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FACILITATION OF SPONTANEOUS ACETYLCHOLINE RELEASE INDUCED BY ACTIVATION OF cAMP IN RAT NEUROMUSCULAR JUNCTIONS

Adriana Losavio and S. Muchnik

Instituto de Investigaciones Médicas A. Lanari, Universidad de Buenos Aires,
Combatientes de Malvinas 3150,
1427 Buenos Aires, Argentina.

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Summary

Regulation of neurotransmitter release is thought to involve modulation of the release probability by protein phosphorylation. Activation of the cAMP-protein kinase A (PKA) pathway has been shown to facilitate synaptic transmission in mammalian neuromuscular synapses, although the relevant phosphorylation targets are mostly unknown. We found that the inhibitor of the phosphodiesterase aminophylline (1 mM AMIN), the membrane-permeable analog of cAMP, 8-Br-cAMP (5 mM) and, the direct adenylate cyclase activator, forskolin (20 μM), induced an increase of miniature end-plate potentials (MEPPs) frequency in rat neuromuscular junctions. We investigated the possible involvement of the voltage-dependent calcium channels (VDCC), since these proteins are known to be phosphorylated by PKA. But this possibility was ruled out, since the increase in MEPPs frequency was not attenuated by the VDCC blocker Cd^{2+} (100 μM) and it was observed when AMIN was studied on hyperosmotic response, which is independent of $[Ca^{2+}]_o$ and of Ca^{2+} influx through the VDCC. The lack of action of AMIN on MEPPs frequency when $[Ca^{2+}]_i$ was diminished by exposing the preparations to zero Ca^{2+}-EGTA solution (isotonic condition) or when nerve terminals were loaded with a permeant Ca^{2+} chelator (BAPTA-AM) (hypertonic condition), indicate that cAMP-mediated presynaptic facilitation is a function of nerve terminal Ca^{2+} concentration. We also found that AMIN exerted a comparable increase in MEPPs frequency in control and high K^+ (10 and 15 mM), suggesting a single mechanism of action for spontaneous and K^+-induced secretion.

Key Words: presynaptic modulation, cAMP, aminophylline, 8-Br-cAMP, forskolin, rat neuromuscular transmission, acetylcholine

Corresponding author: Dr. Adriana Losavio, Instituto de Investigaciones Médicas A. Lanari, Combatientes de Malvinas 3150, 1427 Buenos Aires, Argentina. FAX: 54-11-4567-9217. E-mail: alosavio@mail.retina.ar

Neurotransmitters stored in synaptic vesicles within nerve terminal, are released by exocytosis and modulated by a variety of regulatory processes, such as occur during the different forms of short-term synaptic plasticity (1), modulation by presynaptic receptors (2-4), and long-term potentiation and depression (5-7). Several pieces of evidence suggest that protein phosphorylation events play an important role in the regulation of the secretory process. It seems to be a generalized characteristic, since in endocrine cells exocytosis is also modulated by phosphorylation (8-10). In many preparations, activation of the cAMP-protein kinase A (PKA) pathway has been shown to facilitate synaptic transmission (11-15). In mammalian neuromuscular synapses, it has been demonstrated that the inhibitors of phosphodiesterase aminophylline (AMIN) and theophylline, and cAMP and its derivatives facilitate acetylcholine release apparently by a presynaptic mechanism (16-19). In *Drosophila* mutant *dunce*, defective in different steps of the cAMP cascade, voltage-clamp analysis of neuromuscular transmission revealed impaired synaptic facilitation and post-tetanic potentiation as well as abnormal responses to direct application of dibutyryl cAMP (20).

The aim of the present work was to determine the mechanisms through which agents acting on the cAMP/PKA cascade facilitate spontaneous neurotransmitter release in mammalian neuromuscular junctions. One possibility, is that PKA could phosphorylate the voltage-dependent calcium channels (VDCC) related to spontaneous release (21), leading to an increase in the intracellular Ca^{2+} concentration as consequence of enhancement of the amplitude of voltage-dependent Ca^{2+} currents. Alternatively, PKA might phosphorylate proteins involved in the trafficking, docking and/or fusion of the synaptic vesicles to the presynaptic membrane. Although, in other preparations, there is evidence in support of both mechanisms, a clear causal relationship between phosphorylation of a specific protein and consequent facilitation of neurotransmitter release remains to be established. Chetkovich et al (22) reported that cAMP and NMDA receptor activation increased the probability of opening of VDCC in rat CA1 pyramidal neurons. This phenomenon has also been postulated to occur in area CA3 of hippocampus, where cell-permeant analogs of cAMP modulated VDCC function (23). In contrast, in rat CA3 pyramidal cells, it was found that PKA-induced potentiation of spontaneous release was not attenuated by the VDCC blocker Cd^{2+}(11), and in rat hippocampal interneurons, it was shown that forskolin induced an enhancement of the secretory response in presence of ruthenium red, a tool that stimulates exocytosis independently of Ca^{2+} influx (14).

We have first confirmed that AMIN, 8-Br-cAMP and forskolin, which activate the cAMP-PKA pathway, increase miniature end-plate potential (MEPPs) frequency in rat diaphragm muscles. We suggest that the secretory process is facilitated at a step down-stream from Ca^{2+} influx and that this modulation is a function of nerve terminal Ca^{2+} concentration. Furthermore, we find that AMIN appeared to exert a comparable increase in MEPPs frequency in basal conditions and in K^+-induced neurotransmitter secretion.

