Role of Stored Calcium in the Regulation of Neurotransmitter Quantum Size

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Release of stored calcium ions during activation of ryanodine receptors with ryanodine or caffeine elevates the mean amplitude of spontaneous miniature end-plate potentials. Blockade of these receptors with selective antagonists abolishes this effect. Preliminary loading of the motor nerve terminals with intracellular calcium buffer EGTA-AM, but not with BAPTA-AM, also completely prevented the ryanodine-induced increment of miniature end-plate potential amplitude probably induced by the release of stored calcium. Vesamicol, a selective blocker of acetylcholine transport into vesicles, prevented the ryanodine-induced increment of the mean amplitude of miniature end-plate potentials. This increment was 2-fold more pronounced after preliminary blockade of protein kinase C with chelerythrine and was completely abolished by blockade of protein kinase A with H-89.

Key Words: *neurotransmitter quantum size; stored calcium; Ca^{2+}-signal; protein kinase C; protein kinase A*

The mechanism regulating the neurotransmitter quantum size is an actual but little examined problem of modern physiology. There are data attesting to an increase in the quantum size of secreted neurotransmitter in synapses of central nerve terminals induced by enhanced release of stored calcium triggered by ryanodine receptors (RR) [8]. We have previously demonstrated that ryanodine (RR agonist) increased the amplitude of miniature end-plate potentials (MEPP) in mouse nerve-muscle synapses [1]. However, the mechanisms underlying these changes are still unknown.

Our aims were *1*) to examine the effect of RR agonists on MEPP amplitude, *2*) to study the modulating action of Ca^{2+}-buffers (fast and slow) and a selective blocker of acetylcholine pumping into the nerve terminal vesicles on the effects of ryanodine, and *3*) to investigate the dependence of ryanodine effects on activity of protein kinase A (PKA) and protein kinase C (PKC).

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MATERIALS AND METHODS

The experiments were carried out on intact *m. diaphragma – n. phrenic* neuromuscular preparation isolated from adult outbred mice weighing ~20 g. The preparation was perfused with oxygenated Laily solution (95% O_2 and 5% CO_2) containing (in mM): 135.0 NaCl, 4.0 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 1.0 NaH_2PO_4, 16.0 $NaHCO_3$, and 11.0 glucose, pH 7.2-7.4.

The transmembrane potentials were recorded using standard microelectrode technique. The amplitudes of intracellularly recorded MEPP were analyzed.

Caffeine in a concentration of 2.5 mM and ryanodine in a low concentration of 1 μM were used as RR agonists. Ryanodine in a high concentration (of 10 μM) and dantrolene (100 μM) were used as RR blockers. Acetomethoxy derivatives of calcium buffers crossing the cell membrane 1,2-bis-(o-2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) and ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA-AM) in a concentration of 50 μM were also used. Both substances have similar calcium buffering capacity and Ca^{2+} affinity, but differ in binding

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kinetics. BAPTA is a fast buffer, which binds Ca^{2+} ions 100-fold more rapidly than EGTA buffer [10]. The buffers were dissolved in DMSO to a concentration of 10^{-2} M in the stock solutions. Concentration of DMSO in the test solutions did not exceed 0.1%. The nerve-muscle preparation was incubated (for 2 h) in Ca-free Laily solution (0 mM Ca^{2+}, 3.0 mM Mg^{2+}) containing BAPTA-AM or EGTA-AM (50 μM). Then this solution was replaced with normocalcemic (standard) Laily solution (2.0 mM Ca^{2+}, 1.0 mM Mg^{2+}) and the recordings was started in 20-30 min [12]. Vesamicol (5 μM) was used to block vesicle pumping with the neurotransmitter. PKC and PKA were inhibited with chelerythrine (4 μM) and H-89 (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide hydrochloride, 1 μM).

The data were analyzed statistically using Microsoft Excel and Statistica software. Significance of differences was assessed by Student's *t* test and Mann–Whitney *U* test.

