Spontaneous acetylcholine release in mammalian neuromuscular junctions

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Losavio, Adriana, and S. Muchnik. Spontaneous acetylcholine release in mammalian neuromuscular junctions. Am. J. Physiol. 273 (Cell Physiol. 42): C1835–C1841, 1997.—Spontaneous secretion of the neurotransmitter acetylcholine in mammalian neuromuscular synapses depends on the Ca²⁺ content of nerve terminals. The Ca²⁺ electrochemical gradient favors the entry of this cation. We investigated the possible involvement of three voltage-dependent Ca²⁺ channels (VDCC) (L-, N-, and P/Q-types) on spontaneous transmitter release at the rat neuromuscular junction. Miniature end-plate potential (MEPP) frequency was clearly reduced by 5 µM nifedipine, a blocker of the L-type VDCC, and to a lesser extent by the N-type VDCC blocker, ω-conotoxin GIVA (ω-CgTx, 5 µM). On the other hand, nifedipine and ω-CgTx had no effect on K⁺-induced transmitter secretion. ω-Agatoxin IVA (100 nM), a P/Q-type VDCC blocker, prevents acetylcholine release induced by K⁺ depolarization but failed to affect MEPP frequency in basal conditions. These results suggest that in the mammalian neuromuscular junction Ca²⁺ enters nerve terminals through at least three different channels, two of them (L- and N-types) mainly related to spontaneous acetylcholine release and the other (P/Q-type) mostly involved in depolarization-induced neurotransmitter release. Ca²⁺-binding molecule-related spontaneous release apparently binds Ca²⁺ very rapidly and would probably be located very close to Ca²⁺ channels, since the fast Ca²⁺ chelator (BAPTA-AM) significantly reduced MEPP frequency, whereas EGTA-AM, exhibiting slower kinetics, had a lower effect. The increase in MEPP frequency induced by exposing the preparation to hypertonic solutions was affected by neither external Ca²⁺ concentration nor L-, N-, and P/Q-type VDCC blockers, indicating that extracellular Ca²⁺ is not necessary to produce hyperosmotic neurosecretion. On the other hand, MEPP frequency was diminished by BAPTA-AM and EGTA-AM to the same extent, supporting the view that hypertonic response is promoted by “bulk” intracellular Ca²⁺ concentration increases.

spontaneous transmitter release; L-type voltage-dependent calcium channel; nifedipine; ω-agatoxin IVA; ω-conotoxin GVIA; 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxyethyl ester; ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid-acetoxyethyl ester; hypertonic response

that multiple types of channels may coexist at a given individual synapse. Synapses differ in their channel types, and the same kind of synapse uses different VDCCs in different species. In this regard, ω-conotoxin GIVA (ω-CgTx), an N-type VDCC blocker, was shown to inhibit neuromuscular transmission in the frog (10, 13, 16, 34) but was reported to be ineffective in mammals (34, 48). In contrast, it has been shown that mammalian-evoked neuromuscular transmission is blocked by P/Q-type VDCC antagonists such as funnel-web spider toxin, ω-conotoxin MVIIIC, and ω-agatoxin IVA (ω-Aga) (5, 18, 28, 46).

Intracellular free Ca²⁺ concentration ([Ca²⁺]i) seems to play a relevant role in spontaneous neurotransmitter secretion (3, 23, 30), particularly under hypertonic conditions (36). However, Tanabe and Kijima (40) have questioned the putative relationship between miniature end-plate potential (MEPP) frequency and [Ca²⁺]i in hypertonicity (40).

The aim of the present work was to identify the VDCCs responsible for spontaneous quantal secretion in mammalian synapse and its relationship with [Ca²⁺]o and [Ca²⁺]i. Experiments were performed in isotonic and hypertonic conditions and in the presence of ω-Aga, ω-CgTx, or nifedipine (P/Q-, N-, and L-type VDCC blockers, respectively). To study the effect of [Ca²⁺]i on the kinetics of spontaneous acetylcholine release, nerve terminals were loaded with buffers having similar equilibrium Ca²⁺ affinities but different binding kinetics. The effect of the slowly binding Ca²⁺ buffer ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)-acetoxyethyl ester (AM) was compared with the rapidly binding homologue 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA)-AM (1, 44, 45).