Materials and Methods

Wistar rat diaphragm muscles were used. Rats (180-220 g) were anesthetized with sodium thiopental (50 mg/kg) and the left hemidiaphragm was excised and transferred to a chamber filled with Ringer Krebs (mM): (NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11, HEPES 5, pH 7.3-7.4), bubbled with O_2. In some experiments the KCl concentration of the Ringer Krebs was raised to 10 or 15 mM. MEPPs frequency was recorded intracellularly at the end-plate region of the muscle fiber with glass microelectrodes filled with 3 M KCl (resistance 5-10 MΩ). 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 minutes before the collection of the data. In all cases MEPPs

frequency was recorded during 10 seconds from at least 10 different neuromuscular junctions, after observing that MEPPs represented a period of stable spontaneous release. Data frequencies were measured by hand from the screen of the oscilloscope or as MEPPs amplitudes, acquired through an A/D converter controlled by computer and analyzed with a WCP software (Dagan Corp.).

Hyperosmotic media were freshly prepared by adding sucrose to the Ringer solution and their osmolarity checked with a Fiske osmometer before each experiment. To study the time course of hyperosmotic response, 10 junctions were sampled in the control solutions (isotonic) and their values averaged (open squares, Fig 7). Immediately after the change in osmolarity, synapses were sampled repeatedly from the same small area of diaphragm over brief intervals during 75 minutes at the most (filled squares, Fig 7). An effort was made to keep the intervals between sampling as short as possible. In these experiments, tetrodotoxin 10^{-6} M (Sigma, USA) was added to external media to prevent the muscle from twitching violently, which otherwise occurred upon sudden exposure of preparations to hypertonic solutions. In order to render comparable frequency values from different experiments, mean MEPP frequency of the peak hypertonic response was normalized with respect to mean MEPP frequency obtained in isotonic solution (24, 25). The comparison between hypertonic responses in control and test solutions was expressed as the ratio of the areas under their respective curves measured by numerical integration up to 30 minutes after the addition of hyperosmotic solution. To eliminate the inward Ca^{2+} gradient, a Ringer solution containing 0 Ca^{2+}, 2 mM Mg^{2+} and 1 mM EGTA was employed. Other details are described when required in the Results section. In order to decrease $[Ca^{2+}]_i$, nerve terminals were loaded with 1-2-bis-(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM, Molecular Probes, Inc. USA), a cell permeant buffer, as follows: the preparation was immersed in a Ca^{2+}-free Ringer solution containing BAPTA-AM dissolved in dimethylsulphoxide (DMSO) (3 x 10^{-7} mol/30 mg of muscle). Preparations were incubated, under these conditions, for two hours at room temperature (26), then rinsed during 40 minutes with Ca^{2+}-free Ringer solution.

The following compounds were used: aminophylline (Sigma, USA), 8-Bromoadenosine-3':5'-cyclic monophosphate (8-Br-cAMP, Sigma USA) and forskolin (Sigma, USA). In results, figures are mean ± SE , and n expresses number of muscles.

Results

Effect of cAMP on spontaneous acetylcholine release

In Figure 1 is illustrated the increase of spontaneous acetylcholine release through the activation of the cAMP-PKA pathway, observed in three different groups of experiments. Bath application of 1mM AMIN induced a clear increase in MEPPs frequency, to 226.7 ± 13.3 % (n=6) of control values. To obtain independent evidence that cAMP can actually mediate MEPPs frequency increase response, a membrane-permeable analog of cAMP, 8-Br-cAMP (5mM), was used. It enhanced acetylcholine release to 226.4 ± 22.7 %, n=4. Moreover, to stimulate directly cAMP cascade through the activation of the catalytic subunit of adenylate cyclase, 20 μM forskolin was applied to the solution. As can be seen in the figure, forskolin produced an enhancement of the spontaneous neurotransmitter release to 231.2 ± 20.1 % (n=5) of control values.