RESULTS

In preliminary experiments we found that application of caffeine (2.5 mM) to the muscle had no effect on the resting potential of muscle fibers, the control and experimental values being -61.9±0.9 and -60.0±1.2 mV, respectively. Other substances used in this study also had no effect on resting potential of the muscle fibers. In contrast, incubation of the muscle in caffeine solution for 30 min significantly increased MEPP amplitude by 56.2±13.3% (n=17, p<0.05) compared to the normal level of 1.05±0.04 mV (n=28). This effect is illustrated by a shift of the peak of MEPP amplitude histogram to higher amplitudes under the effect of caffeine (Fig. 1, *a*).

Similar changes were produced by more selective agonist of RR, *i.e*. ryanodine in a low concentration of 1 μM, which increased MEPP amplitude by 21.8±6.7% relative to the control level (n=14, p<0.01). Apparently, both caffeine and low ryanodine did not distort symmetrical bell-shaped histogram of MEPP amplitude (Fig. 1, *a*).

In further experiments, RR were preliminary blocked by application of selective antagonists dantrolene (100 μM) and ryanodine in a high concentration of 10 μM to the nerve-muscle preparation. Both antagonists had no effects on MEPP amplitude and its distribution histogram during the observation period of 40-60 min. However, it prevented the effects of both examined agonists. Specifically, the amplitude parameters of MEPP evoked by low ryanodine (1 μM) in dantrolene-treated preparation did not significantly differ from the control values; the same was true for caffeine applied to the muscle treated with high (blocking) concentration of ryanodine (10 μM, Fig. 1, *b*). These data suggest that activation of RR by caffeine or low concentration of ryanodine increased the mean MEPP amplitude.

In further experiments we used only low concentration of ryanodine (1 μM) as RR agonist, because caffeine produces a number of side pre- and postsynaptic effects [6], which complicate analysis of the agonistic effect on RR.

After loading of the motor terminals with any of the used Ca^{2+} buffers, the MEPP amplitude histogram and the mean value of MEPP amplitude did not differ

