Topography and Affinity of Calcium Sensors of Exo- and Endocytosis in Motor Nerve Terminals

A. L. Zefirov and P. N. Grigor'ev

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 146, No. 12, pp. 608-612, December 2008
Original article submitted July 9, 2008

Measurements with extracellular microelectrode technique showed that depolarization of motor nerve terminals in frog cutaneous pectoris muscle with high-potassium solution (40 mM K^+) increased frequency of miniature end-plate currents. Both fast intracellular calcium chelator BAPTA-AM and slow chelator EGTA-AM equally moderated the increase in the frequency of miniature end-plate currents. Intravital fluorescent microscopy with FM 1-43 dye showed that under conditions of stimulation of neurotransmitter exocytosis and secretion with high-potassium solution, internalization of the dye into newly-formed endocytotic synaptic vesicles proceeded both in the control and in the presence of EGTA-AM. In contrast, internalization of the dye was not observed in the presence of BAPTA-AM. It was concluded that asynchronous exocytosis of synaptic vesicles goes on in the active zones enriched with Ca-channels due to activation of high-affinity Ca-site in Ca-macrodomain. Endocytosis of vesicles is probably initiated by Ca-microdomain during activation of low-affinity Ca-site in the immediate proximity to the Ca channel.

Key Words: *motor nerve terminals; exo-endocytosis; high-potassium solutions; BAPTA-AM; EGTA-AM*

The work of chemical synapse is closely related to secretion of neurotransmitter quanta from nerve terminals (NT) resulting from fusion (exocytosis) of synaptic vesicles with the presynaptic membrane in the active zone [11,12]. After exocytosis, new vesicles are formed from the presynaptic membrane (endocytosis); they accumulate the neurotransmitter and again participate in neurotransmitter secretion. The chain of these processes are referred to as the vesicular cycle [1]. Evidently, exo- and endocytosis are interrelated processes, since endocytosis cannot proceed without exocytosis [1,6].

The key role in the initiation of exocytotic processes is played by intracellular Ca^{2+} ions, which enter NT through voltage-gated Ca-channels and interact with the specific protein sites [12]. High density of Ca-channels was detected in active zones. At present, two basic hypotheses describe affinity and topography of the exocytotic sites. According to the Ca-microdomain hypothesis, the low-affinity endocytotic site is situated in the immediate proximity (about 10 nm) to the Ca-channel activated by a short-living cloud of high calcium concentration (about 100 μM) formed during channel opening. By contrast, the macrodomain hypothesis implies the existence of a high-affinity site at a greater distance from Ca channel (about 100-200 nm). In this case, exocytosis is activated at calcium concentration <10 μM and is caused by opening of many closely located channels [8,10]. It is hypothesized that endocytosis is also controlled by intracellular Ca^{2+} ions [1,3,14], although the topography, nature, and properties of endocytotic Ca-binding site remain unknown.

Department of Normal Physiology, Kazan State Medical University, Tatarstan, Russia. ***Address for correspondence:*** zefiroval@rambler.ru. A. L. Zefirov

0007-4888/08/1466-0667

In this study, we combined electrophysiological and fluorescent methods to assess affinity and location of the exo- and endocytotic sites relatively to Ca channels in the presynaptic membrane. To this end, we used Ca-buffers BAPTA-AM and EGTA-AM, which are converted into BAPTA and EGTA upon entering NT. These chelators are characterized by equal affinity to Ca^{2+} ions, although Ca^{2+} binding rate of BAPTA is higher than that of EGTA [7]. Exocytosis (and, consequently, endocytosis) of synaptic vesicles was stimulated by high-potassium solution, which depolarizes the membrane, opens Ca channels, and finally induces Ca^{2+} entry into NT.

MATERIALS AND METHODS

Experiments were carried out on isolated nerve-muscle preparations of cutaneous pectoris muscle of frog *Rana ridibunda* in summer-autumn period (from August to November). The muscle was stretched and fixed in a glass chamber filled with standard Ringer solution (in mM): 117 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 2.4-2.7 $NaHCO_3$, pH 7.2-7.4, 20°C. Osmotic balance in high-potassium solution ($[K^+]$=40 mM) was maintained with corresponding changes in sodium concentration. BAPTA-AM and EGTA-AM were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mM and immediately before the experiments the stock solutions were diluted with Ringer solution to final concentrations of 50 μM (buffer) and 0.1% DMSO. To load the buffers into NT, the preparations were incubated in this solution for 1 h, thereafter they were washed in standard Ringer solution. To promote buffer loading, the incubation solution was supplemented with Pluronic (0.01%). BAPTA-AM and EGTA-AM were from Invitrogen, other chemicals were from Sigma.

