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# Calcium Transients and Transmitter Secretion in Different Parts of Frog Nerve Endings in Different Conditions of Calcium Ion Influx

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Experiments on frog neuromuscular preparations were performed to study the characteristics of the calcium response and the quantum secretion of acetylcholine in different pats of extended nerve terminals in different conditions of calcium influx. A calcium-sensitive fluorescent dye was used to analyze $Ca^{2+}$ influx ($Ca^{2+}$ transients) into the proximal and distal parts of nerve endings in conditions of increased $K^+$ ion content, in response to blockers of N- and L-type calcium channels, and on blockade of calcium-activated potassium channels. These studies showed that at a uniform distribution density of voltage-gated calcium channels along nerve endings, the proximal-to-distal decrement in calcium transients and quantum secretion intensity persisted in conditions of additional opening of voltage-gated calcium channels by potassium depolarization, on "thinning" of these channels using specific blockers, but changed on blockade of calcium-activated potassium channels.

**Keywords:** neuromuscular junction, calcium transient, proximal-to-distal decrement, quantum composition, calcium channels, potassium channels.

Neuromuscular synapses in vertebrates are classical and widely used system for studying the mechanisms underlying the realization and modulation of synaptic transmission of excitation in fast chemical synapses. It is well known that $Ca^{2+}$ influx after presynaptic action potentials triggers the secretion and determines the intensity of release of neurotransmitter quanta [1]. However, it is quite difficult to obtain accurate evaluations of the amount of $Ca^{2+}$ entering nerve endings in response to stimuli. This difficulty is due to the very small sizes of nerve terminals, preventing the use of microelectrodes for measuring the calcium influx current [2, 3]. $Ca^{2+}$ influx in response to the first stimulus is presently evaluated by recording calcium transients – fluorescent signals reflecting changes in the emission intensity of a calcium-sensitive dye in response to binding $Ca^{2+}$ [4].

Frog nerve endings are quite characteristic in terms of size and the number of terminal branches [5, 6]. Action potential shape changes along extended terminals, from a predominantly negative direction to a completely positive signal [7–10]. Changes in presynaptic action potentials are accompanied by changes in the probability of neurotransmitter release in different parts of the terminal [11–14]. The causes of differences in measures of acetylcholine quantum release along the terminal may be the morphological features of different areas, particularly the cross-sectional diameter of the terminal, which determines the length of the synaptic contact, the number of active secretion zones in each part [15, 16], differences in the density and conductivity of voltage-gated ion (potassium, sodium, and calcium)

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channels, the involvement of calcium channels of different types, differences in the functioning of the active secretion zones in different parts of the nerve terminal, and differences in the operation of the systems maintaining the intracellular $Ca^{2+}$ concentration [7].

Our previous studies using calcium imaging demonstrated the existence of a proximal-to-distal decrement in the amplitude of calcium transients, apparent as decreases in $Ca^{2+}$ influx into nerve terminals in frogs going from the proximal part of the nerve ending to the distal [17, 18]. However, immunohistochemical analysis did not identify any differences in the distribution density of calcium channels along the nerve ending [19]. This raises the question of why, with identical calcium channel densities, there is a decrease in the calcium response and a corresponding decrease in the probability of acetylcholine quantum secretion. Seeking to answer this question, we analyzed calcium influx into the proximal and distal parts of frog extended motor nerve endings and compared this with changes in the intensity of evoked quantum secretion in these areas on modulation of the activity of voltage-gated calcium channels by blockade of individual subtypes. Experiments using a $Ca^{2+}$-specific fluorescent dye were run to analyze the influx of these ions ($Ca^{2+}$ transients) and the parameters of evoked acetylcholine quantum release in the proximal and distal parts of the nerve terminal in controls and in conditions of blockade of N- and L-type calcium channels, as well as when the $K^+$ content in the solution was elevated.

**Methods.** Experiments on isolated thoracic musculocutaneous preparations from lake frogs (*Rana ridibunda*) were performed in compliance with the ethical principles and normative documents recommended by the European Science Foundation and the declaration of the humane treatment of animals. Each series of experiments used 3–5 animals, in which 1–3 synapses were analyzed. Preparations were made and muscle was cleared of connective tissue in Ringer's solution containing 113.0 mM NaCl, 2.5 mM KCl, 3.0 mM $NaHCO_3$, and 1.8 mM $CaCl_2$; pH was maintained at 7.2–7.4.