MATERIALS AND METHODS

Wistar rat diaphragm muscles were used. Rats (180–220 g) were anesthetized with thiopental sodium (50 mg/kg), and the left hemidiaphragm was excised and transferred to a chamber filled with Krebs-Ringer ([in mM] 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 11 glucose, 5 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.3–7.4) and bubbled with O₂. Hyperosmotic media were freshly prepared by adding sucrose to the Ringer solution, and their osmolarity was checked with a Fiske osmometer before each experiment. MEPP frequency was recorded intracellularly at the end-plate region of the muscle fiber with glass microelectrodes filled with 3 M KCl (resistance of 5–10 MΩ). Muscles fibers with a resting membrane potential less negative than ~60 mV or MEPP with a rise time >1 ms were rejected. To study the time course of hyperosmotic response, 10 junctions were sampled in the control solutions (isotonic) and their values were averaged (see Figs. 3 and 5). Immediately after the change in osmolari-
ity, at least 11 synapses were sampled repeatedly from the same small area of diaphragm over brief intervals for 40 min at the most (see Figs. 3 and 5). An effort was made to keep the intervals between sampling as short as possible. In these experiments, tetrodotoxin (10⁻⁶ M; Sigma) was added to external medium to prevent the muscle from twitching violently, which otherwise occurred when preparations were suddenly exposed to hypertonic solutions. 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 (9, 12). 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. When addition of drugs to the saline significantly increased osmolality (Co²⁺, 13.5 mM), the control solution was corrected to the same tonicity as the test solution. To eliminate the inward Ca²⁺ gradient, a Ringer solution containing 0 Ca²⁺, 2 mM Mg²⁺, and 1 mM EGTA was employed. Other details are described when required in RESULTS. To decrease [Ca²⁺], nerve terminals were loaded with BAPTA-AM (Molecular Probes) as follows: the preparation was immersed in a Ca²⁺-free Ringer solution containing BAPTA-AM dissolved in dimethyl sulfoxide (3 × 10⁻⁷ mol/30 mg of muscle). Preparations were incubated, under these conditions, for 2 h at room temperature (14, 44, 45) and then rinsed during a 40-min period with Ca²⁺-free Ringer solution. In some experiments, an equal concentration of EGTA-AM (Molecular Probes), a buffer with similar equilibrium Ca²⁺ affinity but slower binding kinetics, was used, following a similar procedure. Both buffers are cell permeant. Nifedipine (Sigma; 5 µM, in darkness), ω-CgTx (Alomone Laboratories; 5 µM), and ω-Aga (Peptide Institute; 100 nM) were used as L-, N-, and P/Q-type VDCC blockers, respectively.

In results, n represents number of fibers per number of muscles.

RESULTS

Effect of ω-Aga on spontaneous acetylcholine release. It is known that, in mice neuromuscular junctions, 100 nM ω-Aga abolishes the presynaptic Ca²⁺ currents and acetylcholine release induced by electrical stimulation or by K⁺ depolarization (28). In isotonic conditions (Fig. 1), MEPP frequency was unaltered by 100 nM ω-Aga [results (means ± SD) are 1.9 ± 0.6 for control, n = 38/4, and 2.0 ± 0.5 for ω-Aga, n = 42/4]. However, when nerve terminals were depolarized by increasing extracellular K⁺ concentration to 10 and 15 mM (Fig. 2A), ω-Aga exerted a clear drop in the K⁺-induced increase in spontaneous release [results (means ± SD) are 8.4 ± 0.1 (n = 38/4) with 10 mM K⁺ and 23.2 ± 1.3 (n = 37/4) with 15 mM K⁺ for control and 2.9 ± 0.4 (n = 40/4) with 10 mM K⁺ and 8.4 ± 0.4 (n = 40/4) with 15 mM K⁺ for ω-Aga; P < 0.0001 and P < 0.0001, respectively]. These results suggest that the P/Q-type VDCCs at the mammalian neuromuscular junction are not involved in spontaneous acetylcholine release but are related to the K⁺-evoked MEPPs. When Ringer solution tonicity was raised 35%, measurements of the hypertonic response area remained similar to control values (ratio of areas in control and ω-Aga = 1.0 ± 0.2; n = 4). Figure 3A shows a typical experiment.