The analyzed signals were spontaneous miniature end plate currents (MEPC). Each MEPC is a result of interaction of neurotransmitter quantum with postsynaptic membrane during exocytosis of the synaptic vesicle. The signals were recorded with extracellular glass microelectrodes (tip diameter ~1 μ and resistance 1-2 MΩ) filled with 2 M NaCl. At the beginning of the experiment, we positioned the microelectrode successively at 3-5 different synapses of the same muscle under visual control (interference-polarization BIOLAR microscope, ×400). In each site, 50-100 MEPC were recorded and their frequency was calculated. Then the standard solution was rapidly replaced with high-potassium solution and after a 5-min incubation period, MEPC were again recorded in several synapses. The signals were amplified and fed into PC via an L-CARD 1250 digitizer. MEPC frequency was determined as the mean of two neighboring reverse intervals. The data were processed statistically using Student's *t* test.

Fluorescent dye FM 1-43 (Biotium) was applied in a concentration of 6 μM. The dye reversible binds to the presynaptic membrane and during endocytosis it became internalized into newly formed synaptic vesicles (the process of dye loading into NT). The dye can be used to examine both exo- and endocytosis of vesicles. Endocytosis is assessed by intensity of NT fluorescence during stimulation of exocytosis, for example, by high-potassium solution [2,3]. The intensity of exocytosis is evaluated by a decrease in fluorescence of preliminary loaded NT, because vesicle carries the dye out from the axoplasm [2,3]. The corresponding optical tools analyzing fluorescence in our experiments were described elsewhere [3]. The intensity of fluorescence recorded with Olympus confocal microscope and CCD-camera was measured in relative units assuming the maximum pixel fluorescence (256) for 1 unit. The baseline fluorescence was determined as the mean intensity of fluorescence in the square of 50×50 pixels located away from NT. This value was subtracted from each pixel in the image obtained with CCD-camera.

RESULTS

The frequency of MEPC increased from 0.23±0.03 Hz (*n*=28) to 7.25±0.91 Hz (*n*=23, Fig. 1, *a*) 5 min after application of high-potassium solution. In preparations, where NT were preloaded with Ca-buffers, application of high-potassium solution also increased MEPC frequency, but this increase was less pronounced than in the control. In NT loaded with BAPTA-AM, MEPC frequency increased from 0.21±0.03 Hz (*n*=18) to 4.9±0.80 Hz (*n*=18, Fig 1, *b*). In NT loaded with EGTA-AM, it increased from 0.20±0.03 Hz (*n*=16) to 5.33±0.72 Hz (*n*=18, Fig. 1, *b*). These data suggest that application of high-potassium solution sharply increased spontaneous neurotransmitter secretion from motor NT, which can be explained by elevation of intracellular concentration of Ca [3]. The intracellular Ca-buffers moderate the potassium-induced secretion increment, and both fast (BAPTA-AM) and slow (EGTA-AM) buffers exert similar stabilizing effects.

Application of high-potassium solution with FM 1-43 dye (5 min) induced the appearance of bright luminescent spots in NT, which indicated clusters of synaptic vesicles passed through the cycle of endo- and exocytosis and containing the dye. The mean fluorescence of NT was 0.31±0.02 rel. units (*n*=25, Fig. 2, *b*, *c*). In preparations where