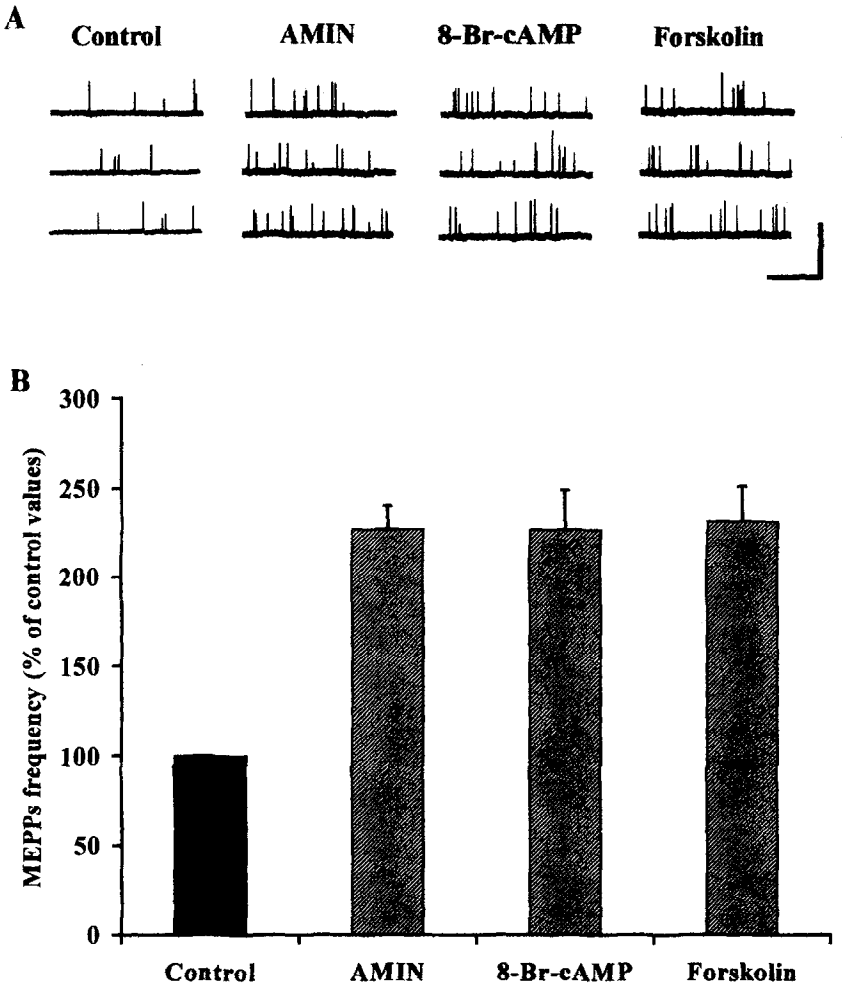

Fig. 1

A: Miniature end-plate potentials (MEPPs) recorded in rat diaphragm muscles fibers in control Ringer solution (Vm= -73.5 mV) and 20 minutes after bath application of the inhibitor of the phosphodiesterase aminophylline (AMIN 1mM, Vm= -74.8 mV), the membrane-permeable analog of cAMP, 8-Br-cAMP (5 mM, Vm= -74.1 mV) or the direct adenylate cyclase activator, forskolin (20 μM, Vm= -74.3 mV). Calibration: 3.6 mV, 1.8 s. *B*: Summary graph bar of the presynaptic facilitation induced by activation of cAMP-PKA pathway. Significant increase in MEPPs frequency was obtained when AMIN ($p < 0.0001$), 8-Br-cAMP ($p < 0.002$) or forskolin ($p < 0.0007$), were included in the normal Ringer solution (control). Data are expressed as % of control values (black bar). Error bars indicate SE.

Effect of cAMP on K^+- induced acetylcholine release

We next studied the effect of the same AMIN concentration on nerve terminals sustainedly depolarized by increasing external K^+ concentration to 10 and 15 mM, in order to determine whether kinase activation can enhance both, spontaneous and K^+-induced acetylcholine release. Figure 2 shows that the changes in MEPPs frequency were quantitatively comparable in all solutions (% of change: 5 mM K^+ 120.7 ± 14.4, 10 mM K^+ 93.4 ± 18.4, 15 mM K^+ 96.5 ± 24.5, n=3), suggesting that the mechanism underlying cAMP effect does not depend on the form of secretion, spontaneous or evoked.

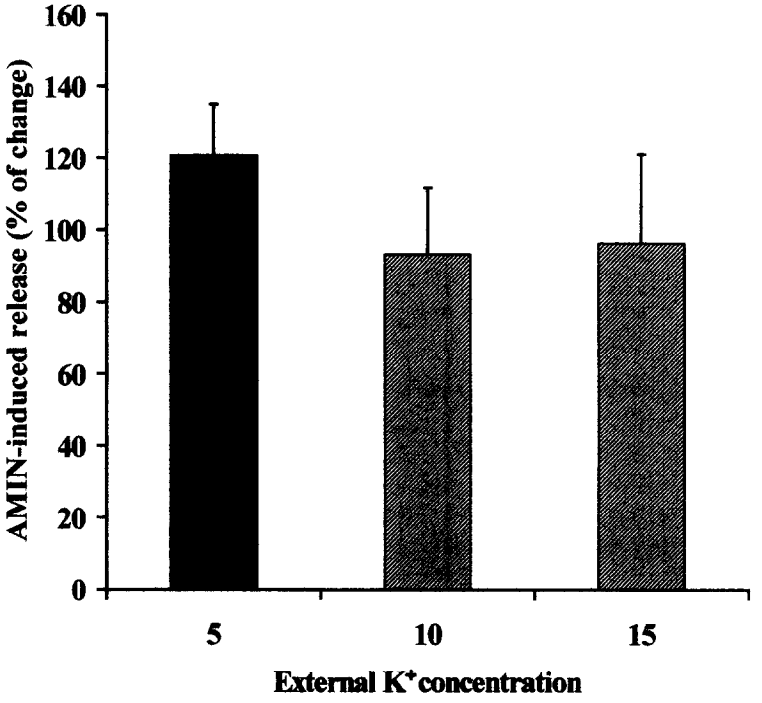

Fig. 2
Percentage of change in MEPPs frequency at different K^+ concentrations induced by 1 mM AMIN. Black bar shows AMIN-induced release in control Ringer (5 mM K^+). Differences between means are not statistically significant. Error bars indicate SE.

AMIN-induced acetylcholine release is independent on voltage-dependent calcium channels

To elucidate whether activation of the cAMP-PKA pathway could enhance the efficacy of secretory process as a result of the direct modulation of the VDCC, the effect of AMIN was tested in zero Ca^{2+}-EGTA solution. As shown in Figure 3, MEPPs frequency in zero Ca^{2+}-EGTA fell to 32.0 ± 4.4 % (n=4) of the control values. Further addition of AMIN did not significantly modify these values (39.4 ± 4.1%). These results may have two interpretations: VDCC would be considered the main target of PKA and the lack of facilitation observed when AMIN was added to zero Ca^{2+}-EGTA solution, could be explained by the virtual absence of external calcium concentration. Alternatively, AMIN-induced release would depend on intraterminal Ca^{2+}

concentration. The decreased effectiveness of AMIN in zero Ca^{2+}-EGTA solution, may be due to a reduced $[Ca^{2+}]_i$ in conditions in which the Ca^{2+} electrochemical gradient is reversed by absence of external Ca^{2+} (27, 28).

