Neuroscience 154 (2008) 1324–1336

EFFECT OF PURINES ON CALCIUM-INDEPENDENT ACETYLCHOLINE RELEASE AT THE MOUSE NEUROMUSCULAR JUNCTION

M. VEGGETTI, S. MUCHNIK AND A. LOSAVIO*

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

Abstract—At the mouse neuromuscular junction, activation of adenosine A_1 and P2Y receptors inhibits acetylcholine release by an effect on voltage dependent calcium channels related to spontaneous and evoked secretion. However, an effect of purines upon the neurotransmitter-releasing machinery downstream of Ca^{2+} influx cannot be ruled out. An excellent tool to study neurotransmitter exocytosis in a Ca^{2+}-independent step is the hypertonic response. Intracellular recordings were performed on diaphragm fibers of CF1 mice to determine the action of the specific adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyl-adenosine (CCPA) and the $P2Y_{12\text{-}13}$ agonist 2-methylthio-adenosine 5′-diphosphate (2-MeSADP) on the hypertonic response. Both purines significantly decreased such response (peak and area under the curve), and their effect was prevented by specific antagonists of A_1 and $P2Y_{12\text{-}13}$ receptors, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and *N*-[2-(methylthioethyl)]-2-[3,3,3-trifluoropropyl]thio-5′-adenylic acid, monoanhydride with dichloromethylenebiphosphonic acid, tetrasodium salt (AR-C69931MX), respectively. Moreover, incubation of preparations only with the antagonists induced a higher response compared with controls, suggesting that endogenous ATP/ADP and adenosine are able to modulate the hypertonic response by activating their specific receptors. To search for the intracellular pathways involved in this effect, we studied the action of CCPA and 2-MeSADP in hypertonicity in the presence of inhibitors of several pathways. We found that the effect of CPPA was prevented by the calmodulin antagonist *N*-(6-aminohexil)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) while that of 2-MeSADP was occluded by the protein kinase C antagonist chelerythrine and W-7. On the other hand, the inhibitors of protein kinase A (*N*-(2[pbromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide, H-89) and phosphoinositide-3 kinase (PI3K) (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride, LY-294002) did not modify the modulatory action in hypertonicity of both purines. Our results provide evidence that activation of A_1 and $P2Y_{12\text{-}13}$ receptors by CCPA and 2-MeSADP inhibits ACh release from mammalian motor nerve terminals through an effect on a Ca^{2+}-independent step in the cascade of the exocytotic process. Since presynaptic calcium channels are intimately associated with components of the synaptic vesicle docking and fusion processes, further experiments could clarify if the actions of purines on calcium channels and on secretory machinery are related.

Key words: CCPA, 2-MeSADP, Ca^{2+}-independent mechanism, hypertonic response, mammalian neuromuscular junction.

Synaptic transmission is under the control of extracellular purines, which through an interplay between their own receptors and the steps involved in the process of exocytosis modulate the neuronal activity. At mammalian neuromuscular junction, ATP is co-released with the neurotransmitter 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). It was demonstrated that both purines, ATP and adenosine, inhibit transmitter release operating via presynaptic P2 and P1 receptors, respectively (Sebastião and Ribeiro, 2000; Sokolova et al., 2003; De Lorenzo et al., 2004, 2006). On the other hand, it is interesting to note that purines could also be released from activated muscle fibers (Santos et al., 2003; Smith, 1991) and from peri-synaptic Schwann cells (Liu et al., 2005; discussed in Todd and Robitaille, 2006).

The mechanism of the depressant action of the purines differs depending on presynaptic terminals and on species. At frog motor nerve terminals, the inhibitory effect of adenosine upon evoked and spontaneous neurotransmitter release is not related to a reduction in calcium influx through N-type voltage-dependent calcium channels (VDCC), suggesting that adenosine exerts its action at a site distal to the locus of calcium entry (Silinsky, 1984; Silinsky and Solsona, 1992; Redman and Silinsky, 1994; Huang et al., 2002). On the other hand, it was demonstrated that in this species, the presynaptic action of ATP is mediated by the inhibition of Ca^{2+} channels and by a mechanism acting downstream of Ca^{2+} entry (Grishin et al., 2005). At mammalian neuromuscular junctions, adenosine was found to decrease the extracellularly recorded Ca^{2+} current in response to nerve stimulation (Hamilton and Smith, 1991; Silinsky, 2004) and we have demonstrated that the nucleoside and the specific adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyl-adenosine (CCPA) exert their modulatory role by decreasing the nitrendipine-sensitive component of miniature end-plate potential (MEPP) frequency through a mechanism related to the action of Ca^{2+}-calmodulin (De Lorenzo et al., 2004). However, Silinsky (2005) found that modulation of calcium currents by

*Corresponding author. Tel: +54-11-4514-8701x160; fax: +54-11-4523-8947.
E-mail address: idimneurofisio@gmail.com or adrianalosavio@gmail.com (A. Losavio).

Abbreviations: AR-C69931MX, *N*-[2-(methylthioethyl)]-2-[3,3,3-trifluoropropyl]thio-5′-adenylic acid, monoanhydride with dichloromethylenebiphosphonic acid, tetrasodium salt; CCPA, 2-chloro-N^6-cyclopentyl-adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; H-89, *N*-(2[p-bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide; LY-294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride; MEPP, miniature end-plate potential; PI3K, phosphoinositide-3 kinase; PKA, protein kinase A; PKC, protein kinase C; VDCC, voltage-dependent calcium channel; W-7, *N*-(6-aminohexil)-5-chloro-1-naphthalenesulfonamide hydrochloride; 2-MeSADP, 2-methylthio-ADP; $\beta\gamma$-imido ATP, adenosine 5′-(β,γ-imido)triphosphate tetralithium salt hydrate.

0306-4522/08$32.00+0.00 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2008.04.056

adenosine cannot occur when the SNARE syntaxin is cleaved. Recently, we have found that ATP and the slowly hydrolysable ATP analog adenosine 5′-(β,γ-imido)triphosphate tetralithium salt hydrate($\beta\gamma$-imido ATP) decreased spontaneous secretion by a mechanism that involves the reduction of Ca^{2+} entry through the calcium channels related to spontaneous secretion, L-type and N-type VDCC (Losavio and Muchnik, 1997) by activating P2Y receptors. Both, Cd^{2+} and the combined application of nitrendipine and ω-conotoxin GVIA, occluded the effect of the nucleotide (De Lorenzo et al., 2006).

Independently of the effects induced by adenosine and adenine nucleotides on calcium channels at mammalian motor nerve terminals, a concomitant action of the purines on the neurotransmitter-releasing machinery downstream of Ca^{2+} influx cannot be ruled out. An excellent tool to study neurotransmitter exocytosis in a Ca^{2+}-independent step is the hypertonic response. When nerve terminals are exposed to hypertonic solutions a marked increase in spontaneous quantal neurotransmitter release occurs (Fatt and Katz, 1952; Hubbard et al., 1968; Kita and Van der Kloot, 1977; Niles and Smith, 1982; Bourque and Renaud, 1984; Doherty et al., 1986; Brosius et al., 1992; Yu and Miller, 1995; Rosenmund and Stevens, 1996; Losavio and Muchnik, 1997; Mochida et al., 1998). This enhancement is not dependent on Ca^{2+} entry (Furshpan, 1956; Hubbard et al., 1968; Blioch et al., 1968; Quastel et al., 1971; Kita and Van der Kloot, 1977). We have demonstrated that at rat neuromuscular junctions, hypertonic response is not affected by nifedipine, ω-conotoxin-GVIA or ω-agatoxin-IVA, selective antagonists of L type-, N type- and P/Q type VDCC, respectively (Losavio and Muchnik, 1997).

In order to understand the mechanism/s involved in the depressant action of purines on mouse motor nerve endings, we have examined the effect of the specific adenosine A_1 receptor agonist CCPA and the $P2Y_{12\text{-}13}$ agonist 2-methylthio-ADP (2-MeSADP) upon hypertonicity-induced transmitter release. The results demonstrated that both purinergic agents decreased the hypertonic response suggesting that a mechanism acting in a Ca^{2+}-independent step in the cascade of the exocytotic process is also involved in their modulatory action.

EXPERIMENTAL PROCEDURES

Preparations and solutions

CF1 mouse diaphragm muscles were used. Mice (30–40 g) were anesthetized with sodium thiopental (50 mg kg^{-1}) intraperitoneally and the left hemidiaphragm was excised and transferred to a 5 ml chamber superfused (3 ml min^{-1}) with Ringer Krebs solution (mM: NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 11, Hepes 5, pH 7.3–7.4, bubbled with O_2). Hyperosmotic media were freshly prepared by adding 100 mM sucrose to Ringer solutions and their osmolarity was checked with a Fiske osmometer before each experiment.

Electrophysiological recordings

MEPPs were recorded at the end-plate region of the muscle fibers using borosilicate glass microelectrodes (WP Instruments, Sarasota, FL, USA) with a resistance of 5–10 MΩ filled with 3 M KCl. Muscle fibers with a resting membrane potential less negative than −60 mV or MEPPs with a rise time greater than 1 ms were rejected. In each experimental group, the muscles were allowed to equilibrate in the respective solution for at least 20 min after observing that MEPPs represented a period of stable spontaneous release.