Effect of nifedipine on spontaneous acetylcholine release. L-type VDCCs are sensitive to 1,4-dihydropyridine compounds (8, 26, 27), some of which, like nifedipine, act as blockers and whose effect on spontaneous neurotransmitter release was studied. As shown in Fig. 1, in isotonic conditions, 5 µM nifedipine reduced MEPP frequency by roughly 53% (3.6 ± 1.8 for control, n = 34/5, 1.7 ± 0.9 for nifedipine, n = 32/5; P < 0.005). The effect was not evident under hyperosmotic conditions (ratio of areas in control and nifedipine = 1.0 ± 0.1; n = 3; Fig. 3B). These results indicate that nifedipine-blockable channels play a major role in resting spontaneous acetylcholine release and, furthermore, that they do not participate to any appreciable extent in osmotic response.

Figure 2B shows the effect of the same nifedipine concentration on nerve terminals depolarized by increasing external K⁺ concentration to 10 and 15 mM. Interestingly enough, nifedipine appeared to exert a selective inhibitory effect on spontaneous acetylcholine release only in basolateral conditions (5 mM K⁺), without interfering with the Ca²⁺ current associated with nerve terminal depolarization (10 and 15 mM K⁺).

A nifedipine effect independent of Ca²⁺ channel blockade was ruled out, since MEPP frequency in the presence of nifedipine + zero Ca²⁺-EGTA was similar to values obtained in zero Ca²⁺-EGTA solution (percent of control values: 35.1 ± 2.6% for zero Ca²⁺-EGTA, n = 40/4, and 37.8 ± 4.9% for 5 µM nifedipine + zero Ca²⁺-EGTA, n = 42/4).

Effect of ω-CgTx on spontaneous acetylcholine release. To determine whether L-type VDCCs were the only channels involved in spontaneous secretion in mammals, MEPP frequency was recorded in zero Ca²⁺-EGTA solution. In Fig. 1, the decrease in spontaneous transmitter release produced by 5 µM nifedipine (47 ± 12%, n = 32/5) can be compared with that observed in zero Ca²⁺-EGTA solution (33.5 ± 3.8%, n = 60/6).
Values obtained in zero Ca\textsuperscript{2+}-EGTA were significantly lower than those observed after addition of nifedipine ($P < 0.01$). These results suggest that more than one type of VDCC is involved in spontaneous release.

The application of 5 µM $\omega$-CgTx reduced MEPP frequency to 71.5 ± 6.9% ($n = 42/4$), suggesting that the N-type VDCCs also play a role in the modulation of spontaneous neurotransmitter release. It is worthwhile pointing out that the combined application of nifedipine and $\omega$-CgTx reduced MEPP frequency to 41.5 ± 3.1% ($n = 50/5$) (see Fig. 1), clearly lower than the sum of the individual effect of the two drugs (~25%).

Similar to nifedipine, $\omega$-CgTx did not affect either K\textsuperscript{+}-evoked MEPPs (Fig. 2C) or osmotic response (ratio of areas in control and $\omega$-CgTx = 1.1 ± 0.2, $n = 4$, Fig. 3C).