Thoracic musculocutaneous preparations with nerve fragments were placed in Petri dishes for loading with the calcium fluorescent dye. Drops of 0.1–0.3 μl of 50 mM Oregon Green 488 BAPTA-1 hexapotassium salt, cell impermeant, were applied to short nerve segments of length 2–3 mm, after which preparations were incubated in two stages. At the first stage, preparations were held in Ringer's solution at room temperature for 4–6 h in a humid chamber. The second stage was for 15–20 h in a refrigerator at 8 ± 2°C. Dye in contact with the outside of preparations was washed away in this step and loading with the calcium dye occurred as a result of diffusion and anterograde axonal transport [20–22]. Using this loading method, dye not penetrating the cell membrane was loaded only into the cytosol of the nerve ending, such that calcium signals could be recorded only from the presynaptic nerve cell. After loading fluorescent dye into nerve terminals, preparations were placed in a 5-ml bath perfused with Ringer's solution with a decreased $Ca^{2+}$ content and an elevated $Mg^{2+}$ content, containing 113.0 mM NaCl, 2.5 mM KCl, 3.0 mM $NaHCO_3$, 6.0 mM $MgCl_2$, and 0.9 mM $CaCl_2$, pH 7.2–7.4, 20.0 ± 0.5°C). Preparations were illuminated with monochromatic light with a wavelength of 488 nM to excite the dye and recordings were made of increases in fluorescence in response to stimulation of the motor nerve with square-wave electrical pulses of suprathreshold amplitude and duration 0.2 msec. Terminals of at least 100 μm in length were selected; the first 30% of the length of the whole terminal from the ending of the myelin sheath of the nerve was taken as the proximal part, and the last 30% was taken as the distal part.

Changes in dye fluorescence emission in terminals were recorded using a high-speed neuroCCD camera (Redshirt Imaging), which provided for local assessment of low-amplitude fast calcium signals in different parts of the nerve terminal. The amplitudes of $Ca^{2+}$ transients were assessed as $100\% \times \Delta F/F_0$, where $\Delta F$ is the change in fluorescence intensity in response to stimulation relative to baseline fluorescence $F_0$ without stimulation [23].

The mean quantum composition of postsynaptic responses was assessed by recording extracellular nerve ending currents and endplate currents using standard microelectrode methods. Extracellular microelectrodes with tip diameter 2.5–3.5 μm, filled with Ringer's solution or NaCl (0.5 mM) had impedance 1.0–1.5 MΩ. The recorded signals were filtered at up to 10 kHz, amplified, passed to the input of a 16-bit ADC, and digitized with an interval of 10 μsec. Currents were recorded using Ringer's solution with a decreased $Ca^{2+}$ content: 113.0 mM NaCl, 2.5 mM KCl, 3.0 mM $NaHCO_3$, 4.0 mM $MgCl_2$, and 0.3 mM $CaCl_2$. The mean quantum composition of endplate currents was assessed by the "dropout" method [24].

Experiments used the following substances (all from Sigma): conotoxin GVIA, nitrendipine, iberiotoxin, and d-tubocurarine. As some reagents were soluble in DMSO (dimethylsulfoxide), the final concentration of which in solution was no greater than 0.1%, preliminary experiments were run to analyze the effects of solvent on the processes of interest. These showed that DMSO had no significant effect on the amplitude of $Ca^{2+}$ transients over a period of more than 2 h.

Experimental $Ca^{2+}$ transient signals were processed statistically using standard methods for computation of mean values, standard errors, the parametric paired Student's $t$ test, and the Mann–Whitney test. Significant differences between mean values were identified at $p < 0.05$.

**Results.** In control conditions, the amplitude of $Ca^{2+}$ transients decreased from proximal to distal by 39.9 ± 5.6% ($n$ = 22, $p < 0.05$). The quantum composition in controls was 0.45 ± 0.06 ($n$ = 10) in the proximal part and 0.31 ± ± 0.09 ($n$ = 7) in the distal part.