To study the time course of hyperosmotic response, first 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 (Sigma, St. Louis, MO, USA) was added to hypertonic solutions to prevent the muscle from twitching violently, which otherwise occurred upon sudden exposure of preparations to hypertonic solutions. Data frequencies were measured by hand from the screen of the oscilloscope or acquired through an A/D converter (Digidata 1322A; Axon Instruments Inc. Sunnyvale, CA, USA) controlled by computer and analyzed using pClamp-8.2 (Axon Instruments). The experiments were carried out at room temperature (22–23 °C). At this range of temperature MEPP frequency does not show important changes, while above 30 °C MEPP frequency rapidly increases with temperature, so that small changes in the bath temperature are associated with a big jump in secretion, making the measurements more variable (Liley, 1956; Li, 1958; Hubbard et al., 1971).

Data analysis

In results, figures represent mean±S.E.M. and n expresses number of animals (only the left hemidiaphragm was used from each mouse for a given experiment). To overcome the problem of variability associated with spontaneous release of different muscles, in each experiment the peak of the hypertonic response and the area under the curve of the test solution were expressed as percentage of the same parameters obtained in control response. Areas under the curve were calculated using Prism (version 3.02). Statistical significance of differences between means was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered to be significant when $P<0.05$ (*).

Chemicals

CCPA (500 nM), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1 μM), 2-MeSADP (150 nM), *N*-(2[p-bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide (H-89, 1 μM), chelerythrine (5 μM), *N*-(6-aminohexil)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, 10 μM) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY-294002, 100 μM) were purchased from Sigma, whereas forskolin (20 μM) was acquired from Alomone Laboratories, Jerusalem, Israel. *N*-[2-(methylthioethyl)]-2-[3,3,3-trifluoropropyl]thio-5′-adenylic acid, monoanhydride with dichloromethylenebiphosphonic acid, tetrasodium salt (AR-C69931MX, 1 μM) was kindly provided by The Medicines Company, MA, USA.

RESULTS

Activation of adenosine A_1 receptors decreases the hypertonic response

In a previous paper we have found that adenosine (100 μM) and the specific AD A_1 receptor agonist CCPA (500 nM) reduce spontaneous secretion and asynchronous acetylcholine release induced by 10 mM K^+ at mice motor nerve terminals via a mechanism that involves the L-type and P/Q-type VDCC, respectively (De Lorenzo et al., 2004). In order to

investigate whether the depressant action of adenosine occurs concomitantly by inhibition of a step related to the neurotransmitter-releasing machinery downstream of Ca^{2+} influx, we studied the effect of CCPA 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 so-

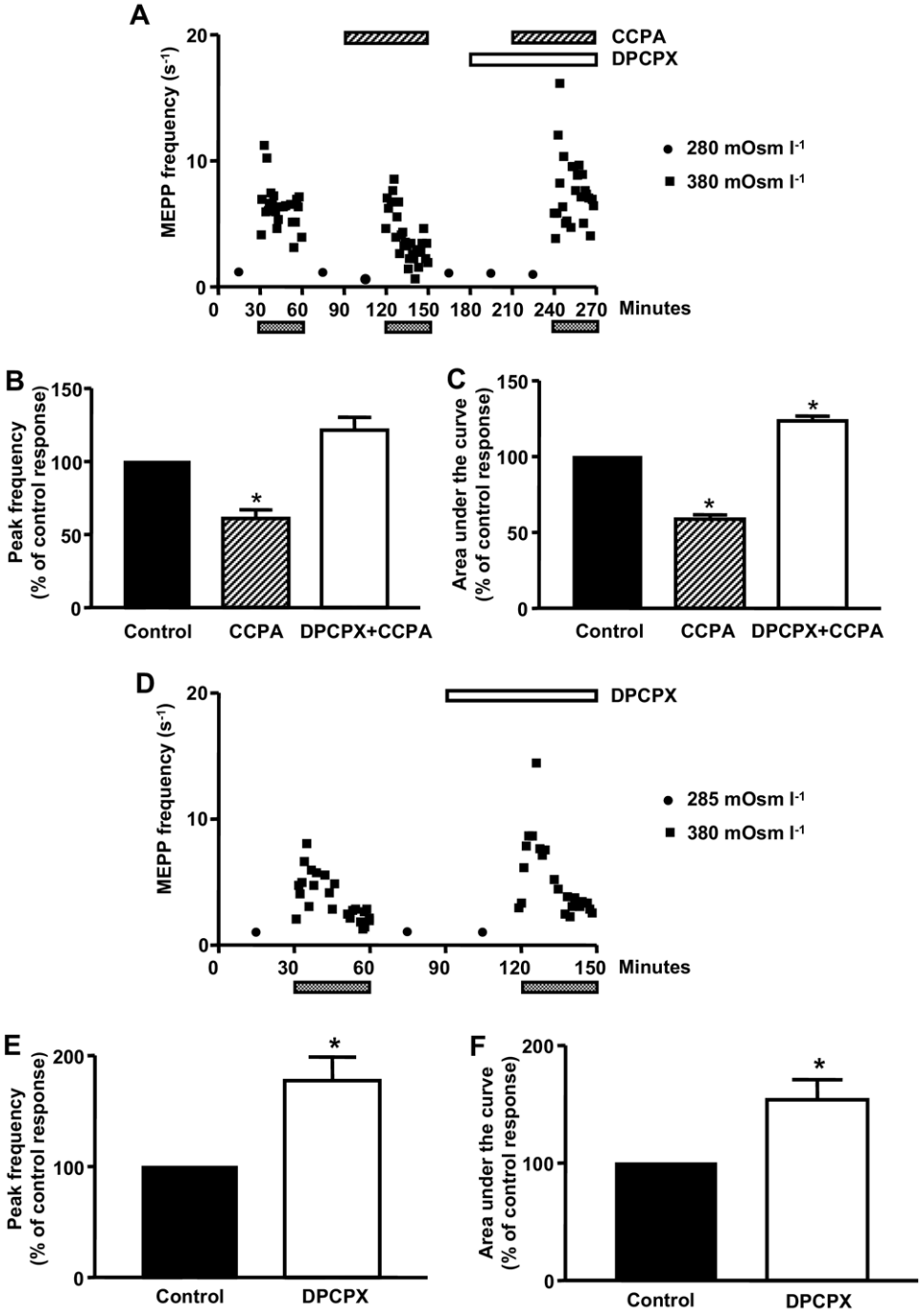

Fig. 1. (A) Effect of CCPA and DPCPX+CCPA on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic condition. In this and in the next figures circle symbols indicate mean values from 10 synapses obtained after exposing the preparations to isotonic conditions and square symbols represent the time course of hypertonic response (each point represents averaged value of MEPP frequency recorded during 10 s from a single synapse). Hypertonic Ringer solution containing sucrose was applied during the period indicated by the horizontal bars below *x* axis. (B, C) Summary histograms showing the depressant effect of CCPA on the peak frequency and area under the curve of the hypertonic response and their reversion in the presence of DPCPX. Data are expressed as percentage of control response. (D) Effect of DPCPX on ACh release when a preparation was exposed to isotonic and hypertonic condition. (E, F) Summary histograms showing the increase of the peak and area under the curve when muscles were incubated in DPCPX. * $P<0.05$.

lution was applied to diaphragm muscles, MEPP frequency increased from a frequency of 1.07±0.03/s in isotonic condition to a peak of 10.3±0.3/s, and declined gradually during continuous application of the hypertonic solution, being the area under the curve of 165.6±11.6 (n=4, see Fig. 1A). After complete washout of the preparation with isotonic solution MEPP frequency returned to control values. The addition of CCPA to the preparations induced a decrease of MEPP frequency in isotonic as well as in hypertonic condition. In the first situation, CCPA reduced spontaneous secretion to 0.56±0.03/s (52.6±1.9% of control values, P<0.05), and in hypertonic condition, CCPA depressed the peak of the response to 6.17±0.88/s (59.8±6.8% of the control responses, P<0.05) and the area under the curve to 98.3±10.7 (58.9±2.8% of the control responses, P<0.05). To verify that this effect was due to the activation of adenosine A_1 receptors, now the preparations were washed and then incubated with CCPA in the presence of the selective A_1 receptor antagonist DPCPX (0.1 μM). DPCPX prevented the effect of CCPA in isotonicity (96.2±6.9% of the control values) and hypertonicity, being the peak of the hypertonic response 121.4±8.9% of control response and the area under the curve 123.4±3.2%, P<0.05 (Fig. 1A, B and C). In order to rule out that the reduced peak during the second application of hypertonic solution could simply indicate rundown of releasing machinery, two consecutive hypertonic responses in control solutions were performed. No statistically significant differences were found when analyzing the peak and the area under the curve of both responses indicating a genuine effect of the nucleoside in hypertonicity. Moreover, a third hypertonic response in control solution showed similar results (peak of the hypertonic response: 2nd 92.3±4.9% and 3rd 100.2±4.2% of the 1st response; area under the curve: 2nd 103.4±7.8% and 3rd 107.0±3.8% of the 1st response).