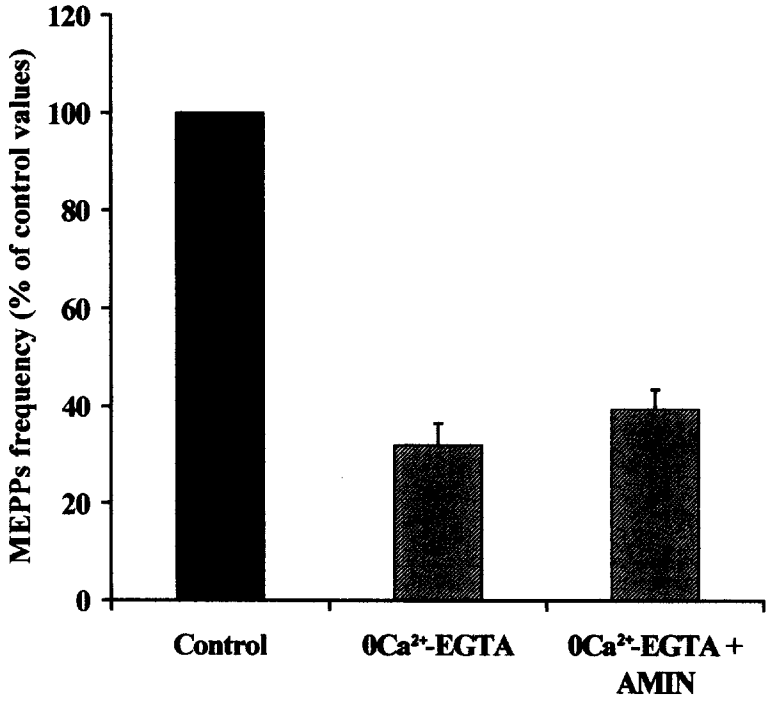

Fig. 3

Effect of 1 mM AMIN on spontaneous acetylcholine release when the preparations were bathed in 0 Ca^{2+}-1 mM EGTA solution. No significant increase in MEPPs frequency was observed. Data are expressed as % of control values (black bar). Error bars indicate SE.

To clarify this point, we took advantage of the universal blocking action of Cd^{2+} on the VDCC, in order to prevent the efflux of Ca^{2+} from nerve terminals when exposing the preparation to zero Ca^{2+}-EGTA solution (28). In Figure 4, it can be observed that 100 μM Cd^{2+} reduced MEPPs frequency to 51.0 ± 1.0 % (n=4) of the control values and to 35.3 ± 1.3 % when preparations were then exposed to zero Ca^{2+}-EGTA. The difference between Cd^{2+} in normal Ringer and Cd^{2+} in zero Ca^{2+}-EGTA solution may be on account of Ca^{2+} remaining efflux from nerve terminal (see discussion). Greater Cd^{2+} concentration did not show additional effect (data not shown). Interesting enough, further addition of AMIN, in these conditions, produced an increase of MEPPs frequency to 90.0 ± 4.8 % of the control values, suggesting that its effect is independent of VDCC and that it might modulate the efficiency of neurotransmitter release as a function of nerve terminal Ca^{2+} concentration.

To further confirm that the increase of the facilitatory effect of AMIN is independent of the VDCC, we then performed experiments with 100 μM Cd^{2+} in normal Ringer solution. These results can be observed in Figure 5, upper panel. As shown above, Cd^{2+} reduced MEPPs frequency to 50.8 ± 1.8 % (n=4) of the control values. Application of AMIN, in the presence of Cd^{2+}, induced an increase of MEPPs frequency to 151.8 ± 5.1 % of control, not significantly different

than the 126.7 ± 13.3 % increase observed in the absence of Cd^{2+} (see above). On switching solutions (Fig 5, lower panel), the facilitating action of AMIN on spontaneous transmitter release was not modified by the addition of the blocker (AMIN 201.9 ± 7.2 %; AMIN + Cd^{2+} 195.6 ± 4.9 %, n= 5).