Depolarization of nerve endings increases the probability of calcium channel opening [1]. We might expect de-

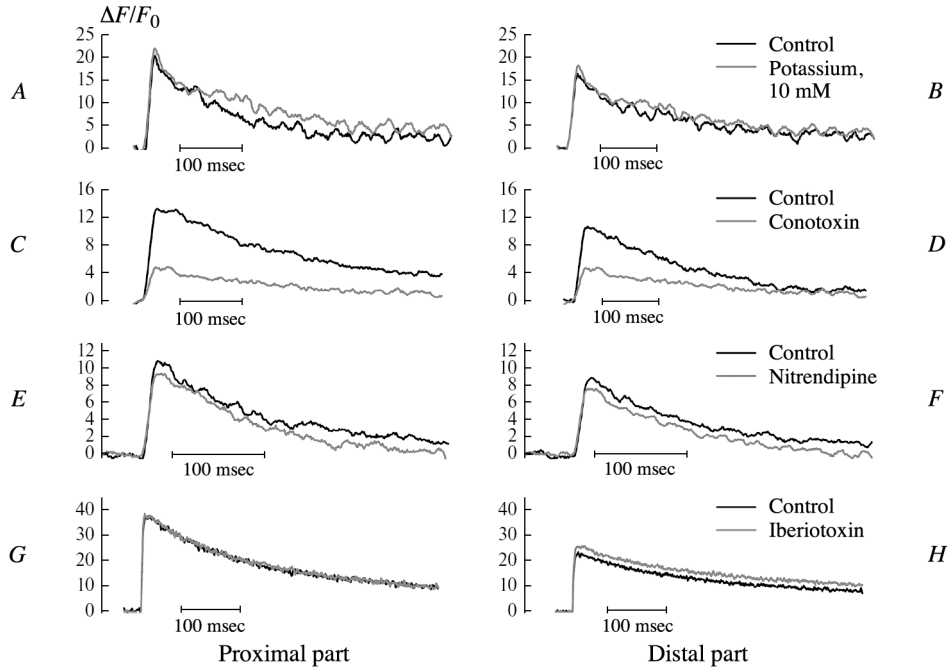


Fig. 1. $Ca^{2+}$ transients in the proximal and distal parts of nerve endings in controls and in the presence of an elevated $K^+$ ion concentration (10 mM, *A*, *B*), conotoxin (*C*, *D*), nitrendipine (*E*, *F*), and iberiotoxin (*G*, *H*).

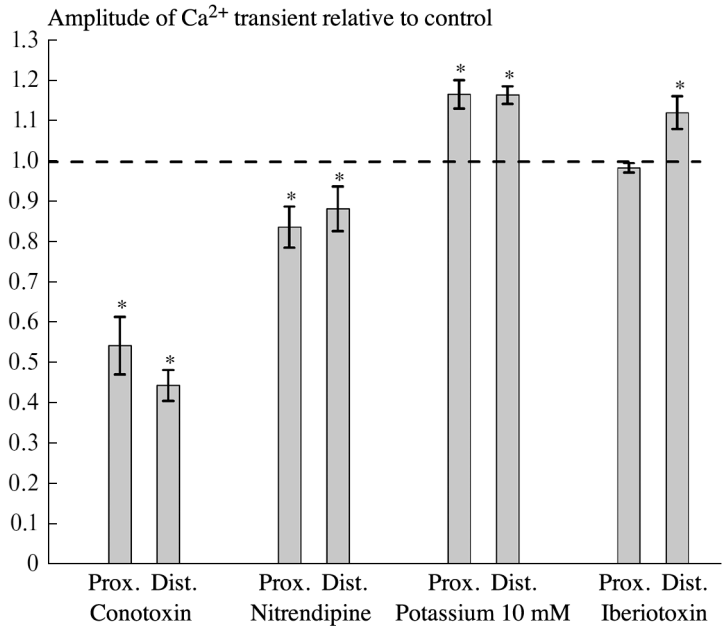


Fig. 2. Changes in the amplitude of the $Ca^{2+}$ transient compared with controls (taken as unity) measured in the proximal and distal parts of nerve endings on exposure to an N-type calcium channel blocker (conotoxin), an L-type calcium channel blocker (nitrendipine), elevation of the $K^+$ ion concentration (10 mM), and iberiotoxin.