It is interesting to note that the hypertonic responses in CCPA+DPCPX seem to be greater than those in control conditions. Thus, we studied the effect of DPCPX in the absence of CCPA upon the hypertonic response. DPCPX increased the peak of the response to 177.3±21.5% (n=4, P<0.05) of control response and the area under the curve to 153.9±17.2% (P<0.05, Fig. 1D, E and F). Taken together, our data suggest that CCPA has an effect on a Ca^{2+}-independent step of transmitter exocytosis by activating A_1 receptors and that the endogenous adenosine accumulated in the synaptic space during the hypertonic response exert a modulatory action on neurotransmitter release.

Activation of $P2Y_{12/13}$ receptors decrease the hypertonic response

Recently, we have demonstrated that, at mouse neuromuscular junction, ATP and the slowly hydrolysable ATP analog $\beta\gamma$-imido ATP induced presynaptic inhibition of spontaneous ACh release via a mechanism that involves the calcium channels related to this kind of secretion (De Lorenzo et al., 2006). Since this effect was abolished by pertussis toxin and N-ethylmaleimide, it was suggested that the P2Y receptors involved are coupled to $G_{i/o}$ protein. Among the P2Y receptors, $P2Y_{12,13,14}$ coupled to $G_{i/o}$ proteins (Communi et al., 2001; Abbracchio et al., 2003), being $P2Y_{12}$ and $P2Y_{13}$ activated by adenine nucleotides, while $P2Y_{14}$ is activated by UDP-glucose. Thus, we studied the effect of 2-MeSADP, the preferential agonist for $P2Y_{12/13}$ receptors (Communi et al., 2001; Kubista et al., 2003) on the hypertonic response. As shown in Fig. 2A, B and C, in control solution the increase in tonicity enhanced MEPP frequency from 1.04±0.02/s to a peak of 11.05±1.2/s (n=4), whereas in the presence of 150 nM 2-MeSADP, MEPP frequency was from 0.57±0.02/s (54.8±1.6% of control values, P<0.05) to a peak of 5.1±0.46/s (48.5±7.5% of the control response, P<0.05). The area under the curve was 143.6±9.3 in control Ringer and 88.6±6.4 in 2-MeSADP. This area was 61.8±3.0% of that in the absence of 2-MeSADP (P<0.05). The addition of the specific $P2Y_{12/13}$ antagonist AR-C69931MX (1 μM, Takasaki et al., 2001; Marteau et al., 2003; Fumagalli et al., 2004) to the solution containing 2-MeSADP prevented the action of the nucleotide in isotonic (96.1±2.1% of the control values) and in hypertonic condition. In hypertonicity the peak and the area of the response were 99.0±2.8% and 107.5±7.7% of the control responses. These results suggest the inhibition of the transmitter-releasing machinery as a mechanism of adenine nucleotides-induced presynaptic depression when $P2Y_{12/13}$ receptors are activated.

To evaluate the action of endogenous ATP/ADP on hypertonicity-induced enhancement of exocytosis, we studied the effect of the $P2Y_{12/13}$ antagonist on hypertonic response (Fig. 2D, E and F). AR-C69931MX (1 μM) produced an increase of the response (peak 126.8±11.4% of control response, P<0.05; area under the curve 131.8±3.7%, P<0.05, n=4) indicating that endogenous adenine nucleotides also contribute to the regulation of Ca^{2+}-independent exocytosis.

Mechanism of action of adenosine and adenine nucleotides acting downstream of Ca^{2+} entry

Presynaptic inhibition of ACh release induced by adenosine and ATP/ADP is mediated by A_1 adenosine receptors and $P2Y_{12/13}$ receptors which are coupled to pertussis toxin-sensitive G proteins ($G_{i/o}$ class) (Nyce, 1999; Hamilton and Smith, 1991; Sokolova et al., 2003; De Lorenzo et al., 2006). In an attempt to determine the transduction mechanisms involved in the modulation of hypertonic response, we analyzed their effects in the presence of inhibitors of several pathways.

One possibility is that purines decrease the hypertonic response by inhibiting the protein kinase A (PKA) pathway via G_i protein. Thus, we investigated whether a specific PKA inhibitor such as H-89 (Dezaki et al., 1996) modified (mimicked or prevented) the hypertonic response. The concentration of H-89 used in the experiments (1 μM) was able to reduce significantly the stimulatory effect of 20 μM forskolin on MEPP frequency via the activation of PKA, suggesting that H-89 inhibits PKA in our system (MEPP frequency: forskolin 165.6±11.8% of control values, P<0.05, n=4; H-89+forskolin: 85.2±2.0%, forskolin vs. forskolin+H-89: P<0.05, n=4). When evaluating the effect of H-89 on hypertonic response, we found that the PKA inhibitor did not significantly modify the response (see Table 1) and did not prevent the inhibitory action of CCPA or 2-MeSADP in isoto-

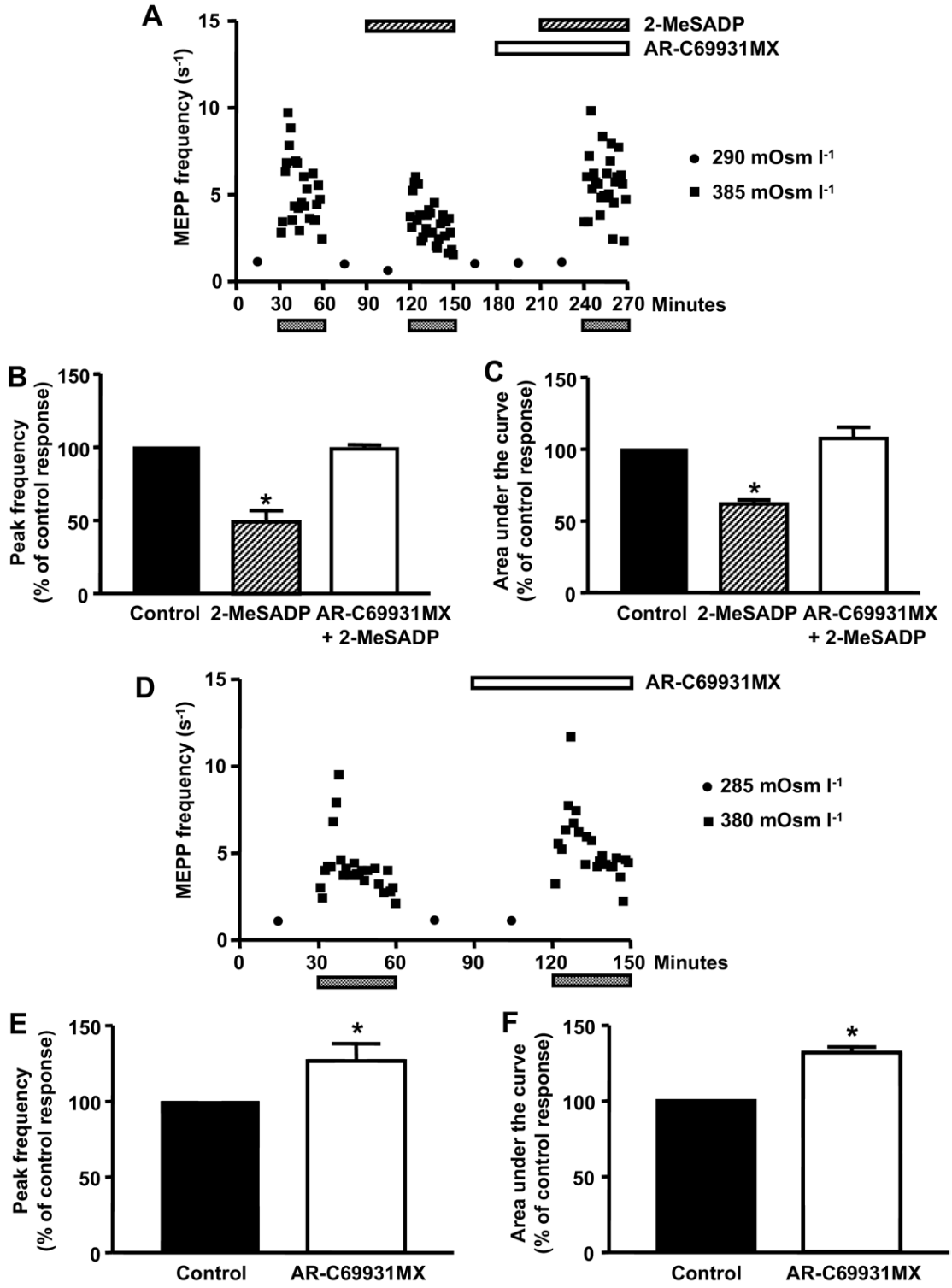

Fig. 2. (A) Effect of 2-MeSADP and AR-C69931MX+2-MeSADP on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic condition. (B, C) Summary histograms showing the depressant effect of 2-MeSADP on the peak frequency and area under the curve of the hypertonic response and their reversion in the presence of AR-C69931MX. (D) Effect of AR-C69931MX on ACh release when a preparation was exposed to isotonic and hypertonic condition. (E, F) Summary histograms showing the increase of the peak and area under the curve when muscles were incubated in AR-C69931MX. * $P<0.05$.

nicity (H-89 102.0±1.4% of control values, H-89±CCPA 58.1±2.2%, $P<0.05$, $n=4$; H-89 103.4±5.4% of control values, H-89±2-MeSADP 57.7±10.8%, $P<0.05$, $n=4$) and hypertonicity (H-89+CCPA: peak 64.0±1.2% of control re-

Table 1. Effect of inhibitors of second-messenger pathways on hypertonic response

Drug	Peak (% of control values)	Area under the curve (% of control values)
H-89 $n=3$	94.4±10.4	102.8±15.8
Chelerythrine $n=5$	86.0±7.7	107.0±10.2
W-7 $n=5$	115.8±10.8	122.3±10.6
LY-294002 $n=4$	261.8±36.9*	277.8±33.6*

Values are means±S.E.M.
* $P<0.05$.

sponse, $P<0.05$, area under the curve 81.7±3.7% of control responses, $P<0.05$, $n=4$; H-89+2-MeSADP: peak 59.4±4.2% of control responses, $P<0.05$, area 68.8±4.2%, $P<0.05$, $n=4$, see Fig. 3). We conclude that at mammalian neuromuscular junction, the effect of CCPA and 2-MeSADP on hypertonic response is not dependent on the inhibition of PKA.

