromuscular junction. Am J Physiol 273:C1835–C1841.
Meissner G (1986) Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. J Biol Chem 261: 6300–6306.
Meriney SD, Grinnell AD (1991) Endogenous adenosine modulates stimulation-induced depression at the frog neuromuscular junction. J Physiol 443:441–455.
Mochida S, Charles T, Yokoyama CT, Kim DK, Itoh K, Catterall WA (1998) Evidence for a voltage-dependent enhancement of neurotransmitter release mediated via the synaptic protein interaction site of N-type Ca^{2+} channels. Proc Natl Acad Sci U S A 95: 14523–14528.
Mouton J, Marty I, Villaz M, Feltz A, Maulet Y (2001) Molecular interaction of dihydropyridine receptors with type-1 ryanodine receptors in rat brain. Biochem J 354:597–603.
Nachshen DA (1984) Selectivity of the Ca binding site in synaptosome Ca channels. Inhibition of Ca influx by multivalent metal cations. J Gen Physiol 83:941–967.
Nilson R, Volle RL (1976) Blockade by cadmium (Cd2+) of transmitter release at mouse neuromuscular junction. Fed Proc Fed Am Soc Exp Biol 35:696.
Nishimura M, Tsutsui I, Yagasaki O, Yanagiya I (1984) Transmitter release at the mouse neuromuscular junction stimulated by cadmium ions. Arch Int Pharmacodyn Ther 271:106–121.
Oliveira L, Timóteo LA, Correia-de-Sá P (2004) Tetanic depression is overcome by tonic adenosine A_{2A} receptor facilitation of L-type Ca^{2+} influx into rat motor nerve terminals. J Physiol 560:157–168.
Ouardouz M, Nikolaeva MA, Coderre E, Zamponi GW, McRory JE, Trapp BD, Yin X, Wang W, Woulfe J, Stys PK (2003) Depolarization-induced Ca^{2+} release in ischemic spinal cord white matter involves L-type Ca^{2+} channel activation of ryanodine receptors. Neuron 40:53–63.
Protti DA, Uchitel OD (1993) Transmitter release and presynaptic Ca^{2+} currents blocked by the spider toxin omega-Aga-IVA. Neuroreport 5:333–336.
Rahamimoff R, Alnaes E (1973) Inhibitory action of ruthenium red on neuromuscular transmission. Proc Natl Acad Sci U S A 70: 3613–3616.
Redman RS, Silinsky EM (1994) ATP relesed together with acetylcholine as the mediator of neuromuscular depression at frog motor nerve endings. J Physiol 477:117–127.
Ribeiro JA, Sebastião AM (1987) On the role, inactivation, and origin of endogenous adenosine at the frog neuromuscular junction. J Physiol 384:571–585.
Robitaille R, Adler EM, Charlton MP (1990) Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. Neuron 5:773–779.
Rosenmund C, Stevens CF (1996) Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron 16: 1197–1207.
Santafé MM, Urbano FJ, Lanuza MA, Uchitel OD (2000) Multiple types of calcium channels mediate transmitter release during functional recovery of botulinum toxin type A-poisoned mouse motor nerve terminals. Neuroscience 95:227–234.
Schneider MF, Chandler WK (1973) Voltage dependent charge movement of skeletal muscle: a possible step in excitation-contraction coupling. Nature 242:244–246.
Shindou T, Nonaka H, Richardson PJ, Mori A, Kase H, Ichimura M (2002) Presynaptic adenosine A_{2A} receptors enhance GABAergic synaptic transmission via a cyclic AMP dependent mechanism in the rat globus pallidus. Br J Pharmacol 136:296–302.
Silinsky EM (1975) On the association between transmitter secretion and the release of adenine nucleotides from mammalian motor nerve terminals. J Physiol 247:145–162.
Silinsky EM (2004) Adenosine decreases both presynaptic calcium currents and neurotransmitter release at the mouse neuromuscular junction. J Physiol 558:389–401.
Silinsky EM (2005) Modulation of calcium currents is eliminated after cleavage of a strategic component of the mammalian secretory apparatus. J Physiol 566:681–688.

Smith DO (1991) Sources of adenosine released during neuromuscular transmission in the rat. J Physiol 432:343–354.

Tempia F, Alojado ME, Strata P, Knopfel T (2001) Characterization of the mGluR(1)-mediated electrical and calcium signaling in Purkinje cells of mouse cerebellar slices. J Neurophysiol 86: 1389–1397.

Thastrup O, Cullen PJ, Drøbak BK, Hanley MR, Dawson AP (1990) Thapsigargin, a tumor promoter discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci U S A 87:2466–2470.

Tsien RW, Lipscombe D, Madison DV, Bley KR, Fox AP (1988) Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci 11:431–438.

Veggetti M, Muchnik S, Losavio A (2008) Effect of purines on calcium-independent acetylcholine release at the mouse neuromuscular junction. Neuroscience 154:1324–1336.

(Accepted 5 October 2010)
(Available online 2 November 2010)