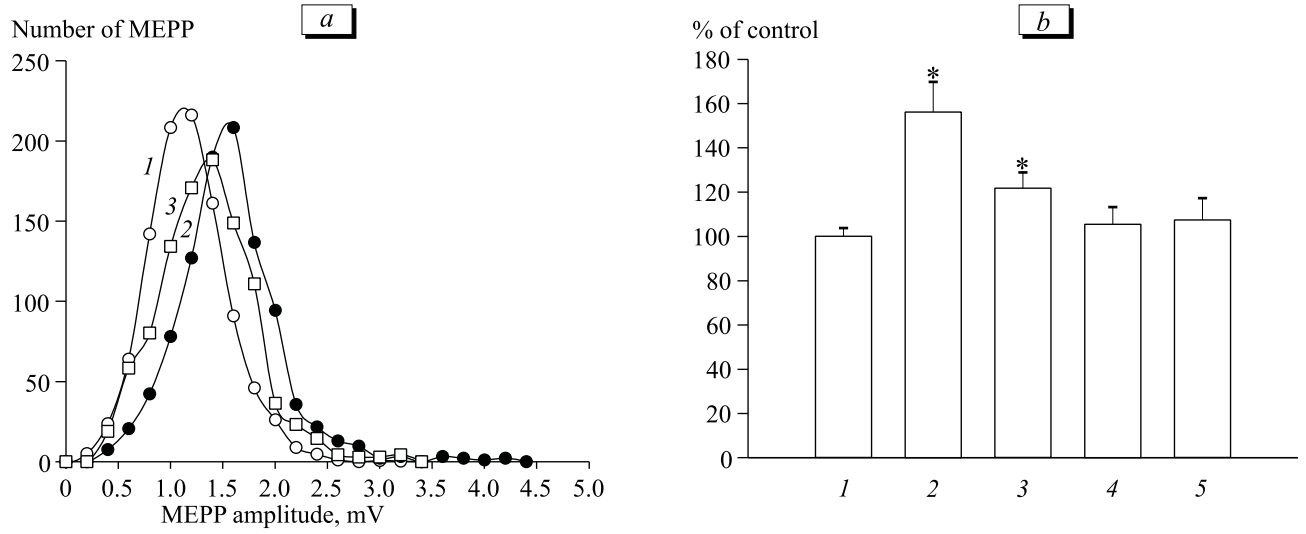

Fig. 1. Amplitude distribution and the mean amplitude of MEPP. *a*) the envelope of the MEPP amplitude histograms in control (1), during application of RR agonists caffeine (2.5 mM, *2*) or low ryanodine (1 μM, *3*); *b*) The mean MEPP amplitude in control (*1*), during application of caffeine (2.5 mM, *2*) low ryanodine (1 μM, *3*), 2.5 mM caffeine after preliminary incubation with RR blocker (10 μM ryanodine, *4*), and low ryanodine (1 μM) after preliminary incubation with RR blocker dantrolene (100 μM, *5*). *p<0.01 compared to the control.

from the control values. Application of ryanodine to the terminals loaded with fast Ca^{2+} buffer BAPTA-AM (50 μM) increased the mean amplitude of MEPP by 19±6% (n=12, p<0.05), which did not significantly differ from the ryanodine–induced increase of the mean MEPP amplitude in buffer-free preparations (p>0.05). In contrast, loading the terminals with slow Ca^{2+} buffer EGTA-AM (50 μM) almost completely prevented the ryanodine–induced increase in the mean MEPP amplitude (Fig. 2). The capacity of EGTA-AM to prevent ryanodine–induced increase of MEPP amplitude attests to dependence of this ryanodine effect on Ca^{2+} released from calcium depot during activation of presynaptic RR with ryanodine.

To test the hypothesis that the above Ca^{2+}-dependent ryanodine–induced increase of the mean MEPP amplitude resulted from elevation of the size of the secreted neurotransmitter quanta, we used vesamicol, a specific blocker of acetylcholine transport into vesicles [9].

In intact nerve-muscle preparation, vesamicol (5 μM) produced no significant changes in the amplitude histogram of MEPP, and, specifically, in the mean amplitude of MEPP (Fig. 3, *a*). However, application of ryanodine (1 μM) to vesamicol-treated preparation produced no significant increase in the mean amplitude of MEPP and no corresponding shift of the histogram to the right, in contrast to the effects observed with individually applied ryanodine (Fig. 3, *a*).

In the next experimental series, we examined the modulating effect of preliminary blockade of PKC with chelerythrine (4 μM) on the effect of ryanodine.

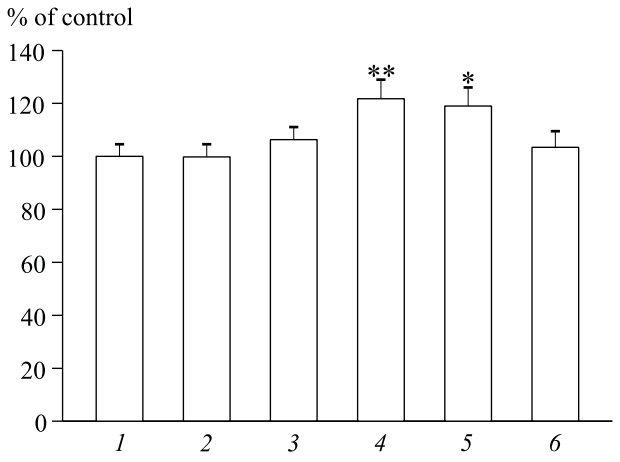

Fig. 2. Mean amplitude of MEPP in control (*1*), after loading the terminal with 50 μM BAPTA-AM (*2*), 50 μM EGTA-AM (*3*), and during application of 1 μM ryanodine (*4*) alone or after preliminary loading the terminals with 50 μM BAPTA-AM (*5) or* 50 μM EGTA-AM (6). p^*<0.05, $^{**}p$<0.01 in comparison with the control.

Blockade of this enzyme produced no significant changes in the mean values and histogram of MEPP amplitude. However, application of ryanodine (1 μM) to chelerythrine-treated preparation unexpectedly increased the mean amplitude of MEPP by 58±9% in comparison with the control (n=17, p<0.01) while ryanodine alone produced only a 22±7% increase of this value (n=14, p<0.01). Then we examined possible modulation of ryanodine effects on MEPP amplitude by H-89 (1 μM), a selective inhibitor of PKA. Incubation of the preparation with H-89 produced no significant changes in the examined parameters of MEPP.