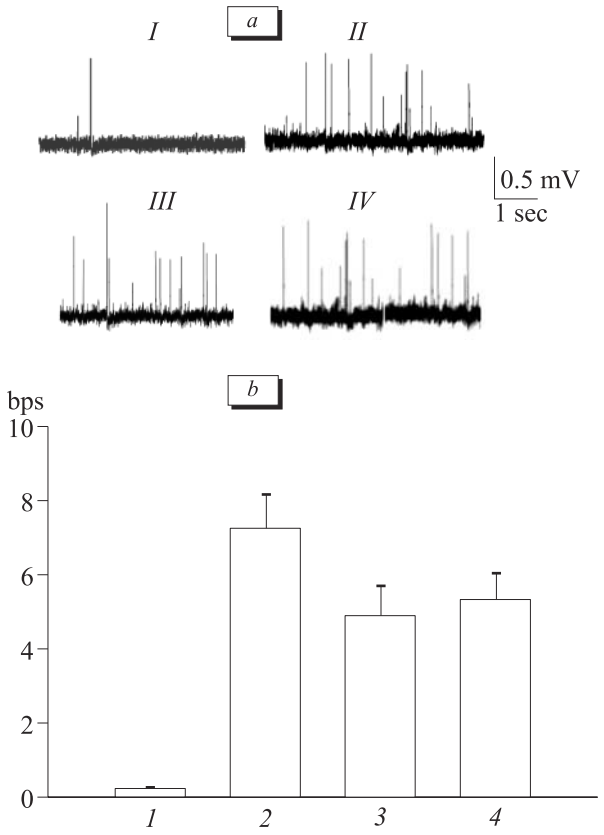

Fig. 1. Effect of membrane-permeable Ca-buffers on frequency of MEPC in high-potassium (HP) solution. *a*) Extracellular MEPC: *I*) normal Ringer solution, no Ca-buffers in NT; *II*) HP, no Ca-buffers in NT; *III*) HP+BAPTA-AM in NT; *IV*) HP+EGTA-AM in NT. *b*) MEPC frequency. Here and in Fig. 2, *c*: *1*) Ringer solution; *2*) HP; *3*) BAPTA-AM; *4*) HP+EGTA-AM.

NT were preliminary loaded with EGTA, stimulation of exocytosis with high-potassium solution also induced the appearance of luminescent spots, which attest to integrity of endocytotic processes and intake of the dye. The level of intensity was 0.24±0.03 rel. units (n=17). In contrast, similar experiments with BAPTA produced no luminescent spots (Fig. 2, *b*, *c*), which indicates disturbance in the processes of endocytosis.

For evaluation of the dynamics of vesicular exocytosis, NT were preliminary loaded with the dye. To this end, the preparations were placed in high-potassium solution with FM 1-43 (5 min), thereafter they were washed with standard solution for 40 min (Fig. 3, *a*). Then the dynamics of the decrease in fluorescence intensity (the loss of dye due to exocytosis) was assessed during long-term (15 min) application of high-potassium solution. In the control, application of high-potassium solution dramatically decreased the intensity of fluorescence and the spots disappeared after 4-5 min (Fig. 3, *b*, *c*). In contrast, when NT were preloaded with Ca-buffers (Fig. 3, *c*), the decrease in fluorescence was slower, which can be explained by weaker stimulation of exocytosis with high potassium solution under these conditions (Fig. 1, *c*).

The presented data on the effect of various Ca-buffers on secretion of neurotransmitter and on exo- and endocytosis led us to certain conclusions about affinity and location of Ca-sensors responsible for fusion and recycling of synaptic vesicles. BAPTA binds Ca^{2+} ions approximately 150-fold more rapidly than EGTA, so the fast buffer BAPTA more strictly limits the size of calcium "cloud" near the Ca channel than slow buffer EGTA [8]. Within the framework of Ca-microdomain hypothesis, this peculiarity should result in a more pronounced action of BAPTA on the intensity of vesicular exocytosis and secretion of neurotransmitter [5,9]. However, our electrophysiological data showed that both