Effect of BAPTA-AM and EGTA-AM on spontaneous acetylcholine release. It is known that the slowly binding Ca\textsuperscript{2+} buffer EGTA fails to block evoked neurotransmitter release, whereas a rapidly binding homologue, BAPTA, efficiently blocks release (1, 35, 47). However, scarce information is available concerning the kinetics of spontaneous transmitter release. Figure 4 shows that nerve terminals loaded with BAPTA-AM display a reduction in MEPP frequency to 49.2 ± 3.6% ($n = 40/4$) and to 16.7 ± 3.2% ($n = 58/6$) when the recordings were...
performed in muscles exposed to normal Ringer (2 mM Ca^{2+}) and to zero Ca^{2+} Ringer solutions, respectively. Control values obtained in normal Ringer solution before BAPTA-AM loading are expressed as 100%. It is evident that the efficacy of the chelator decreases when Ca^{2+} is reentering the terminal during the recording period. The effect of BAPTA-AM was also demonstrated when the MEPP frequency recorded in zero Ca^{2+} solution from loaded end plates was compared with that obtained in zero Ca^{2+} solution from not loaded preparations [16.7 ± 3.2% for loaded (n = 58/6) and 43.4 ± 8.6% for not loaded (n = 38/4), P < 0.0002]. These results are different from those found in frog (40).

To study the kinetics of exocytosis related to spontaneous neurotransmitter secretion, we compared the effect of BAPTA-AM on MEPP frequency recorded in normal Ringer and in zero Ca^{2+} to the slowly binding Ca^{2+} buffer EGTA-AM (Fig. 4; 75.1 ± 19.1% in normal Ringer, n = 40/4; 30.7 ± 5% in zero Ca^{2+} solution, n = 40/4). The reduction in MEPP frequency induced by EGTA-AM was significantly lower than that observed in BAPTA-AM (P < 0.018 and P < 0.0005).

Osmotic response dependence on [Ca^{2+}]_{o} and [Ca^{2+}]_{i}. With the assumption that nerve terminals behave in a similar way to muscle fibers, resting membrane potential was recorded from the latter during exposure to hypertonic solutions. No difference in resting membrane potential was found from the latter during exposure to hypertonic solutions. No difference in resting membrane potential was found between isotonic and hypertonic conditions (72.5 ± 1.5 for isotonic and 73.2 ± 0.8 for hypertonic, n = 8); similar results were also reported by Hubbard et al. (12). To study osmotic response dependence on [Ca^{2+}]_{o}, MEPP frequency was evaluated in zero Ca^{2+-}EGTA solution to preclude Ca^{2+} influx. In Fig. 5A, a single experiment is illustrated, showing that, by increasing tonicity from 280 to 420 mosM, MEPP frequency was raised both in control Ringer solution and in a Ringer made without Ca^{2+} and containing 1 mM EGTA. Although the magnitude of the hypertonic response in control solution was higher than in zero Ca^{2+-}EGTA solution (ratio of areas in control and zero Ca^{2+-}EGTA = 4.8 ± 0.3; n = 4), extracellular Ca^{2+} is hardly necessary to produce hyperosmotic neurosecretion, as shown by the similar ratios of peak osmotic response and mean isotonic MEPP frequencies in control and test solutions (8 ± 1.9 for control and 9.1 ± 3.5 for zero Ca^{2+-}EGTA; n = 4). The relative decrease in MEPP frequency in zero Ca^{2+-}EGTA may be

Fig. 4. Effect of 1,2-bis(2-aminophenoxy)ethane-N,N',N'N'-tetraacetic acid (BAPTA)-acetoxyethyl ester (AM) and EGTA-AM on spontaneous transmitter release. MEPP frequency was recorded in normal (2 mM Ca^{2+}) and in 0 Ca^{2+} Ringer solutions after loading the nerve terminals for 2 h with the chelator. Results obtained in control Ringer solution before chelator loading (open control bars) were expressed as 100%. Error bars indicate SE.

