Effect of Fast and Slow Calcium Buffers on Induced Secretion of Neurotransmitter

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Loading of mouse motor nerve terminals with EGTA-AM, but not with BAPTA-AM, inhibited the release of the neurotransmitter in response to stimulation of the nerve with rare (0.3 Hz) "single" pulses. During rhythmic stimulation with short (50 EPP) high-frequency (20 Hz) series, BAPTA-AM buffer modified burst pattern in a dose-dependent manner: it replaced the phase of initial facilitation by persistent depression of secretion and decreased its plateau level at the end of the burst. In contrast, loading of the nerve terminals with EGTA-AM buffer produced no effect on the phase of initial facilitation, but decreased the plateau level to the same degree as BAPTA-AM did. Probably, the different effects of both buffers on secretion of neurotransmitter reflect peculiarities of involvement of fast and slow Ca^{2+} signals of motor terminals in single and rhythmic release of the neurotransmitter.

Key Words: *BAPTA-AM; EGTA-AM; quantum content of endplate potentials; rhythmic activity; Ca^{2+} signal*

In synapses, the nerve terminals employ Ca^{2+} signals whose parameters vary in dependence of the source supplying calcium ions into the axoplasm and the mode of its removal or binding. The role of buffer systems in shaping calcium signals and secretion of neurotransmitter is examined by loading cells with exogenous Ca^{2+} buffers, *i.e.*, organic Ca^{2+}-binding molecules such as EGTA and BAPTA [10,11]. These substances are characterized by similar capacity and affinity to Ca^{2+} ions, but differ by kinetics of binding of bivalent ions. BARTA is a fast buffer: it binds Ca^{2+} ions 100-times more rapidly than EGTA [11]. In nerve terminals, BAPTA buffer can reduce the magnitude of rapidly emerging and strictly local elevations of calcium concentration (so-called Ca^{2+} microdomains) formed around the intracellular mouth of a single open Ca^{2+} channel. In contrast, EGTA contributes to diminution of slow and diffuse elevations of Ca^{2+} concentration (Ca^{2+} macrodomains, [8]). The role of fast and slow calcium signals in the regulation of neurotransmission secretion in various types of synapses is an important problem in synaptology [7]. Examination of this problem using BAPTA and EGTA in neuromuscular synapses yielded contradictory results [1,6,10]. Our aim was to compare the dose-dependent influences of BAPTA and EGTA on the processes of evoked quantum secretion of acetylcholine in mouse motor synapses during single excitation or rhythmic activity.

MATERIALS AND METHODS

Experiments were carried out on dissected nerve-muscle preparations of the phrenic muscle (*m. diaphragm n. phrenicus*) isolated from adult random-bred mice weighing 20 g on the average. The preparation was placed into a chamber with normal Lilly solution for warm-blooded animals containing (in mM): 135 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 NaH_2PO_4, 11 glucose, 16 $NaHCO_3$ (pH 7.2-7.4) aerated with carbogen (95% O_2 and 5% CO_2).

The routine microelectrode technique for recording of membrane potentials was employed through-

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out the experiments. Miniature end-plate potentials (MEPP) and evoked end-plate potentials (EPP) were recorded in response to stimulation of the nerve with single pulses at a rate of 0.3 Hz or short rhythmic trains of pulses at 20 Hz evoking 50 EPP. Quantum content (QC) of single EPP was calculated by direct method as the ratio of the mean EPP amplitude to the mean amplitude of mEPP: $QC=A(EPP)_{mean}/A(mEPP)_{mean}$. Initial facilitation was assessed as the amplitude ratio of the second to the first EPP in the burst, $A(EPP_2)/A(mEPP_1)$. The plateau level was calculated as the mean amplitude of the last 30 EPP in the burst in percentage of $A(mEPP_1)$.