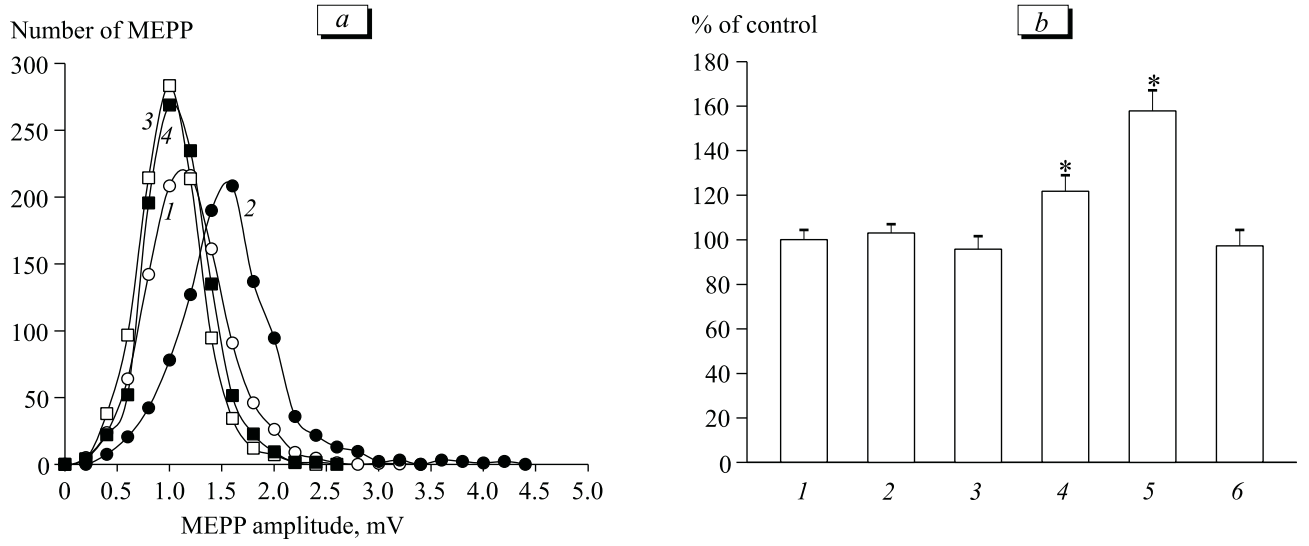

Fig. 3. Amplitude distribution and the mean amplitude of MEPP. *a*) The envelope of the MEPP amplitude histograms in control (*1*), during application of 1 μM ryanodine (*2*), 5 μM vesamicol (*3*), and during application of 4 μM chelerythrine against the background of vesamicol treatment (*4*); *b*) the mean amplitude of MEPP in control (*1*), during application of 4 μM chelerythrine (*2*), 1 μM H-89 (*3*), 1 μM ryanodine (*4*), and during application of ryanodine after preliminary loading the terminals with chelerythrine (*5)* or H-89 M (*6*). p^*<0.01 in comparison with the control.

Similarly, application of ryanodine (1 μM) to the preparation preliminary treated with H-89 produced no effects on amplitude parameters of MEPP (Fig. 3, *b*).

Thus, the examined RR-agonists significantly increased the mean amplitude of MEPP without distorting symmetrical shape of the amplitude histogram, which was only shifted to the right. These results agree with the data previously obtained on mouse motor synapses [1] and on central neurons [8]. Similar phenomena were observed in nematodes: ryanodine increased the mean amplitude of miniature postsynaptic currents in the nerve-muscle synapses, but this effect was not observed in RR-gene knockout mice [7]. The capacity of RR blockers to prevent the potentiating effects of caffeine and ryanodine on MEPP amplitude proves that increment of MEPP amplitude results from activation of RR, and it is not related to possible side effects of the above agonists.