Fig. 5. A: effect of zero Ca^{2+}-EGTA solution on spontaneous release when a preparation was exposed to isotonic and hypertonic solutions. Mean values from 10 synapses obtained 30 min after exposing preparation to an isotonic solution. B: Time course of osmotic response (each point indicates averaged values of MEPP frequency recorded during 10 s from a single synapse). B and C: effect of BAPTA-AM (B) and EGTA-AM (C) on spontaneous release when preparations were exposed to isotonic and hypertonic solutions. Open and solid squares represent MEPP frequency as described in A. MEPP frequency in EGTA-AM or BAPTA-AM was recorded in a Ca^{2+}-free Ringer solution containing the chelator and rinsing during a 40-min period with the recording solution (indicated by broken horizontal axes). Control MEPP frequency in isotonic and hypertonic solutions was recorded in normal saline (2 mM Ca^{2+}). Resting membrane potential (mean ± SE, in mV) is indicated in parentheses.
due to a reduced [Ca\(^{2+}\)] in conditions in which the Ca\(^{2+}\) electrochemical gradient is reversed by the virtual absence of external Ca\(^{2+}\) (30, 49). Such an effect was not discernible when 13.5 mM Co\(^{2+}\) (a Ca\(^{2+}\) channel blocker) was added to the zero Ca\(^{2+}\)-EGTA solutions (ratio of areas in control and zero Ca\(^{2+}\)-EGTA + Co\(^{2+}\) = 1.1 ± 0.1; n = 4). To investigate further the role of free Ca\(^{2+}\) concentration within nerve terminals during hypertonic response, presynaptic terminals were loaded with BAPTA-AM or EGTA-AM, two membrane-permeant Ca\(^{2+}\) chelators. After chelator loading, MEPP frequency in isotonic and hypertonic conditions was recorded in Ca\(^{2+}\)-free solution and compared with the response obtained in control Ringer solution (2 mM Ca\(^{2+}\)) before BAPTA-AM or EGTA-AM loading. Figure 5 SB shows the effect of BAPTA-AM on hyperosmotic neurosecretion. The magnitude of such response, 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 and BAPTA-AM = 8.1 ± 14; n = 4). When the early transient increase in MEPP 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 before chelator treatment (ratio of peak osmotic response and mean isotonic MEPP frequency was 9.1 ± 0.8 for control and 5.6 ± 2.4 for BAPTA-AM; n = 4, P < 0.01), suggesting that the presence of Ca\(^{2+}\) within terminals is a basic requirement for hyperosmotic response. It is worthwhile pointing out that both BAPTA-AM and EGTA-AM (see Fig. 5C) had a similar effect on the response of MEPP frequency to hypertonicity (ratio of peak osmotic response and mean isotonic MEPP frequency was 7.6 ± 0.8 for control and 4.0 ± 1.4 for EGTA-AM; n = 4, P < 0.002), which contrasts with the greater potency of BAPTA-AM compared with EGTA-AM on spontaneous transmitter release observed in isotonic conditions (see DISCUSSION).