The study used acetomethoxy derivatives of calcium buffers: 1,2-bis-(o-2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) in concentrations of 1, 10, 50, and 100 μM and ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA-AM) in concentrations of 10 and 50 μM. The calcium buffers were dissolved in DMSO; their concentrations in stock solution were 10^{-1} and 10^{-2} M. The concentration of DMSO in the test solutions did not surpass 0.1%. The muscle was incubated for 2 h in calcium-free Lilly solution (0 mM Ca^{2+}, 3 mM Mg^{2+}) containing (in μM) 1, 10, 50, or 100 BAPTA-AM and 10 or 50 EGTA-AM. Then this incubation solution was replaced for normal calcium solution containing 2.0 mM Ca^{2+} and 1.0 mM Mg^{2+}. Recording of the signals was started after 20-30 min [14]. Calculations were performed with Microsoft Excel and Statistica software. The data were processed statistically using the Student and Mann–Whitney tests and presented as *m*±*SEM*.

RESULTS

In the control, the mean value of resting potential (RP) of muscle fibers in dissected preparation was -44.2±0.8 mV (*n*=65). The control value of QC was 20.6±0.3 (*n*=65). Both parameters did not change throughout the experiments. When the terminals were loaded with the fast buffer BAPTA-AM, RP was -43.2±1.6 mV ($p>0.05$, *n*=14). The corresponding value of RP in experiments with EGTA-loaded terminals was -43.6±1.2 mV ($p>0.05$, *n*=25).

In the first experimental series, we examined the dose-dependent effect of 2-h loading of the nerve terminals with BAPTA-AM and EGTA-AM on QC of single EPP. When applied in concentrations of 10, 50, and 100 μM, BAPTA-AM produced no significant changes in QC of EPP (Fig. 1, *a*).

Loading of motor terminals with slow buffer EGTA-AM in a low concentration of 10 μM also produced no effect on QC in EPP. However, elevation of this concentration to 50 μM significantly decreased QC in EPP by 16% relative to the control value (Fig. 1, *b*).

In the second series of experiments, we examined the effects of loading of nerve terminals with calcium buffer on evoked EPP bursts. Loading the nerve terminals with BAPTA-AM in concentrations of 1, 10, 50, and 100 μM produced dose-dependent changes in the initial facilitation in the bursts. At low concentrations (1 and 10 μM), the buffer inhibited the development of initial facilitation and decreased the amplitude and duration of this process (Fig. 2). Elevation of concentration of this buffer to 50 and 100 μM qualitatively modified burst pattern and replaced the initial facilitation by initial depression. In addition, high concentrations of the buffer aggravated this depression. Thus, loading the nerve terminals with BAPTA-AM buffer inhibited initial facilitation in a dose-dependent manner and augmented initial depression at the large concentration of 100 μM.

When the nerve terminals were loaded with BAPTA-AM at low concentrations, the plateau phase in the