We next studied whether the activation of A_1 and $P2Y_{12/13}$ receptors could activate protein kinase C (PKC), leading to the decrease of the hypertonic response. So, the effect of CCPA and 2-MeSADP in hypertonicity was investigated in the presence of 5 μM chelerythrine, a specific PKC blocker. The agent did not alter the hypertonic response (Table 1), but as is depicted in Fig. 4, it has a differential effect on the action of the purines. Chelerythrine did not prevent the effect of CCPA on MEPP frequency in isotonic condition (chelerythrine 91.5±6.4% of control values, chelerythrine±CCPA 60.5±3.1%, $P<0.05$, $n=3$) and in hypertonic condition (chelerythrine±CCPA: peak 63.1±7.3% of control response, $P<0.05$, area under the curve 73.5±2.2% of control responses, $P<0.05$, $n=3$), suggesting that this intracellular pathway is not involved in the effect of CCPA. On the contrary, chelerythrine occluded the effect of 2-MeSADP on MEPP frequency in isotonicity (chelerythrine 95.3±3.7% of control values, chelerythrine±2-MeSADP 104.5±4.1%, $n=5$) and in hypertonicity (chelerythrine±2-MeSADP: peak 110.2±6.9% of control response, area under the curve 113.4±5.0% of control responses, $n=5$), indicating that PKC is related to the Ca^{2+}-independent effect of 2-MeSADP on neurotransmitter release.

In our previous studies the inhibition of spontaneous ACh release induced by CCPA and $\beta\gamma$-imido ATP was prevented when preparations were bathed with W-7, an antagonist of calmodulin (De Lorenzo et al., 2004, 2006). In the present investigation, W-7 (25 μM) did not modify the hypertonic response (Table 1), but occluded the inhibition exerted by both purines in hypertonicity. In Fig. 5, it is shown that W-7 prevented the effect of CCPA (W-7+CCPA, peak 113.6±8.6%, area 117.4±14.2% of control response, $n=4$) and 2-MeSADP (W-7+2-MeSADP, peak 93.3±5.6%, area 111.7±14.0% of control response, $n=4$), suggesting that calmodulin is also involved in the action of CCPA and 2-MeSADP on the hypertonic response.

Since in others systems, it was shown that ADP stimulates phosphoinositide-3 kinase (PI3K) through $P2Y_{12}$ receptors, and this pathways have been implicated in integrin activation (see discussion, Kauffenstein et al., 2001; Dorsam and Kunapuli, 2004; Jackson et al., 2004; Sun et al., 2005), we studied the effect of LY-294002, a reversible inhibitor of PI3K and casein kinase II, upon the modulation of the hypertonic response induced by CCPA and 2-MeSADP. At frog neuromuscular junctions, it was found that LY-294002 increases MEPP frequency through a mechanism independent of internal calcium concentration (Rizzoli and Betz, 2002; Searl and Silinsky, 2005), this action being transient at high concentrations of the drug (Rizzoli and Betz 2002). Thus, we first tested the time-dependent action of LY-294002 on MEPP frequency. We found that immediately after the exposure to 100 μM LY-294002 MEPP frequency increased from 1.03±0.01/s to 3.23±0.15/s ($n=3$, $P<0.05$), and values obtained every 5 min during 120 min did not show statistical differences between them (data not depicted). Similar results were found by Hong and Chang (1999) at mice diaphragms for 5 h. Next we tested the effect of LY-294002 in hypertonicity. We found that LY-294002 also increased the hypertonic response in the magnitude of the peak as well as in the area under the curve with respect to the control response (see Table 1 and Fig. 6A, D and E), suggesting that the increase of MEPP frequency induced by LY-294002 is not related to that evoked by the hypertonicity. We then evaluated the effect of purines in the presence of LY-294002. As can be seen in Fig. 6B and C, in isotonic condition, the addition of CCPA or 2-MeSADP to solutions containing LY-294002 decreased MEPP frequency compared with those recorded in LY-294002 without the purines, although the values were not different from the control ones (MEPP frequency: LY-294002 280.6±20.1% of control values, $P<0.05$; LY-294002+CCPA 140.5±14.8%, LY-294002 vs. LY-294002+CCPA $P<0.05$, $n=4$; LY-294002 271.6±23.2% of control values, $P<0.05$, LY-294002+2-MeSADP 160.9±9.3%, LY-294002 vs. LY-294002+2-MeSADP $P<0.05$, $n=3$). The same behavior was observed in hypertonic condition: CCPA or 2-MeSADP decreased significantly the peak of the response and the area under the curve if we compared the results with those observed in LY-294002 without the purines, while the responses were similar to those found in control solutions (LY-294002+CCPA: peak 123.7±9.2% of control response, area under the curve 180.4±22.7%, $n=4$; LY-294002+2-MeSADP: peak 147.8±19.5%, area under the curve 177.5±13.9%, $n=3$, Fig. 6B, C, D and E). In order to evaluate whether PI3K and casein kinase II are involved in the inhibition of the hypertonic response induced by the purines, we took together data obtained with CCPA and 2-MeSADP in control solution (see Figs. 1 and 2) and in the saline containing LY-294002 in isotonic and hypertonic condition (see Fig. 6). As is depicted in Fig. 7A and B, the percentage of inhibition induced by CCPA and 2-MeSADP in the presence of LY-294002 is not statistically different from those observed in control solution, suggesting that the modulatory effect of CCPA and 2-MeSADP on hypertonic response is not dependent on these kinases.