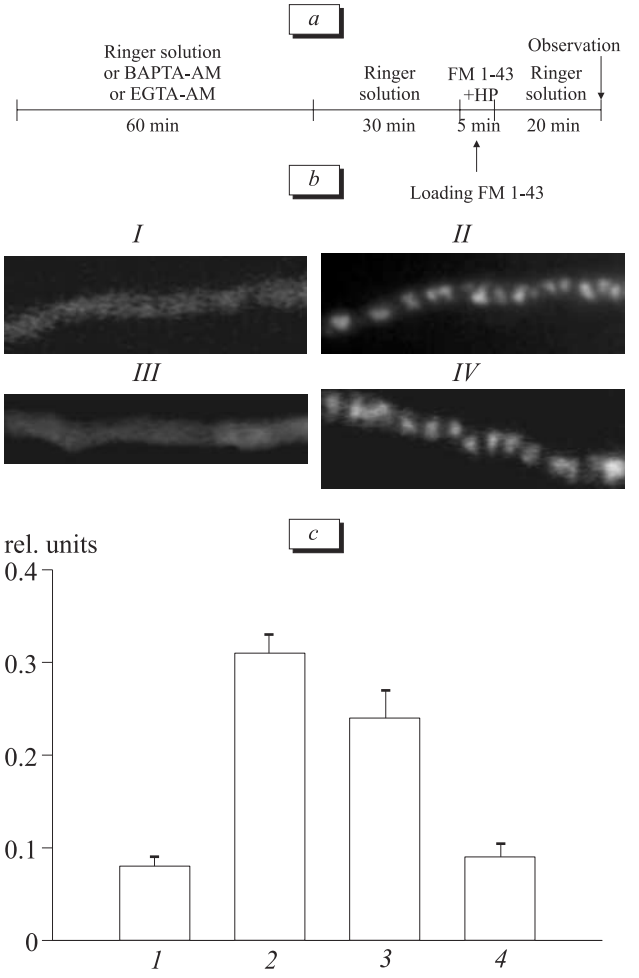

Fig. 2. Effect of membrane-permeable Ca-buffers on endocytosis of synaptic vesicles. *a*) Experimental protocol; *b*) fluorescence of NT segment after a 5-min application of FM 1-43: *I*) Ringer solution; *II*) intact NT, high-potassium (HP) solution; *III*) HP+BAPTA; *IV*) HP+EGTA. The dye was internalized only under high potassium (*II*) or high potassium with slow Ca-buffer (*IV*). *c*) mean intensity of fluorescence in NT.

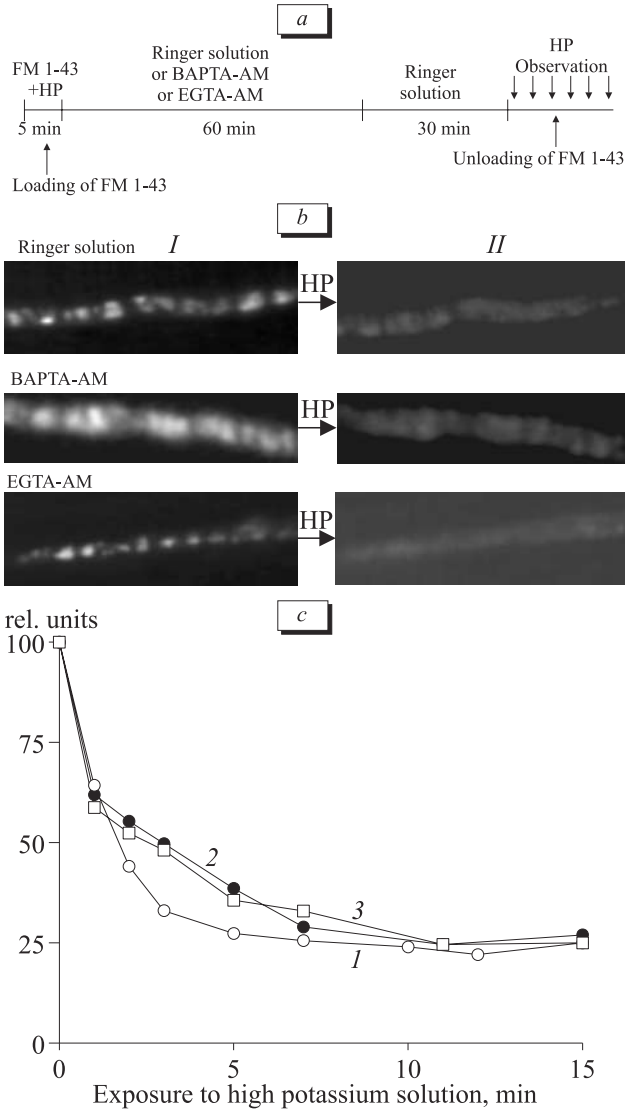

Fig. 3. Effect of membrane-permeable Ca-buffers on exocytosis of synaptic vesicles. *a*) experimental protocol; *b*) fluorescence of NT preliminary loaded with dye FM 1-43 prior to (*I*) and after (*II*) a 15-min exposure of the preparation to high-potassium solutionin (HP) intact (control) NT and NT loaded with BAPTA or EGTA; c) the dynamics of mean intensity of fluorescednce of NT during bating in HP solution; *1*) control; *2*) EGTA-AM; *3*) BAPTA-AM.