polarization to lead to different extents of changes to transients in different parts of the terminal because of the characteristics of the different parts – different cross-sectional diameters of terminals, different numbers of active secretion zones in each part, different densities and conductivities of voltage-gated ion channels, the involvement of different types of calcium channels, and differences in the functioning of active secretion zones. Elevated extracellular potassium concentrations induce a decrease in cell membrane potential [25]. Increases in the potassium ion concentration in the solution washing the preparation from 2.5 to 10 mM increased the amplitude of $Ca^{2+}$ transients to the same extent

in the proximal part, by 16.7 ± 3.5% ($n = 7$, $p < 0.05$), and the distal part, by 16.6 ± 2.2% ($n = 6$, $p < 0.05$) (Fig. 1, *A*, *B*; Fig. 2), indicating a uniform increase in the $Ca^{2+}$ concentration and equal quantities of additionally opening calcium channels in the proximal and distal parts of terminals.

The main type of calcium channels responsible for calcium influx into frog nerve endings after development of presynaptic action potentials are N-type voltage-gated channels [26]. Treatment of neuromuscular preparations with the specific N-type calcium channel antagonist conotoxin GVIA at a concentration of 300 nM led to decreases in the amplitude of $Ca^{2+}$ transients by 45.6 ± 7.2% ($n = 7$, $p < 0.05$) in the proximal part and 55.5 ± 3.9% ($n = 6$, $p < 0.05$) in the distal part (Fig. 1, *A*; Fig. 2, *A*, *B*); there were no significant differences in the extents of reductions in the calcium response in different parts. Conotoxin GVIA significantly decreased quantum composition, by 57.4 ± 5.2% ($n = 11$, $p < 0.05$) in the proximal part and by 46.4 ± 8.8% ($n = 7$, $p < 0.05$) in the distal part (Fig. 1, *C*, *D*; Fig. 2). There were also no significant differences in decreases in the mean quantum composition in response to the N-type calcium channel blocker in the proximal and distal parts of the synapse.

We have previously established that apart from the main N-type calcium channels, frog nerve endings also bear L-type channels [27]. The specific L-type calcium channel blocker nitrendipine at a concentration of 5 μM decreased $Ca^{2+}$ transients in the proximal part by 16.2 ± 5.1% ($n = 13$, $p < 0.05$) and the distal part by 11.7 ± 5.5% ($n = 11$, $p < 0.05$) as compared with controls, without any significant differences in different parts (Fig. 1, *E*, *F*; Fig. 2). In the presence of nitrendipine, the quantum composition decreased relative to controls by 28.3 ± 4.9% ($n = 13$, $p < 0.05$) in the proximal part and by 34.1 ± 3.2% ($n = 12$, $p < 0.05$) in the distal part. There were no significant differences in the effects of nitrendipine on the quantum composition in different parts of nerve terminals.

Calcium-activated calcium channels in nerve endings take part in forming the amplitude and duration of the presynaptic action potential and, as a result, determine the level of depolarization of the presynaptic membrane [28]. The calcium-activated potassium channel blocker iberiotoxin at a concentration of 100 nM did not alter $Ca^{2+}$ transients in the proximal part (–1.5 ± 1.2%, $n = 17$, $p > 0.05$) and significantly increased $Ca^{2+}$ transients in the distal part of the nerve ending, by 12.2 ± 4.1% ($n = 17$, $p < 0.05$) as compared with controls (Fig. 1, *G*, *H*; Fig. 2).