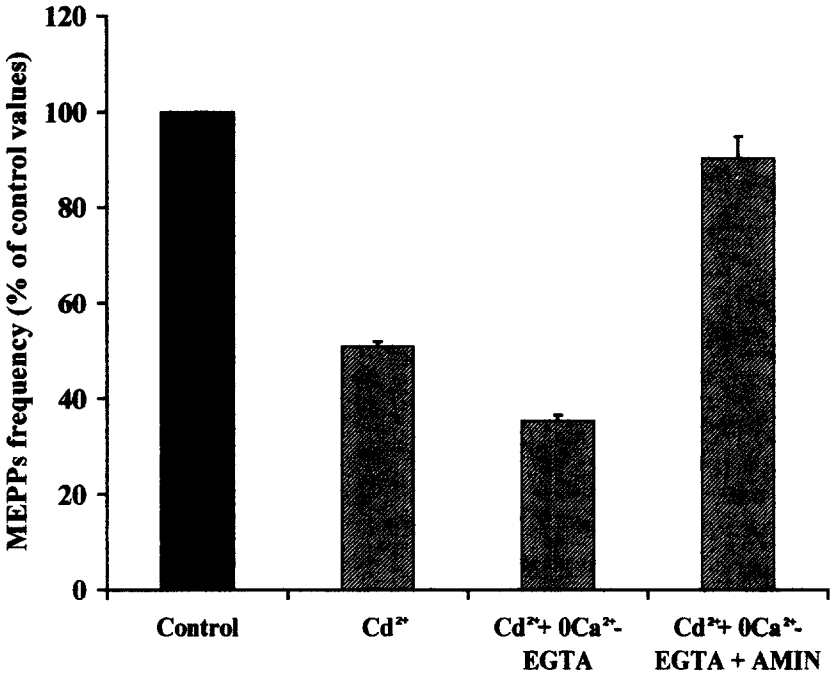

Fig. 4

Effect of 1 mM AMIN on spontaneous acetylcholine release when the preparations were bathed in 0 Ca^{2+}-1 mM EGTA + Cd^{2+} solution. 100 μM Cd^{2+} was first applied to normal Ringer solution, in order to prevent the efflux of Ca^{2+} from nerve terminals when exposing the preparation to 0 Ca^{2+}-EGTA solution. Significant increase in MEPPs frequency was obtained when AMIN was then added in this condition ($p < 0.0001$). Data are expressed as % of control values (black bar). Error bars indicate SE.

In Figure 6 is summarized the effect induced by AMIN upon the spontaneous neurotransmitter release in different salines. In control Ringer solution 1 mM AMIN by itself produced an increase of MEPPs frequency of 126.7 ± 13.3 %, but this facilitation was drastically reduced to 15.0 ± 6.6 % when the preparations were previously incubated in zero Ca^{2+}-EGTA solution. In the latter condition, previous exposure of the muscles to 100 μM Cd^{2+}, in this case to prevent Ca^{2+}eflux, reverted the results; the secretory response to AMIN was enhanced to 154.9 ± 4.3 %, suggesting that its effect is function of Ca^{2+} ions within the nerve terminals. As expected if the facilitation of acetylcholine release was down-stream from Ca^{2+} channels, the AMIN-induced facilitation was unhampered in the presence of Cd^{2+} (199.2 ± 19.1 %).

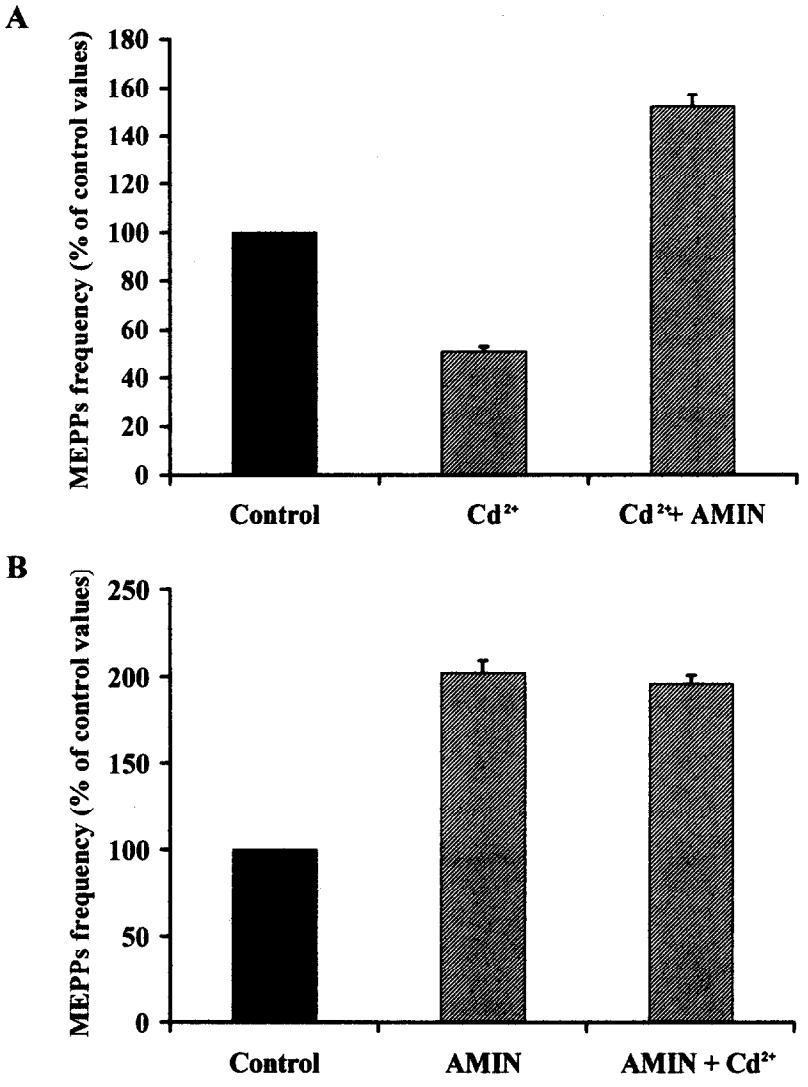

Fig. 5

A: Effect of 1 mM AMIN on spontaneous acetylcholine release in presence of 100 μM Cd^{2+}. AMIN increased significantly MEPPs frequency ($p < 0.0001$). Data are expressed as % of control values (black bar). Error bars indicate SE. *B*: Effect of Cd^{2+} in preparations previously bathed with AMIN. Cd^{2+} did not reduce the facilitation induced by AMIN.