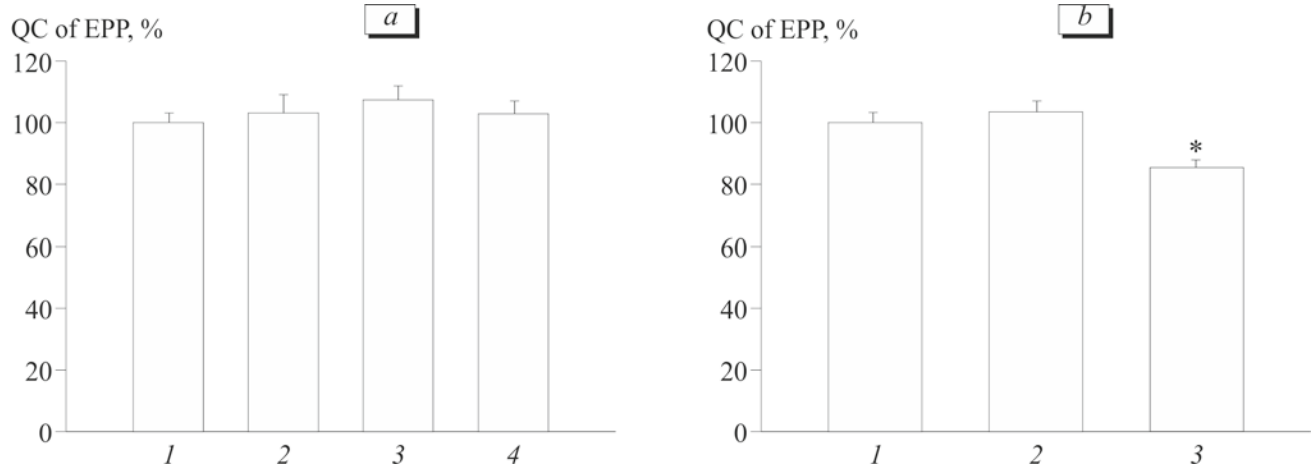

Fig. 1. Quantum content in single EPP under the control (*1*) conditions and after loading of nerve terminals with BAPTA-AM (*a*) and EGTA-AM (*b*) in concentrations of 10 μM (*2*), 50 μM (*3*), and 100 μM (*4*). Here and in Figs. 2 and 3: *$p<0.05$ in comparison with the control.

burst did not change. However, higher concentrations of 50 and 100 μM significantly decreased the plateau level to 93.4 and 86.5% of the control value, respectively. Thus, the processes of initial facilitation and depression as well as the level of plateau differed in sensitivity to BAPTA-AM. Consequently, it seemed important to compare the described changes in the burst patterns with those produced after loading of motor terminals with slow buffer EGTA-AM.

When the nerve was stimulated at a rate of 20 Hz, loading of terminals with 10 μM EGTA-AM produced no effect on the examined parameters of the bursts. Under these conditions, initial facilitation and the plateau level were 100.1±0.9 and 100.6±2.1% (p>0.05, n=19) of the control value, respectively. Elevation of concentration of slow buffer to 50 μM produced no effect on initial facilitation, its value or the rate of development (Fig. 3). In contrast, it augmented depression and decreased the plateau level to 93% of control value. Thus, the single parameter that equally changed by either buffer was the plateau level during rhythmic nerve stimulation.

The above comparative analysis of presynaptic effects of BAPTA-AM and EGTA-AM concludes that the fast and slow Ca^{2+} buffers differently affect the evoked release of the neurotransmitter. The use of BAPTA-AM in any concentration produced no effect on QC of single EPP, while loading of terminals with EGTA-AM decreased this parameter only at a concentration of 50 μM. There are published reports that loading of nerve terminals with BAPTA-AM differently affected QC of the postsynaptic signals depending of examined onject. This buffer inhibited evoked secretion of neurotransmitter in giant squid axon [2], hippocampal synapses [11], and motor synapses in frogs [1,9]. However, similar concentrations of BAPTA-AM did not decrease EPP in motor synapses in frogs [13] or phrenic synapses in mice [14]. The effect of EGTA on secretion of neurotransmitter also depends on the test object. This slow buffer does not affect QC of evoked EPP in squid synapses [2] or frog [1,10]. By contrast, in rat CNS neurons, both BAPTA and EGTA were similar efficient to inhibit exocytosis of synaptic vesicles [11]. In our experiments, EGTA but not BAPTA diminished QC of single EPP. This fact can indicate that in mouse motor terminals, binding and saturation of specific exocytotic proteins (so called Ca^{2+}-sensors) with calcium ions, which is a necessary stage for exocytosis of a vesicle, are realized by the formation of Ca^{2+}-macrodomains due to interaction of closely juxtaposed Ca^{2+}-microdomains. This hypothesis is corroborated by the data on close clustering of Ca^{2+}-channels in the vicinity of every vesicle at the release face of presynaptic terminal of these synapses [12] and by the data on larger probability of transmitter release in our experiments in comparison with other species such as frogs.