**Discussion.** Increases in the length of the nonmyelinated part of the axon are accompanied by changes in the parameters of the nerve spike-induced release of acetylcholine quanta in frog neuromuscular synapses. Decreases in the intensity of evoked secretion of quanta have been described, as assessed in terms of the quantum composition [11, 13, 14], increases in the level of synchronization of quantum release [29], and different extents of the influences of physiologically active compounds [29]. As the leading component triggering the process of quantum secretion is $Ca^{2+}$ influx after the development of action potentials [1, 30], it is natural to suggest that this factor is the main item determining the proximal-to-distal decrement in measures of quantum secretion. In fact, recording of the fluorescence intensity of a specific calcium-sensitive fluorescent dye allowed us to show that going from the proximal to the distal part was linked with decreases in calcium influx into the nerve ending in response to spikes [17, 18]. This may be linked with changes in the density of calcium channels along the extended nerve terminal. However, our immunofluorescence studies using specific antibodies to N- and L-type calcium channels [19] and studies reported by Robitaille et al. [31] showed that calcium channels are uniformly distributed along the nerve ending. The experiments presented here showed that the magnitude of the calcium response decreased on blockade of N-type calcium channels by conotoxin GVIA and on exposure to nitrendipine, a blocker of dihydropyridine-sensitive L-type channels. These effects were equally apparent in the proximal and distal parts of the synapse. Additional nerve ending depolarization when the extracellular potassium concentration was increased led to increases in the amplitude of the calcium response, also to the same extents in different parts. Blockade of calcium-activated potassium channels with iberiotoxin increased $Ca^{2+}$ influx only in the distal part of the terminal. This indicates that the proximal-to-distal decrement in $Ca^{2+}$ transients is due to an uneven distribution of voltage-gated and calcium-dependent potassium channels. Changes in $Ca^{2+}$ transients on blockade of channels were accompanied by decreases in the number of quanta released in response to nerve spikes, this being to identical extents in different parts of the synapse. Thus, these data provide evidence that N- and L-type voltage-gated calcium channels are involved in mediating $Ca^{2+}$ influx into frog motor nerve endings and that their activation leads to increases in the intensity of nerve stimulus-evoked release of acetylcholine quanta. These channels are equally represented in different parts of extended terminals and their "thinning" using specific channel blockers leads to decreases in both $Ca^{2+}$ influx into terminals and reductions in the number of transmitter quanta released to identical extents in the proximal and distal parts of extended synaptic contacts. Only blockade of calcium-activated potassium channels showed a difference in changes in calcium influx into nerve endings. This raises the question of the cause of the proximal-to-distal decrement in $Ca^{2+}$ transient amplitude.

As demonstrated by Pattillo et al. [32] and Dittrich et al. [33], there is a relationship between action potential shape and the presynaptic calcium current. It is well known that action potential shape changes going from the proximal part of the nerve ending to the distal part [7]. The change in the shape of electrical responses along the nerve ending at different distances from the end of the myelinated segment of the nerve can be explained by the different contributions

of different types of voltage-gated channels located on the membrane surface [7]. Sodium channels have been shown to be present at maximal density in the proximal part of each terminal branch and are almost not detected in the distal part. Potassium channels are located at higher density in the mid parts of nerve endings [7]. Thus, the different levels of activation or different numbers of sodium and potassium channels evidently support different levels of depolarization in different parts and determines the existence of a proximal-to-distal decrement in $Ca^{2+}$ transients. Furthermore, a contribution from changes in the size and architecture of active secretion zones along a decreasing-diameter nerve ending cannot be excluded [7].

Thus, the studies reported here showed that while the distribution density of voltage-gated calcium channels along extended nerve endings was uniform, frog synapses showed a proximal-to-distal decrement in $Ca^{2+}$ transients, and the intensity of quantum secretion persisted on additional opening of calcium channels due to potassium depolarization and on "thinning" of these channels with specific blockers, but changed on blockade of the calcium-activated potassium channels involved in forming presynaptic action potentials. This points to an important role for parameters of the presynaptic action potential in supporting the proximal-to-distal decrement in calcium influx and quantum secretion in the frog neuromuscular synapse.