**DISCUSSION**

This study investigates the control of spontaneous acetylcholine release by Ca\(^{2+}\) influx into nerve terminals through diverse VDCCs at mammalian neuromuscular junctions. Our results in rat diaphragm strongly suggest the involvement of nifedipine-blockable Ca\(^{2+}\) channels, since the addition of 5 µM of this drug to the saline reduced MEPP frequency by roughly 53%. A nifedipine effect other than on VDCCs may be ruled out, since we obtained similar MEPP frequency values in zero Ca\(^{2+}\)-EGTA and in nifedipine + zero Ca\(^{2+}\)-EGTA solutions. It may be assumed that most of the Ca\(^{2+}\) current contributing to resting MEPP frequency in mammalian neuromuscular junctions takes place through L-type VDCCs. On the other hand, a different type of VDCC seems to be involved, since the reduction in MEPP frequency induced by nifedipine was significantly different from that recorded in zero Ca\(^{2+}\)-EGTA Ringer solutions. It was found that ω-CgTx reduced MEPP frequency by ~29%, indicating that N-type VDCCs also play a role in the regulation of spontaneous secretion. The combined application of nifedipine and ω-CgTx reduced MEPP frequency to values lower than the sum of the individual effects of each drug (see Fig. 1), suggesting that both VDCC types act synergistically to control spontaneous release at individual release sites. Remarkably, nifedipine and ω-CgTx did not affect the high K\(^{+}\)–evoked increase in MEPP frequency, suggesting that L- and N-type VDCCs do not interfere with acetylcholine release associated with nerve terminal depolarization. On the other hand, it has been shown that ω-Aga (a P/Q-type VDCC blocker) inhibits presynaptic Ca\(^{2+}\) current and acetylcholine release induced by either electrical or K\(^{+}\) depolarization in mice neuromuscular junction (5, 28). Our results in rat diaphragm muscles are in agreement with those findings (see Fig. 2A). However, our experiments showed that P/Q-type VDCCs were not involved in spontaneous acetylcholine secretion in basal conditions (5 mM K\(^{+}\)). Coexistence of several types of VDCCs at a single synapse has already been described in mammalian central nervous system (6, 17, 32, 39). In the frog, it has been shown that a significant fraction of Ca\(^{2+}\) influx involved in the maintenance of resting MEPP frequency occurs via ω-CgTx–blockable channels; however, in this species more than one type of channel may be involved in spontaneous release, since the action of ω-CgTx is not as effective as zero Ca\(^{2+}\)-EGTA Ringer solution (10).

Free Ca\(^{2+}\) concentration within the terminal depends on Ca\(^{2+}\) coming from the extracellular medium or released from an organelle into the cytosol (3, 31, 37, 43). After removal of all external Ca\(^{2+}\) and in conditions in which free Ca\(^{2+}\) is buffered by permeant Ca\(^{2+}\) chelators, residual secretion of transmitter quanta may depend on Ca\(^{2+}\)-binding sites sensing a Ca\(^{2+}\) transient within a small subcellular volume near the organelle. The fact that MEPP frequency fell more sharply when nerve terminals were loaded with a rapid chelator (BAPTA-AM) than with similar concentrations of a slow one (EGTA-AM) indicates that spontaneous secretion seems to follow a rapid kinetic pattern. It may be speculated that the Ca\(^{2+}\) receptors that trigger spontaneous release bind Ca\(^{2+}\) within the L- and N-type VDCC nanodomains, so that nearby vesicles can promptly fuse (1, 25, 35, 38, 47). Strikingly enough, although BAPTA-AM has a greater potency compared with EGTA-AM on spontaneous secretion, this effect is more readily discernible on evoked neurotransmitter release (1, 25, 35, 38, 47).

To investigate further the contribution of [Ca\(^{2+}\)], to the release of acetylcholine in mammalian diaphragm muscle, we modified its concentration within nerve terminals by exposing the preparations to hypertonic solutions, which produced an associated increase in MEPP frequency (7, 9, 12). This osmotic response appears unrelated to any increase in intracellular K\(^{+}\) concentration, which tends to hyperpolarize nerve terminals, decreasing rather than increasing MEPP frequency (19). Although no direct measurements of resting membrane potential were performed in nerve terminals, our data obtained from muscle fibers in
hypertonic and isotonic salines (see RESULTS) may be extrapolated to nerve terminals.

Increase in MEPP frequency promoted by hypertonicity varies widely, but it has been suggested that a solution of a given tonicity increases the frequency by a fixed factor with respect to the frequency recorded in control solution, so that the ratio of the frequency in a hypertonic solution to the control frequency should be roughly constant, at least for the first 15 min following hypertonic exposure. This behavior in zero Ca$^{2+}$ has been shown to be independent of [Ca$^{2+}$]$_i$ when the memory of this condition, both BAPTA-AM and EGTA-AM behave similarly, suggesting that in hypotonic conditions Ca$^{2+}$-regulated release at a chemical synapse.

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