Effect of cAMP on hyperosmotic response

Another tool to assess a direct modulation of the release machinery independently of calcium influx through VDCC has been probed to be the hyperosmotic challenge (21). In Figure 7 (upper panel), the effect of AMIN on hyperosmotic response is illustrated. By increasing tonicity from 250 to 350 mOsm, the magnitude of the hypertonic response was raised (ratio of area under

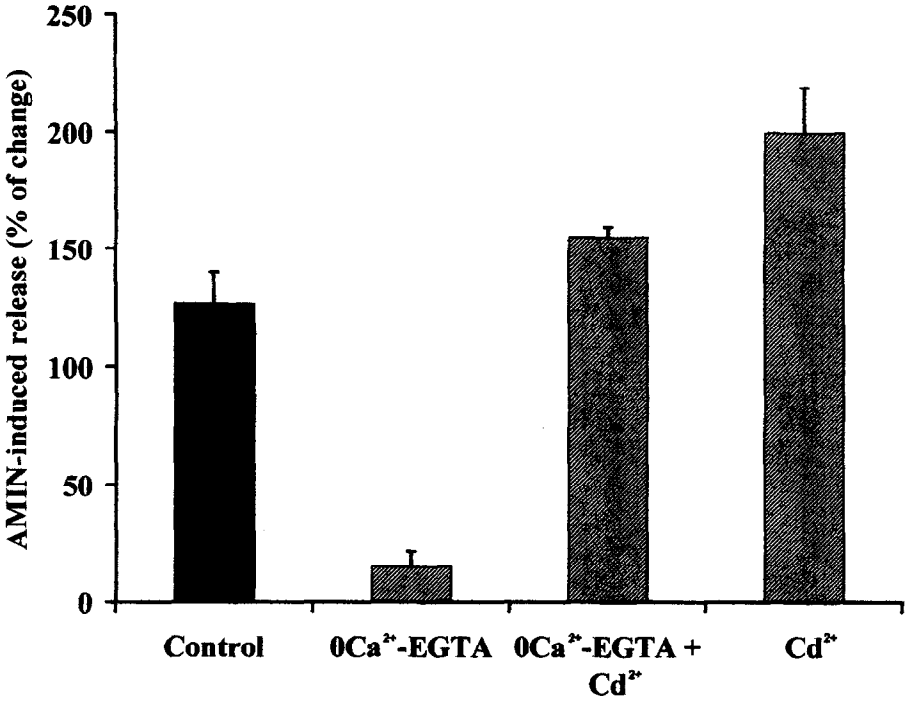

Fig. 6

Summarized bar graph showing % of change in MEPPs frequency induced by AMIN in control solution (black bar) and in 0 Ca^{2+}-EGTA, 0 Ca^{2+}-EGTA + Cd^{2+} and Cd^{2+} solutions. Error bars indicate SE.

the curve in AMIN and control: 2.1 ± 0.2; n = 3). To investigate further the role of free Ca^{2+} concentration within nerve terminals during hypertonic response in presence of AMIN, presynaptic terminals were loaded with BAPTA-AM, a membrane-permeant Ca^{2+} chelator (Fig 7, lower panel). After BAPTA-AM loading, MEPPs frequency in isotonic and hypertonic condition was recorded in Ca^{2+}-free solution. The magnitude of the response after BAPTA-AM loading, in terms of the area under the curve, was markedly reduced compared with the control response obtained in not loaded end plates (ratio of areas in control (36.3 ± 0.04 cm^2, n=3) and BAPTA-AM (8.8 ± 0.01 cm^2, n=3) = 4.1, $p < 0.0015$) and when the early transient increase in MEPPs frequency induced by exposing the preparation to hypertonic solution was related to the mean frequency obtained in isotonic condition, the ratio obtained in end-plates loaded with BAPTA-AM was significantly lower than the ratio obtained in control Ringer solution (ratio of peak osmotic response and mean isotonic MEPPs frequency was 10.4 ± 1.2, n=3 for control and 7.1 ± 0.3, n=3 for BAPTA-AM, $p < 0.017$), suggesting that the presence of Ca^{2+} within terminals is a requirement for hypertonic response. When the preparation loaded with BAPTA-AM were then exposed to AMIN and the hypertonic response were measured, we did not find any significant difference with the values observed before AMIN addition (ratio of area under the curve in AMIN and control : 1.0 ± 0.2; n = 3). The difference between the effect of AMIN upon nerve terminals loaded with BAPTA-AM and nerve terminals in normal Ringer was highly significant (ratio of areas in control (73.8 ± 0.1 cm^2) and BAPTA-AM (8.8 ± 0.01 cm^2) = 8.4, $p < 0.0005$). These results showed that the increase in the activation of PKA induced by AMIN seems to depend on $[Ca^{2+}]_i$ since by

