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

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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

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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.

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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