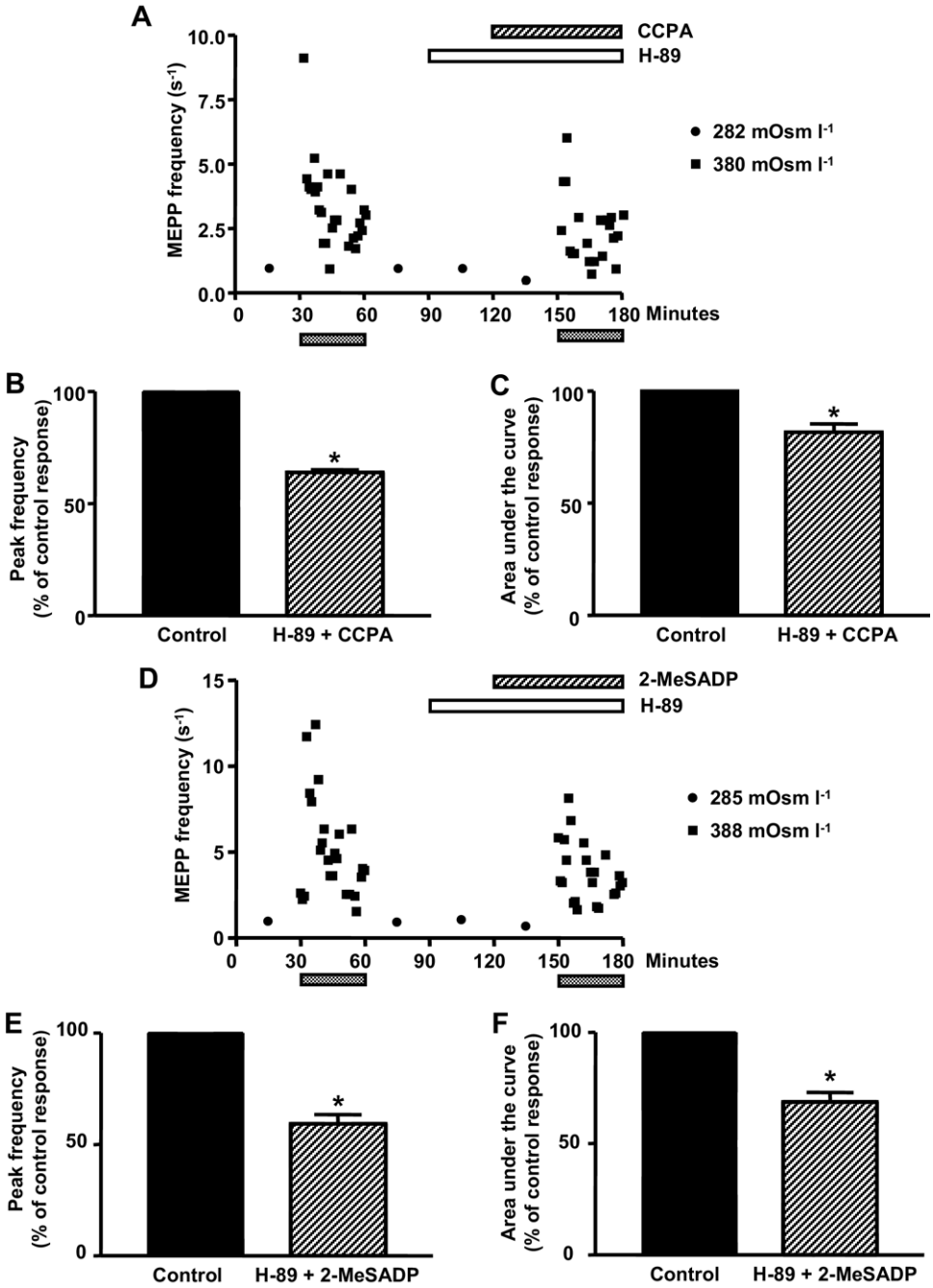

Fig. 3. PKA-dependent pathway is not involved in the modulation induced by CCPA and 2-MeSADP on the hypertonic response. (A) Experiment showing that the PKA inhibitor H-89 did not prevent the inhibition induced by CCPA in isotonic and hypertonic condition. (B, C) Summary histograms depict the decrease of the peak and area under the curve when preparations were incubated with CCPA in the presence of H-89. Data are expressed as percentage of control response. (D) Experiment showing that H-89 did not prevent the inhibition induced by 2-MeSADP in isotonic and hypertonic condition. (E, F) Summary histograms display the decrease of the peak and area under the curve when preparations were incubated with 2-MeSADP in the presence of H-89. * $P<0.05$.

DISCUSSION

The present study provides evidence that at mammalian motor nerve terminals, adenosine and adenine nucleotides are able to modulate ACh secretion by acting on a step downstream of calcium entry, since CCPA and 2-MeSADP decrease the enhancement of transmitter release induced by hypertonicity, a situation shown to bypass the Ca^{2+}-triggering step in exocytosis (Furshpan, 1956; Hubbard et al., 1968; Rosenmund and Stevens, 1996; Losavio and Muchnik, 1997; Mochida et al., 1998; Kashani et al., 2001). The underlying mechanism for hypertonic response is not completely clear. It has been shown that integrins, which are localized in the periactive zone surrounding the release sites (Prokop et al., 1998; Beumer et al., 1999; Sone et al., 2000), mediate part of

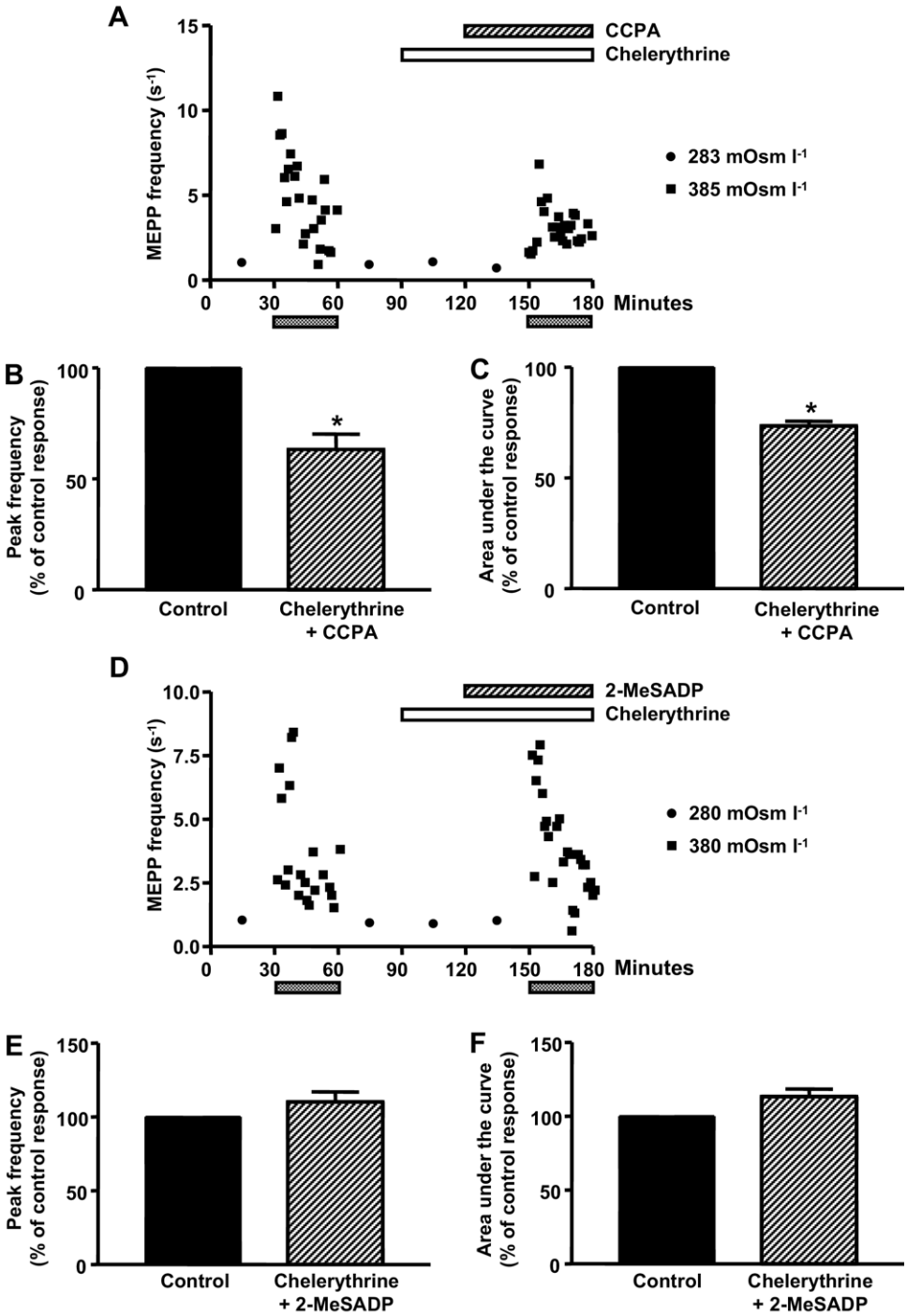

Fig. 4. PKC-dependent pathway is involved in the modulation induced by 2-MeSADP on the hypertonic response but not in that of CCPA. (A) Experiment showing that the specific PKC inhibitor chelerythrine did not prevent the inhibition induced by CCPA in isotonic and hypertonic condition. (B, C) Summary histograms depict the decrease of the peak and area under the curve when preparations were incubated with CCPA in the presence of chelerythrine. Data are expressed as percentage of control response. (D) Experiment showing that chelerythrine prevented the inhibition induced by 2-MeSADP in isotonic and hypertonic condition. (E, F) Summary histograms exhibit the lack of decrease of the peak and area under the curve when preparations were incubated with 2-MeSADP in the presence of chelerythrine. * $P<0.05$.

this response at the frog neuromuscular synapse (Kashani et al., 2001) and at the embryonic *Drosophila* neuromuscular junction (Suzuki et al., 2002), suggesting that the mechanical stress induced by hypertonicity might be transmitted via integrins and the cytoskeleton directly to the vesicle fusion machinery. On the other hand, at mammalian neuromuscular junctions, hypertonicity is no longer effective after cleavage of SNAP-25 or synaptobrevin by clostridial toxins (Dreyer et al.,