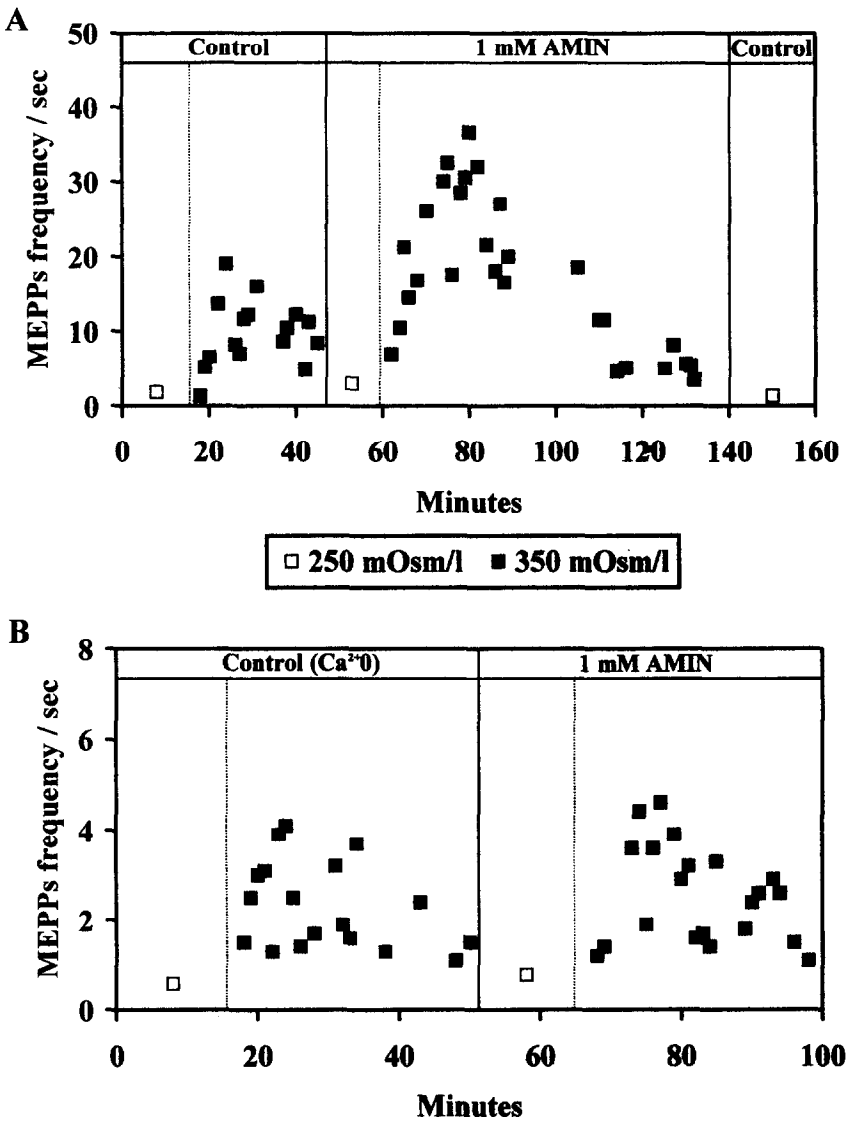

Fig.7

A: Effect of 1mM AMIN on spontaneous acetylcholine release when a preparation was exposed to isotonic and hypertonic solutions. □, Mean values from 10 synapses obtained 20 min after exposing preparation to an isotonic solution. ■, Time course of osmotic response (each point indicates averaged values of MEPPs frequency recorded during 10 s from a single synapse). Radio of areas in control and AMIN= 2.17. AMIN effect was reversible after washout of the drug. *B*: Effect of 1 mM AMIN on spontaneous acetylcholine release when a preparation was previously loaded with BAPTA-AM in isotonic and hypertonic conditions. Open and solid squares represent MEPPs frequency as described in *A*. MEPPs frequency was recorded in a Ca^{2+}-free Ringer solution after incubating the preparation for 2 hs in a Ca^{2+}-free Ringer solution containing BAPTA-AM and rinsing during a 40-min period with the recording solution. Radio of areas in control and AMIN 1.03.

loading nerve terminals with the chelator, the raise of the hypertonic response usually induced by AMIN was not evident.

Discussion

Our observations present evidence for a presynaptic modulatory control of the secretory machinery in mammalian neuromuscular synapses induced by cAMP. Spontaneous acetylcholine release was increased by: a) the inhibitor of the phosphodiesterase AMIN, b) the membrane-permeable analog of cAMP, 8-Br-cAMP and, c) the direct adenylate cyclase activator, forskolin, which activated the cAMP-PKA pathway. Stimulation of such second messenger system enhances neurotransmitter release at peripheral synapses of several species. Okadaic acid, a phosphatase inhibitor, enhances EPPs amplitude by a presynaptic mechanism in frog, crayfish, and lobster neuromuscular junction, since MEPPs frequencies are enhanced whereas MEPPs amplitudes are unaffected (29, 30). Swain et al 1992 (31) have shown that in frog and crayfish, PKA is involved in that effect. In Drosophila mutant dunce, deficient in the cAMP phosphodiesterase, Zhong and Wu 1991 (32), have found larger EPPs and fewer failures in transmission at the larval neuromuscular junction, without significant changes in MEPPs amplitudes, suggesting a presynaptic defect.