fast and slow intracellular Ca-chelators equally attenuate the effect of high-potassium solution on secretion of neurotransmitter and exocytosis of synaptic vesicles (Figs. 1, 3). In other words, they equally compete for binding intracellular Ca^{2+} ions. Therefore, both exocytosis and secretion of neurotransmitter are triggered by an increase in intracellular concentration of Ca^{2+} ions resulting from opening of many closely located Ca channels in the region of active zones in the presynaptic membranes, *i.e.*, they are determined by the events in Ca-microdomain. It can be hypothesized that exocytosis involves a high-affinity Ca-sensor distant from Ca-channel. These considerations can be probably true only for asynchronous spontaneous secretion of the neurotransmitter, because some papers report that fast Ca-buffers inhibit the evoked synchronous secretion of the neurotransmitter to a greater degree than the slow buffers [5,9].

At the same level of exocytosis, endocytosis was preserved in the presence of EGTA, but was completely blocked in the presence of BAPTA (Fig. 2, *b*, *c*). Probably, endocytosis of a synaptic vesicle is determined by Ca-microdomain in the region of a single Ca-channel and proceeds in regions with low density of Ca-channels (outside active zones). It can be hypothesized that endocytosis is triggered by a low-affinity Ca-sensor, whose activation needs high concentration of Ca^{2+} ions.

Thus, molecular sensors of Ca^{2+} ions are different for exo- and endocytosis in the matter of affinity to these ions and location relatively of Ca-channel. Triggering of asynchronous exocytosis during moderate depolarization is effected by Ca-macrodomain activated numerous closely located Ca-channels, while endocytosis is activated by microdomain near a single Ca-channel. The most probable candidates for the role of exocytotic and endocytotic sensors are synaptotagmin [11] and dynamin [6], respectively.

This work was supported by the Russian Foundation for Basic Research (grant No. 02-04-96252); by Leading Scientific Schools Grant of President of Russia (grant No. NSh-1383. 2003); and by Tatarstan Academy of Science (grant No. 03-3.8.-122).

REFERENCES

1. A. L. Zefirov, *Ros. Fiziol. Zh.*, **93**, No. 5, 544-557 (2007).
2. A. L. Zefirov, M. M. Abdrakhmanov, and P. N. Grigor'ev, *Ibid.*, **91**, No. 6, 973-984 (2005).
3. A. L. Zefirov, M. M. Abdrakhmanov, P. N. Grigor'ev, *et al.*, *Ibid.*, **48**, No. 1, 34-41 (2006).
4. J. K. Angleson and W. J. Betz, *J. Neurophysiol.,* **1**, 287-294 (2001).
5. E. M. Adler, G. J. Augustine, S. N. Durry, *et al., J. Neurosci.,* **11,** 1496-1507 (1991).
6. P. De Camilii, V. I. Slepnev, O. Shupiakov, *et al.,* in: *Synapses*, Baltimore (2001), pp. 217-274.
7. M. Naraghi, *Cell Calcium,* **22**, 255-268 (1997).
8. E. Neher, *Neuron,* **20,** 389-399 (1998).
9. E. F. Stanley, *Ibid.,* **11,** 1007-1011 (1993).
10. E. F. Stanley, *Trends Neurosci*, **20,** 404-409 (1997).
11. T. C. Sudhof, *Annu. Rev. Neurosci.,* **27,** 509-547 (2004).
12. W. Van der Kloot and J. Molgo, *Physiol. Rev.*, **74**, 899-991 (1994).
13. A. L. Zefirov, T. Benish, N. Fatkullin, *et al., Nature,* **376,** 393-394 (1995).
14. A. L. Zefirov, M. M. Abdrakhmanov, M. A. Mukhamedyarov, *et al.*, *Neuroscience,* **143**, No. 4, 905-910 (2006).