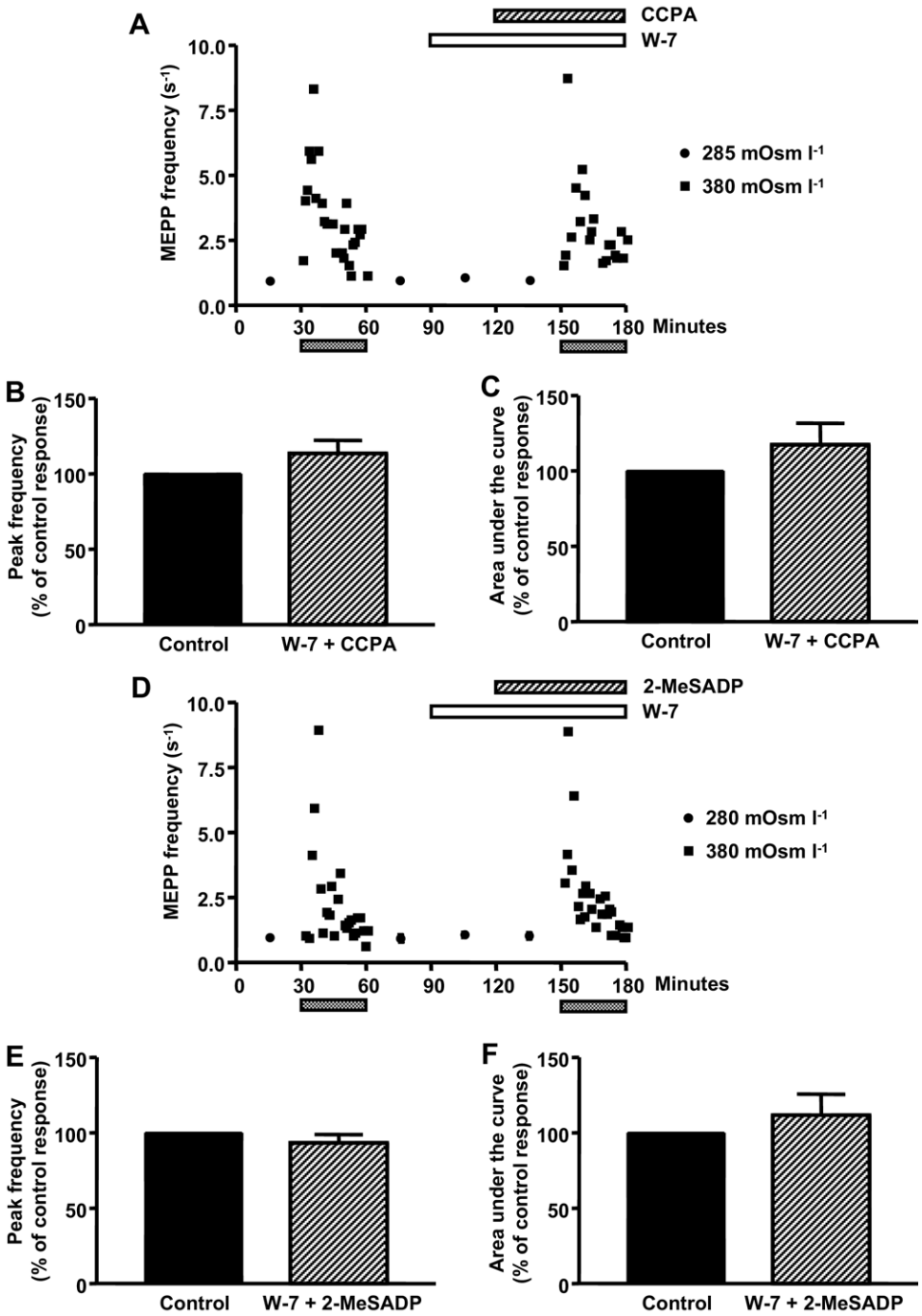

Fig. 5. Intracellular pathway related to calmodulin is involved in the modulation induced by CCPA and 2-MeSADP on the hypertonic response. (A) Experiment showing that the antagonist of calmodulin W-7 occluded the inhibition induced by CCPA in isotonic and hypertonic condition. (B, C) Summary histograms display that W-7 prevented the decrease of the peak and area under the curve induced by CCPA. Data are expressed as percentage of control response. (D) Experiment showing that W-7 precluded the inhibition induced by 2-MeSADP in isotonic and hypertonic condition. (E, F) Summary histograms show the lack of decrease of the peak and area under the curve when preparations were incubated with 2-MeSADP in the presence of W-7. * $P<0.05$.

1987; Gansel et al., 1987), and at *Drosophila* neuromuscular junctions, the hypertonicity effect is eliminated in the absence of synaptobrevin, syntaxin, or Unc-13 (Aravamudan et al., 1999). Therefore, the hypertonic response, while bypassing the Ca^{2+}-sensing mechanism, seems to share the major elements of the basic Ca^{2+} triggered vesicle fusion. Our results demonstrated that the specific adenosine A_1 receptor agonist CCPA, and the preferential agonist for $P2Y_{12/13}$, 2-MeSADP, decrease the peak and the area under the curve of the hypertonic response, suggesting that purines inhibit

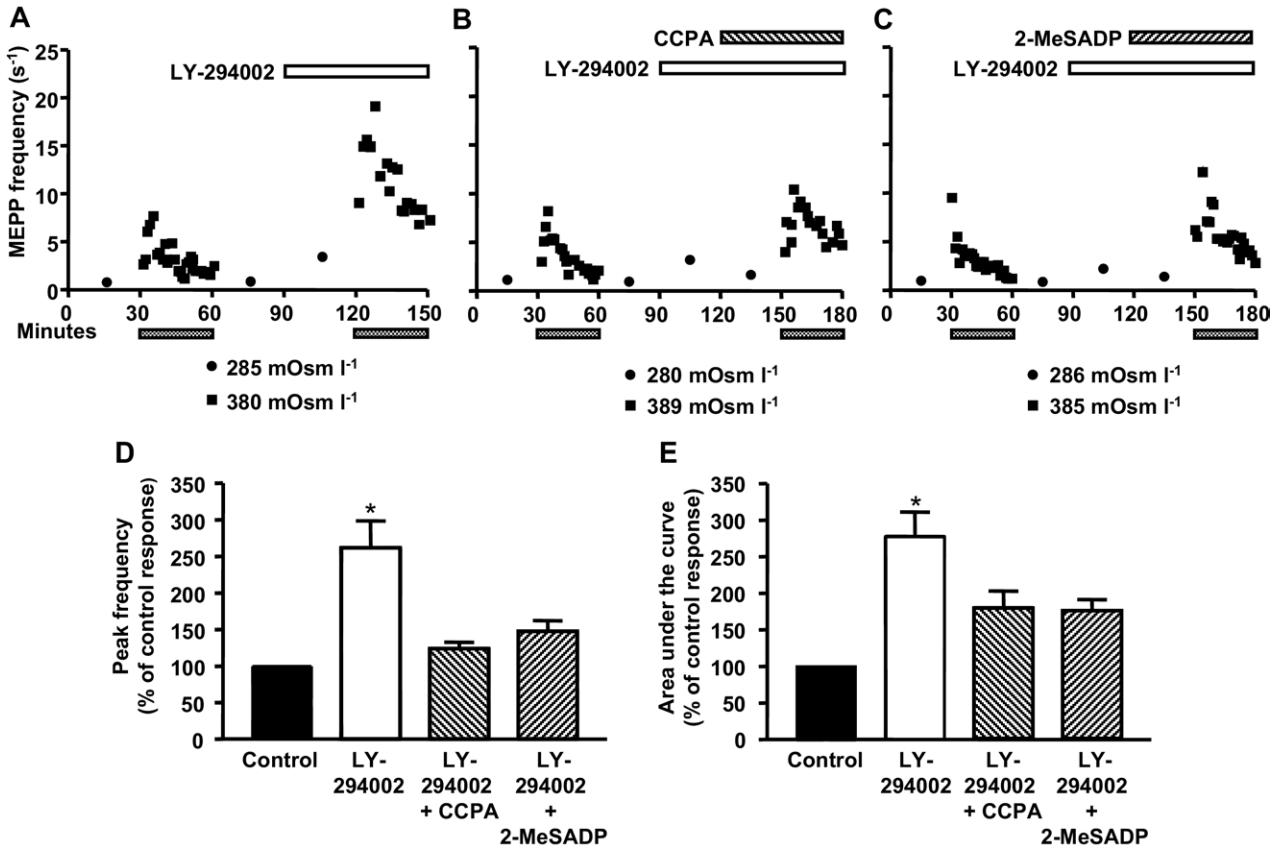

Fig. 6. Effect of the inhibitor of PI3K and casein kinase II LY-294002 upon the modulation of the hypertonic response induced by CCPA and 2-MeSADP. (A) Experiment showing that LY-294002 increased ACh release in isotonic and hypertonic condition. (B, C) Individual experiments showing that CCPA and 2-MeSADP, respectively, decreased the enhancement of ACh release induced by LY-294002 in isotonic and hypertonic condition. (D, E) Summary histograms depict the effect of LY-294002 (LY), LY+CCPA and LY+2-MeSADP on the peak and area under the curve of the hypertonic response. Data are expressed as percentage of control response. * $P<0.05$.

transmitter release by acting at a Ca^{2+}-independent step of the exocytotic process. These effects were prevented when preparations were previously incubated with DPCPX and AR-C69931MX, specific A_1 and $P2Y_{12/13}$ receptor antagonists, respectively, implicating that the activation of these types of receptors were the cause of the modulation of the hypertonic response. Furthermore, application of the antagonists in the absence of CCPA or 2-MeSADP resulted in higher responses compared with controls, suggesting that the enhancement of endogenous ATP/ADP and adenosine induced by hypertonic condition exert a modulatory effect on neurotransmitter release by activating their specific receptors.