The facilitating action of cAMP on neurotransmitter secretion could be at a level ranging from mechanisms regulating intraterminal Ca^{2+} availability to the cascade of events related to exocytosis. PKA could phosphorylate the VDCCs related to spontaneous neurotransmitter release, increasing Ca^{2+} currents through them and intracellular local level of this ion and thereby regulate release. cAMP has been shown to increase current through VDCC in CA1 and CA3 hippocampal pyramidal neurons (22, 23). We have recently found that asynchronous secretion in mammalian diaphragm muscles are mainly related to Ca^{2+} ions influx through L and N-type VDCC, but not through P/Q-type VDCC (21). It is known that dihydropyridine-sensitive calcium channels are substrates for cAMP-dependent proteinkinase (22, 33). The finding that the increase in MEPPs frequency induced by AMIN was similarly facilitated in presence of Cd^{2+}, a broad-spectrum calcium blocker, suggests that the activation of the cAMP transduction pathway does not result from the modulation of the calcium channels. Coherent with this interpretation is the fact that the facilitatory effect of AMIN was observed when nerve terminals were exposed to hypertonic solutions, which produces an associated increase in MEPPs frequency. Early experiments showed that the osmotic response seems to be independent of $[Ca^{2+}]_o$ (25, 34). In agreement with these findings, we have disclosed that osmotic response in zero Ca^{2+}-EGTA solution was similar to that recorded in control solution, since the ratio between the peak osmotic response and the mean isotonic MEPPs frequency was not significantly different in control and in zero Ca^{2+}-EGTA, although the absolute values of both peak response and isotonic MEPPs frequency were considerably lower in the virtual absence of external Ca^{2+}. We have also demonstrated that nifedipine, ω-CgTx-, and ω-Aga-blockable channels are not involved in hyperosmotic response (21). Under such condition, AMIN produced an increase of the area under the curve of hyperosmotic response when compared with control curve. Moreover, we have found no evidence suggesting that the mechanism underlying AMIN effect might depend on the type of secretion, since in spontaneous or K^+-induced neurotransmitter release, related to L- and N-type VDCC, and P/Q-type VDCC respectively, the changes in MEPPs frequency were quantitatively comparable in 5 mM K^+ and in 10 and 15 mM K^+.

An alternative explanation for the facilitatory effect of AMIN is that PKA could phosphorylate one or more proteins, at a step down-stream from Ca^{2+} influx, either associated with, or part of, the protein complex that is necessary for the exocytosis of synaptic vesicles (35, 36). Among them, what would be the substrates of the PKA in the nerve terminals of mammalian

neuromuscular synapses? Synapsin I belongs to a family of synaptic vesicle-associated phosphoproteins that is able to interact with cytoskeletal proteins (37, 38) and is substrate for four protein kinases, Ca^{2+}/calmodulin-dependent protein kinase (CaMK) I and II, PKA, and proline-directed kinases (39). However, phosphorylation of synapsin I by PKA seems not to increase the ability of synaptic vesicles to move to plasma membrane and enhance transmitter release, since it was recently demonstrated that CaMKII, but not PKA, is associated with the inhibition of the binding of synapsin I to the synaptic vesicles (40-42). Phosphorylation by CaMKII is believe to be responsible for the dissociation of synapsin I from the vesicles during high-frequency electrical stimulation or sustained depolarization of nerve terminals (43, 44).

There are other synaptic phosphoproteins that are candidates for mediating regulatory functions in vesicle docking and fusion cycle. For example, Rabphilin-3A is a synaptic vesicle protein that interacts with rab3A in a GTP-dependent manner and binds Ca^{2+} in a phospholipid-dependent manner. It has been shown that rabphilin-3A is an efficient substrate for CaMKII and for PKA and can be a potential regulatory protein in the nerve terminal (45).

Our experiments also show that resting levels of Ca^{2+} within the terminals may play a main role in the facilitation, since in conditions in which the Ca^{2+} electrochemical gradient is reversed by the virtual absence of external Ca^{2+}, the enhancement of MEPPs frequency induced by AMIN was reduced to 15%. Such an effect was not discernible when Cd^{2+} was added to the saline to avoid Ca^{2+} efflux through the VDCC during the exposure to zero Ca^{2+}-EGTA solution (see Fig. 4). Further reduction in MEPPs frequency observed in presence of Cd^{2+} in zero Ca^{2+}-EGTA solution may be explained for the leakage of Ca^{2+} from nerve terminals through voltage-insensitive Ca^{2+} channels, such as those that have been described for smooth muscle (46), heart membranes (47), and cardiac myocytes (48), or for an enhanced activity of the Na^{+}- Ca^{2+} exchanger in situation of inverted Ca^{2+} gradient, even when all VDCC were blocked.

To investigate further the role of $[Ca^{2+}]_i$ on the modulatory effect of AMIN, we modified its concentration within nerve terminals by loading them with the permeant Ca^{2+} chelator BAPTA-AM in hypertonic conditions. Under such condition, the drop in MEPPs frequency was evident whether expressed in absolute values or as a ratio between peak osmotic response and mean MEPPs frequency under isotonic conditions, implying that osmotic response may be dependent on $[Ca^{2+}]_i$. Rosenmund and Stevens (50), in experiments carried out using whole cell recording from rat hippocampal neurons grown in tissue culture, have shown that hypertonic response appeared to be independent on $[Ca^{2+}]_i$. However, these results may reflect important differences in methodological procedures and/or in the type of synapses (i.e. small number of quanta per synapses). Our experiments showed that AMIN did not produce the increase of the hypertonic response observed in control solution, suggesting that the regulation of spontaneous acetylcholine release by protein phosphorylation might be a mechanism to modulate synaptic vesicle exocytosis as a function of Ca^{2+} in nerve terminals.

In summary, our observations indicate that the activation of cAMP-PKA pathway leading to presynaptic facilitation at mammalian neuromuscular junction is down-stream from Ca^{2+} channels, even though this modulatory effect is a function of free $[Ca^{2+}]_i$. Such a mechanism similarly regulates both spontaneous and K^{+}-induced neurotransmitter secretion. Recently, it was found (51) that PKA modulates an early step in the secretory machinery related to calcium sensing to facilitate synaptic transmission.

Acknowledgments

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