The capacity of slow Ca^{2+} buffer to prevent increment of MEPP amplitude caused by ryanodine attests to Ca^{2+}-dependence of the potentiating effect of ryanodine evidently caused by RR-triggered release of stored calcium ions in response to application of ryanodine. EGTA belongs to the so-called slow Ca^{2+} buffers reducing relatively slowly rising and falling Ca^{2+} signals [10]. This peculiarity implies that during the tonic action of ryanodine, the level of intracellular calcium slowly rises in the nerve terminals leading to selective activation of Ca^{2+}-dependent processes that determine MEPP amplitude.

Our data on the capacity of vesamicol to prevent ryanodine-induced increment of MEPP amplitude favors the view that this Ca^{2+}-dependent increment results from an increased size of a neurotransmitter quantum. Up-regulation of pumping and filling of the synaptic vesicles is produced by hormones, enzymes, and other metabolites. Vesamicol can block the transport of acetylcholine into vesicles by binding with its transporter molecule [4,13]. We showed that calcium released from Ca^{2+}-depot during tonic activation of RR in the nerve terminals belongs to the factors stimulating neurotransmitter transport into vesicles.

Numerous Ca^{2+}-dependent cascades and enzymes are coupled with the release of stored calcium ions in neurons and their terminals [3]. In this study, we examined possible involvement of two enzymes, PKA and PKC, into neurotransmission bearing in mind the fact that they can oppositely affect the size of secreted quanta of acetylcholine and other neurotransmitters [13].

It is a common view that in the intact synapses, activity of PKC is suppressed and its effects on secretion of neurotransmitter cannot be detected [11]. We also did not observe changes in MEPP amplitude in the chelerythrine-treated preparations. However, preliminary application of chelerythrine doubled the effect of ryanodine on MEPP amplitude in comparison with that produced with this agonist alone (Fig. 3, *b*).

These data suggest that during stimulation of the release of stored calcium in the nerve terminals by ryanodine, activated PKC inhibits the increase of acetylcholine quantum size. There are data that PKC artificially activated with phorbol ethers can diminish the size of secreted acetylcholine quanta [14]. The mechanism of inhibitory action of PKC on the increase of acetylcholine quantum size is little studied. Hypothetically, activated PKC can inhibit the pumping action of vesicular H^+-ATPase responsible for proton transport into vesicle, and in this way, PKC can modulate the transport of neurotransmitter into vesicle known to be coupled with H^+-gradient across vesicular membrane [2,14]. Our experiments showed that calcium released from Ca^{2+}-depot can selectively activate PKC thereby inhibiting the increase of acetylcholine quantum size.

An unexpected and unknown effect found in this study was prevention of the potentiating effect of ryanodine on MEPP amplitude by a selective PKA blocker H-89. According to literature data, PKA can augment MEPP amplitude and the quantum size of spontaneously secreted acetylcholine provided PKA was stimulated with some hormones and peptides affecting the metabotropic receptors in motor nerve terminals [13]. In this study, PKA that potentiated the acetylcholine quantum size was activated during the release of stored calcium. The coupling mechanism between calcium release and PKA activation is not clear. It cannot be excluded that calcium ions released from Ca^{2+}-depot by ryanodine affect the pool of the Ca/calmodulin-dependent cytoplasmic PKA isoforms recently found on the presynaptic nerve terminals, which are involved in the control of presynaptic plasticity being capable to modulate operation of vacuolar H^+-ATPase and pumping of neurotransmitter into vesicles in a calcium-dependent manner [5,15].

Thus, we have established that release of stored calcium triggered by ryanodine via RR can augment MEPP amplitude and the acetylcholine quantum size in mouse motor nerve terminals via Ca^{2+}-dependent up-regulation of neurotransmitter pumping into vesicles. In some way, this process is modulated by PKA. At the same time, PKC can also be activated during release of the stored calcium and exert the reciprocal effect by diminishing the quantum size of secreted acetylcholine. Probably, calcium ions released from Ca^{2+}-depot trigger the opposite mechanisms controlling acetylcholine quantum size under the modulating effect of various enzymes, the up-regulating pathway playing the major role during their simultaneous activation. The specific mechanisms of PKA- and PKC-dependent reciprocal control of acetylcholine quantum

size triggered by release of stored calcium are the aims of further studies.

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