At the frog neuromuscular junction, it was suggested that adenosine depresses transmitter release acting on a step that includes the fusion process of synaptic vesicle to the plasma membrane (Redman and Silinsky, 1994; Robitaille et al., 1999; Huang et al., 2002) while the presynaptic action of ATP was found to be mediated by inhibition of Ca^{2+} channels and impairment of transmitter-releasing machinery (Grishin et al., 2005). Our results at mammalian motor nerve endings suggest that the depressant action of adenosine and adenine nucleotides on neurosecretion appears to be multimodal, modulating calcium channels related to evoked and spontaneous secretion and acting on a Ca^{2+}-independent step in ACh exocytosis by affecting i.e. the proteins related to the synaptic vesicle fusion (synaptobrevin, SNAP-25, syntaxin, synaptotagmin or others). We have previously found that CCPA and $\beta\gamma$-imido ATP did not exert a further inhibition of spontaneous ACh release after the blockade of Ca^{2+} channels with Cd^{2+} or the combined application of nitrendipine and ω-CgTx, (De Lorenzo et al., 2004, 2006). However, a concomitant effect of the purines on a component of the secretory apparatus cannot be ruled out. It is possible that spontaneous release might have a separate molecular machinery from the SNARE complex, related to fast synchronous release and hypertonic response. At *Drosophila* neuromuscular synapse, it was observed that MEPPs persisted in mutant embryos lacking proteins such as synaptobrevin, SNAP-25 and synaptotagmin I, while the SNARE complex, synaptotagmin I and Unc-13 are indispensable for the Ca^{2+}-independent hypertonicity response (Deitcher et al., 1998; Rao et al., 2001; Broadie et al., 1994; Hua et al., 1998; Aravamudan et al., 1999, see review Kidokoro, 2003). At mammalian motor nerve terminals hypertonicity is no longer effective after cleavage of synaptobrevin or SNAP-25 by

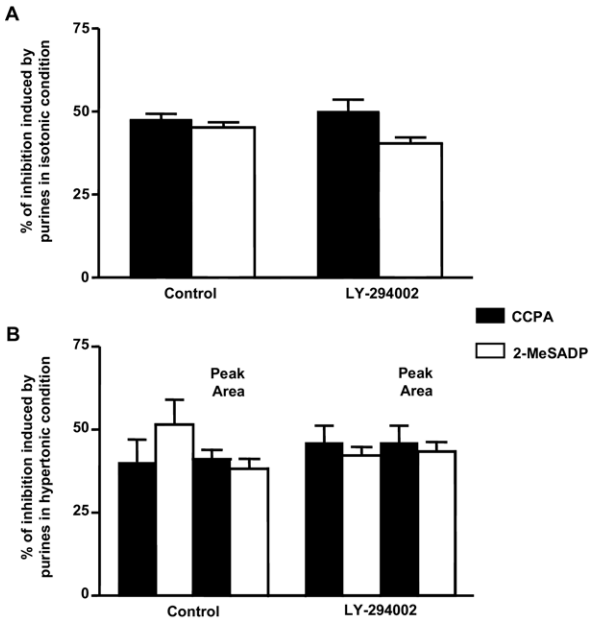

Fig. 7. Percentage of inhibition of ACh release induced by CCPA and 2-MeSADP in isotonic (A) and hypertonic condition (B) when preparations were incubated in control Ringer solutions and in saline containing LY-294002.

clostridial toxins (Dreyer et al., 1987; Gansel et al., 1987). Thus, one can speculate that at isotonic condition the action of purines on the release machinery was not detected when observing spontaneous release, whereas at hypertonicity this modulatory effect is evident. On the other hand, we cannot exclude the possibility that, due to the intimate coupling between Ca^{2+} channels and the SNARE proteins, the effects of adenosine and ATP/ADP on a component of the release machinery could decrease the activation of the Ca^{2+} channels. In this regard, Silinsky (2005) found in the mouse, that the effect of adenosine on Ca^{2+} currents is eliminated when the presynaptic membrane SNARE syntaxin is cleaved by botulinum toxin type C.

Since each of the core components of the exocytosis machinery is individually essential for the hypertonic response (see above), we cannot clarify which is/are the target/s of the action of the purines by cleaving these components via clostridial toxins. So, in order to approach to this question, we decided to investigate the transduction mechanisms implicated in the modulation of hypertonic response. A_1 adenosine receptors and $P2Y_{12/13}$ receptors are coupled to pertussis toxin–sensitive G proteins ($G_{i/o}$ class) (Nyce, 1999; Hamilton and Smith, 1991; Sokolova et al., 2003; De Lorenzo et al., 2006), thus, activation of these receptors by CCPA and 2-MeSADP respectively, promotes the dissociation of α_{i-o}-GTP and $\beta\gamma$ subunits. Both subunits may exert their modulatory effect by regulating second messenger enzymes, ion channels, or other targets (Hamm, 1998; Blackmer et al., 2001). Our results demonstrated that the cAMP/PKA cascade is not involved in the action of the purines on the hypertonic response, since the PKA antagonist H-89 did not prevent the modulatory effect of CCPA and 2-MeSADP on the response. At *Drosophila* neuromuscular junctions it has been suggested that part of the hypertonic response involves the cAMP/PKA cascade (Suzuki et al., 2002). However, treatment of our preparations with H-89 did not modify such response, suggesting that the component of the hypertonic response dependent on cAMP/PKA pathway does not seem to play a relevant role at mammalian neuromuscular junction. When evaluating the participation of PKC in the effect of the purines, we found that the PKC inhibitor chelerythrine occluded the inhibitory action of 2-MeSADP but not the effect of CCPA on the hypertonic response, suggesting that PKC is involved in the Ca^{2+}-independent effect of 2-MeSADP. Activation of PKC via $P2Y_{12/13}$ receptors may lead to phosphorylation of some of the proteins implicated in exocytosis. Many proteins involved in secretion have been identified to contain predicted phosphorylation sites for PKC, but fewer have been shown to be functionally altered by in terms of their effects on exocytosis in cells (SNAP-25 and Munc 18, Morgan et al., 2005). Phosphorylation of SNAP-25 and Munc-18 by PKC has been shown to reduce the affinity of these proteins for syntaxin (Shimazaki et al., 1996; Fujita et al., 1996), thus being able to modify the exocytotic mechanism. On the other hand, the antagonist of calmodulin W-7 precluded the effect of CCPA and 2-MeSADP on hypertonic response, suggesting that calmodulin is involved in the modulatory action of purines on Ca^{2+}-independent ACh release. Among the proteins related to exocytosis that interact with calmodulin, it was shown that the GTP-bound form of Rab3 requires an interaction with Ca^{2+}/calmodulin to inhibit secretion (Coppola et al., 1999).

We then investigated the possibility that LY-294002, a specific inhibitor of PI3K and casein kinase II, which also increases MEPP frequency by a mechanism that is independent of intraterminal calcium, could be involved in the modulation of hypertonic secretion. LY-294002 induced a potentiation of MEPP frequency in isotonicity as well as in hypertonicity (Table 1), suggesting that the mechanisms by which hypertonicity and LY-294002 produce increase of secretion do not interfere each other. When evaluating the action of LY-294002 on the modulatory effect of purines on hypertonic response, we found that the percentage of inhibition induced by CCPA and 2-MeSADP in the presence of LY-294002 was similar to that observed in control solution (isotonic and hypertonic condition, see Fig. 7), suggesting that the kinases inhibited by LY-294002 did not affect the modulation exerted by adenosine and the adenine nucleotides.

Interestingly, in our previous papers (De Lorenzo et al., 2004, 2006), we found that the modulatory effect of the purines upon Ca^{2+} channels depended on the contribution of calmodulin (CCPA and $\beta\gamma$-imido ATP) and PKC ($\beta\gamma$-imido ATP), the same pathways involved in the action of CCPA and 2-MeSADP on the hypertonic response. Since presynaptic CCVD are intimately associated with key elements of the synaptic vesicle docking and fusion mechanisms (see Khanna et al., 2007), it is possible that an action of purines on strategic components of the secretory apparatus could modify the activation of those channels (see above).

CONCLUSION

In summary, ours results suggest that activation of adenosine A_1 and $P2Y_{12-13}$ receptors by CCPA and 2-MeSADP, respectively, inhibits ACh release from mammalian motor nerve terminals by an effect on Ca^{2+}-independent step in the cascade of the exocytotic process, since both purines decreased hypertonicity-induced transmitter release. Furthermore, endogenous ATP/ADP and adenosine were shown to modulate hypertonic response. The depressant action of CCPA was prevented by the inhibitor of calmodulin W-7, whereas the action of 2-MeSADP was occluded by W-7 and the inhibitor of PKC chelerythrine. Further experiments are needed to clarify whether the action of purines on presynaptic calcium channels and on secretory machinery downstream of Ca^{2+} influx is associated or if they are individual targets.

Acknowledgments—We thank Mrs. María Fernanda Rodriguez and Mrs. Raquel Almirón for technical assistance. The research was supported by grants from SECYT (BID 1201/OC-AR-PICT11231) and UBA (M055).

REFERENCES

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

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

Beumer KJ, Rohrbough J, Prokop A, Broadie K (1999) A role for PS integrins in morphological growth and synaptic function at the postembryonic neuromuscular junction of *Drosophila*. Development 126:5833–5846.

Blackmer T, Larsen EC, Takahashi M, Martin TF, Alford S, Hamm HE (2001) G protein betagamma subunit-mediated presynaptic inhibition: regulation of exocytotic fusion downstream of Ca^{2+} entry. Science 292:293–297.

Blioch ZL, Glagoleva IM, Liberman EA, Nenashev VA (1968) A study of the mechanism of quantal transmitter release at a chemical synapse. J Physiol 199:11–35.

Bourque CW, Renaud LP (1984) Activity patterns and osmosensitivity of rat supraoptic neurones in perfused hypothalamic explants. J Physiol 349:631–642.

Broadie K, Bellen HJ, DiAntonio A, Littleton JT, Schwarz TL (1994) Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission. Proc Natl Acad Sci U S A 91:10727–10731.

Brosius DC, Hackett JT, Tuttle JB (1992) Ca^{2+}-independent and Ca^{2+}-dependent stimulation of quantal neurosecretion in avian ciliary ganglion neurons. J Neurophysiol 68:1229–1234.

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

Coppola T, Perret-Menoud V, Lüthi S, Farnsworth CC, John A, Glomset JA, Regazzi R (1999) Disruption of Rab3-calmodulin interaction, but not other effector interactions, prevents Rab3 inhibition of exocytosis. EMBO J 18:5885–5891.