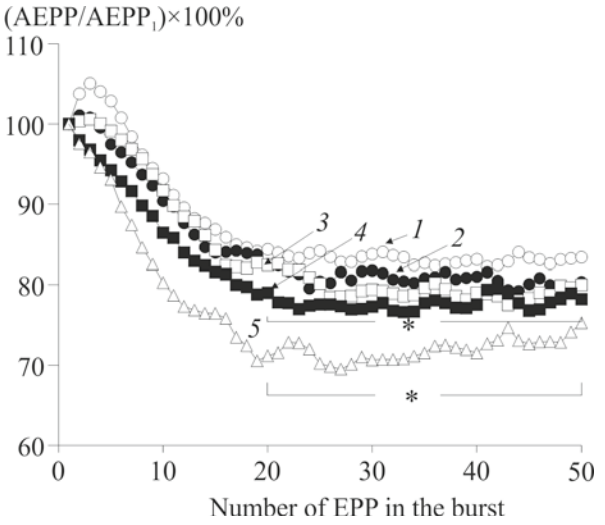

Fig. 2. Envelope of EPP burst during nerve stimulation at the rate of 20 Hz in control (*1*) and after loading the nerve terminals with BAPTA-AM in concentrations of 1 μM (*2*), 10 μM (*3*), 50 μM (*4*), and 100 μM (*5*).

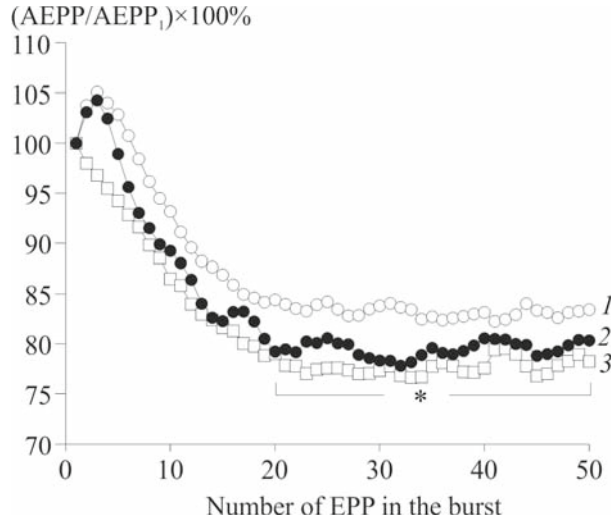

Fig. 3. Envelope of EPP burst during stimulation of the nerve at the rate of 20 Hz in control (*1*) and after loading the nerve terminals with EGTA-AM (*2*) or BAPTA-AM (*3*) in concentration of 50 μM. The asterisk marks significant difference between (*2*) and (*3*).

We describe the effects of both buffers not only on the single acts of multiquantal transmitter secretion, but also on the pattern of burst secretion evoked by nerve stimulation including the stages of initial facilitation, short-term depression, and stabilization of EPP at a lower level in comparison with EPP_1 (the plateau phase). In contrast to a single act of transmitter release reduced by the slow buffer only in large concentrations, secretion of acetylcholine during the burst (at the stage of initial facilitation) was sensitive only to the fast buffer and in concentrations that produced no effect on single EPP. At the same time, the presence of

slow buffer (EGTA) in the nerve terminal produced no significant effect on initial facilitation. Similar data on the effects of slow and fast buffers on initial facilitation were obtained on the dissected frog nerve-muscle preparation [6]. In contrast to slow EGTA buffer, only the fast buffer BAPTA could eliminate initial facilitation in synapses of hippocampal neurons [5]. The stage of initial facilitation in the burst results from summation and action of so-called "residual" calcium from the pool of local Ca^{2+}-microdomain on the highly sensitive Ca^{2+}-sensors involved in exocytosis of vesicles during repetitive excitation of the terminal [11]. In contrast to selective action on the initial phase of the burst, both buffers were equally efficient in decreasing the plateau phase of the burst. According to published reports, the fast and slow buffers with the same capacity were equally effective in elimination of calcium-dependent processes in case of generalized, but not local elevation of Ca^{2+} concentration in the cell [3,4]. The fact that both buffers equally decreased the plateau level in this study suggests that Ca-depending processes determining the plateau level may result from generalized elevation of Ca^{2+} in the terminal.

Thus, analysis of the effects of fast and slow buffers on secretion of neurotransmitter showed that the parameters of Ca^{2+} transients determining single or rhythmic release of acetylcholine at different stages of the burst significantly differ. Probably, Ca^{2+}-transients should group into macrodomains to provide single acts of secretion, and to maintain the capacity of calcium from microdomain to contribute to facilitation of transmitter release in the burst. In addition, generalized elevation of calcium level in the terminals probably plays an important role in the formation of the burst pattern at its later stages.

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