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

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

Dezaki K, Kimura I, Tsuneki H, Kimura M (1996) Enhancement by calcitonin gene-related peptide of non-contractile Ca^{2+}-induced nicotinic receptor desensitization at the mouse neuromuscular junction. Br J Pharmacol 118:1971–1976.

Doherty P, Hawgood BJ, Smith IC (1986) Changes in miniature endplate potentials due to moderate hypertonicity at the frog neuromuscular junction. J Physiol 376:1–11.

Dorsam RT, Kunapuli SP (2004) Central role of the P2Y12 receptor in platelet activation. J Clin Invest 113:340–345.

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.

Fatt P, Katz B (1952) Spontaneous subthreshold activity at the motor nerve endings. J Physiol 117:109–128.

Fujita Y, Sasaki T, Fukui K, Kotani H, Kimura T, Hata Y, Südhof TC, Schelleri RH, Takai Y (1996) Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C. Its implication in regulating the intaction of Munc-18/n-Sec1/rbSec1 with syntaxin. J Biol Chem 271:7265–7268.

Fumagalli M, Trincavelli L, Lecca D, Martini C, Ciana P, Abbracchio M (2004) Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled P2Y13 receptor. Biochem Pharmacol 68:113–124.

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.

Grishin S, Shakirzyanova A, Giniatullin A, Afzalov R, Giniatullin R (2005) Mechanisms of ATP action on motor nerve terminals at the frog neuromuscular junction. Eur J Neurosci 21:1271–1279.

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.

Hamm HE (1998) The many faces of G protein signaling. J Biol Chem 273:669–672.

Hong SJ, Chang CC (1999) Inhibition of quantal release from motor nerve by wortmannin. Br J Pharmacol 128:142–148.

Hua SY, 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.

Huang SM, Kitamura A, Akita T, Narita K, Kuba K (2002) Adenosine depresses a Ca^{2+}-independent step in transmitter exocytosis at frog motor nerve terminals. Eur J Neurosci 15:1291–1298.

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.

Hubbard JI, Jones SF, Landau EM (1971) The effect of temperature change upon transmitter release, facilitation and post-tetanic potentiation. J Physiol 216:591–609.

Jackson SP, Yap CL, Anderson KE (2004) Phosphoinositide 3-kinases and the regulation of platelet function. Biochem Soc Trans 32:387–392.

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.

Kauffenstein G, Bergmeier W, Eckly A, Ohlmann P, Leon C, Cazenave JP, Nieswandt B, Gachet C (2001) The P2Y12 receptor induces platelet aggregation through weak activation of the αIIbβ3 integrin: a phosphoinositide 3-kinase-dependent mechanism. FEBS Lett 505:281–290.

Khanna R, Li Q, Bewersdorf J, Stanley EF (2007) The presynaptic CaV2.2 channel-transmitter release site core complex. Eur J Neurosci 26:547–559.

Kidokoro Y (2003) Roles of SNARE proteins and synaptotagmin I in synaptic transmission: Studies at the *Drosophila* neuromuscular synapse. Neurosignals 12:13–30.

Kita H, Van der Kloot W (1977) Time course and magnitude of effects of changes in tonicity on acetylcholine release at frog neuromuscular junction. J Neurophysiol 40:212–224.

Kubista H, Lechner SG, Wolf AM, Boehm S (2003) Attenuation of the P2Y receptor-mediated control of neuronal Ca^{2+} channels in PC12 cells by antithrombotic drugs. Br J Pharmacol 138:343–350.

Li C (1958) Effect of cooling on neuromuscular transmission in the rat. Am J Physiol 194:200–206.

Liley AW (1956) An investigation of spontaneous activity at the neuromuscular junction of the rat. J Physiol 132:650–666.

Liu GJ, Werry EL, Bennett MR (2005) Secretion of ATP from Schwann cells in response to uridine triphosphate. Eur J Neurosci 21: 151–160.

Losavio A, Muchnik S (1997) Spontaneous acetylcholine release in mammalian neuromuscular junction. Am J Physiol 273:C1835–C1841.

Marteau F, Le Poul E, Communi D, Communi D, Labouret C, Savi P, Boeynaems JM, Suarez Gonzalez N (2003) Pharmacological characterization of the human P2Y13 receptor. Mol Pharmacol 64:104–112.

Meriney SD, Grinnell AD (1991) Endogenous adenosine modulates stimulation-induced depression at the frog neuromuscular junction. J Physiol 443:441–455.

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.

Morgan CA, Burgoyne RD, Barclay JW, Craig TJ, Prescott GR, Ciufo LF, Evans GJO, Graham ME (2005) Regulation of exocytosis by protein kinase. Biochem Soc Tran 33:1341–1344.

Niles WD, Smith DO (1982) Effects of hypertonic solutions on quantal transmitter release at the crayfish neuromuscular junction. J Physiol 329:185–202.

Nyce JW (1999) Insight into adenosine receptor function using antisense and gene knockout approaches. Trends Pharmacol Sci 20:79–83.

Prokop A, Martin-Bermudo MD, Bate M, Brown NH (1998) Absence of PS integrins or laminin A affects extracellular adhesion, but not intracellular assembly, of hemiadherens and neuromuscular junctions in *Drosophila* embryos. Dev Biol 196:58–76.

Quastel DM, Hackett JT, Cooke JD (1971) Calcium: is it required for transmitter secretion? Science 172:1034–1036.

Rao SS, Stewart BA, Rivilin PK, Vilinsky I, Whatson BO, Lang C, Boulianne G, Salpeter MM, Deitcher DL (2001) Two distinct effects on neurotransmission in a temperature-sensitive SNAP-25 mutant. EMBO J 20:6761–6771.

Redman RS, Silinsky EM (1994) ATP released together with acetylcholine as the mediator of neuromuscular depression at frog motor nerve endings. J Physiol 447:117–127.

Ribeiro JA, Sebastião AM (1987) On the role, inactivation, and origin of endogenous adenosine at the frog neuromuscular junction. J Physiol 384:571–585.

Rizzoli SO, Betz WJ (2002) Effects of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one on synaptic vesicle cycling at the frog neuromuscular junction. J Neurosci 22:10680–10689.

Robitaille R, Thomas S, Charlton MP (1999) Effects of adenosine on Ca^{2+} entry in the nerve terminal of the frog neuromuscular junction. Can J Physiol Pharmacol 77:707–714.

Rosenmund C, Stevens CF (1996) Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron 16:1197–1207.

Santos DA, Salgado AI, Cunha RA (2003) ATP is released from nerve terminals and from activated muscle fibres on stimulation of the rat phrenic nerve. Neurosci Lett 338:225–228.

Searl TJ, Silinsky EM (2005) LY 294002 inhibits adenosine receptor activation by a mechanism independent of effects on PI-3 kinase or casein kinase II. Purinergic Signal 1:389–394.

Sebastião AM, Ribeiro JA (2000) Fine-tuning neuromodulation by adenosine. Trends Pharmacol Sci 21:341–346.

Shimazaki Y, Nishiki T, Omori A, Sekiguchi M, Kamata Y, Kozaki S, Takahashi M (1996) Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. J Biol Chem 271: 14548–14553.

Silinsky EM (1984) On the mechanism by which adenosine receptor activation inhibits the release of acetylcholine from motor nerve endings. J Physiol 346:243–256.

Silinsky EM, Solsona CS (1992) Calcium currents at motor nerve endings: absence of effects of adenosine receptor agonists in the frog. J Physiol 457:315–328.

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.

Sokolova E, Grishin S, Shakirzyanova A, Talantova M, Giniatullin R (2003) Distinct receptors and different transduction mechanisms for ATP and adenosine at the frog motor nerve endings. Eur J Neurosci 18:1254–1264.

Sone M, Suzuki E, Hoshino M, Hou D, Kuromi H, Fukata M, Kuroda S, Kaibuchi K, Nabeshima Y, C Hama C (2000) Synaptic development is controlled in the periactive zones of Drosophila synapses. Development 127:4157–4168.

Sun D, Lo SJ, Tsai W, Lin C, Yu M, Chen Y, Chang H (2005) PI3-kinase is essential for ADP-stimulated integrin $\alpha_{IIb}\beta_3$-mediated platelet calcium oscillation, implications for P2Y receptor pathways in integrin $\alpha_{IIb}\beta_3$-initiated signaling cross-talks. J Biomed Sci 12:937–948.

Suzuki K, Grinnell AD, Kidokoro Y (2002) Hypertonicity-induced transmitter release at *Drosophila* neuromuscular junctions is partly mediated by integrins and cAMP/protein kinase A. J Physiol 538:103–119.

Takasaki J, Kamohara M, Saito T, Matsumoto M, Matsumoto S, Ohishi T, Soga T, Matsushime H, Furuichi K (2001) Molecular cloning of the platelet $P2T_{AC}$ ADP receptor: Pharmacological comparison with another ADP receptor, the $P2Y_1$ receptor. Mol Pharmacol 60:432–439.

Todd KJ, Robitaille R (2006) Purinergic modulation of synaptic signaling at the neuromuscular junction. Pflugers Arch 452:608–614.

Yu W, Miller RF (1995) Hyperosmotic activation of transmitter release from presynaptic terminals onto retinal ganglion cells. J Neurosci Methods 62:159–168.

(Accepted 29 April 2008)
(Available online 3 May 2008)