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## REFERENCES


1. N. Burnashev and A. Rozov, "Presynaptic $Ca^{2+}$ dynamics, $Ca^{2+}$ buffers and synaptic efficacy," *Cell Calcium*, **37**, No. 5, 489–495 (2005).
2. J. G. Borst and B. Sakmann, "Calcium influx and transmitter release in a fast CNS synapse," *Nature*, **383**, No. 6599, 431–434 (1996).
3. B. Yazejian, D. A. DiGregorio, J. L. Vergara, et al., "Direct measurements of presynaptic calcium and calcium-activated potassium currents regulating neurotransmitter release at cultured Xenopus nerve-muscle synapses," *J. Neurosci.*, **17**, No. 9, 2990–3001 (1997).
4. R. Y. Tsien, "Fluorescence ratio imaging of dynamic intracellular signals," *Acta Physiol. Scand.*, **582**, Suppl., 6 (1989).
5. M. R. Bennett and N. A. Lavidis, "Quantal secretion at release sites of nerve terminals in toad (*Bufomarinus*) muscle during formation of topographical maps," *J. Physiol.*, **401**, 567–579 (1988).
6. B. M. Nudell and A. D. Grinnell, "Inverse relationship between transmitter release and terminal length in synapses on frog muscle fibers of uniform input resistance," *J. Neurosci.*, **2**, No. 2, 216–224 (1982).
7. A. Mallart, "Presynaptic currents in frog motor endings," *Pflügers Arch.*, **400**, No. 1, 8–13 (1984).
8. B. Katz and R. Miledi, "The effect of local blockage of motor nerve terminals," *J. Physiol.*, **199**, No. 3, 729–741 (1968).
9. M. Braun and R. F. Schmidt, "Potential changes recorded from the frog motor nerve terminal during its activation," *Pflügers Arch. Gesamte Physiol. Menschen Tiere*, **287**, No. 1, 56–80 (1966).
10. A. L. Zefirov and I. A. Khalilov, "Analysis of electrical activity in different parts of amphibian nerve endings. Physiology of mediators. The peripheral synapse," in: *Abstr. 5th All-Union Symp.*, Kazan, June 1984, pp. 97–99.
11. M. R. Bennett and N. A. Lavidis, "Variation in quantal secretion at different release sites along developing and mature motor terminal branches," *Brain Res.*, **281**, No. 1, 1–9 (1982).
12. A. J. D'Alonzo and A. D. Grinnell, "Profiles of evoked release along the length of frog motor nerve terminals," *J. Physiol.*, **359**, 235–258 (1985).
13. M. R. Bennett, P. Jones, and N. A. Lavidis, "The probability of quantal secretion along visualized terminal branches at amphibian (*Bufomarinus*) neuromuscular synapses," *J. Physiol.*, **379**, 257–274 (1986).
14. E. Bukcharaeva, D. Samigullin, E. E. Nikolsky, and F. Vyskocil, "Cyclic AMP synchronizes evoked quantal release at frog neuromuscular junctions," *Physiol. Res.*, **49**, No. 4, 475–479 (2000).
15. D. F. Davey and M. R. Bennett, "Variation in the size of synaptic contacts along developing and mature motor terminal branches," *Brain Res.*, **281**, No. 1, 11–22 (1982).
16. M. R. Bennett, N. A. Lavidis, and F. M. Armson, "Changes in the dimensions of release sites along terminal branches at amphibian neuromuscular synapses," *J. Neurocytol.*, **16**, No. 2, 221–237 (1987).
17. E. Khaziev, A. Golovyahina, E. Bukharaeva, et al., "Action of ATP on $Ca^{2+}$-transient in different parts of the frog motor nerve ending," *BioNanoScience* (2017), doi: 10.1007/s12668-016-0350-6.
18. D. V. Samigullin, A. L. Vasin, E. A. Bukharaeva, and E. E. Nikolsky, "Characteristics of calcium transient in different parts of frog nerve terminal in response to nerve impulse," *Dokl. Biol. Sci.*, **431**, No. 1, 83–85 (2010).
19. L. F. Nurullin, A. R. Mukhitov, A. N. Tsentsevytsky, et al., "Voltage-dependent P/Q-type calcium channels at the frog neuromuscular junction," *Physiol. Res.*, **60**, 815–823 (2011).
20. Y. Y. Peng and R. S. Zucker, "Release of LHRH is linearly related to the time integral of presynaptic Ca2 elevation above a threshold level in bullfrog sympathetic ganglia," *Neuron*, **10**, No. 3, 465–473 (1993).
21. E. Neher, "The use of fura-2 for estimating Ca buffers and Ca fluxes," *Neuropharmacology*, **34**, No. 11, 1423–1442 (1995).
22. V. Shahrezaei, A. Cao, and K. R. Delaney, "$Ca^{2+}$ from one or two channels controls fusion of a single vesicle at the frog neuromuscular junction," *J. Neurosci.*, **26**, No. 51, 13,240–13,249 (2006).
23. D. V. Samigullin, E. F. Khaziev, N. V. Zhilyakov, et al., "Loading a calcium dye into frog nerve endings through the nerve stump: calcium transient registration in the frog neuromuscular junction," *J. Vis. Exp.*, (125) e55122 (2017), doi: 10.3791/55122.
24. J. Del Castillo and B. Katz, "Quantal components of the end-plate potential," *J. Physiol.*, **124**, 560–573 (1954).
25. D. P. Matyushkin, I. A. Shabunova, G. M. Sharovarova, and I. M. Vinogradova, "On potassium functional feedback in neuromuscular junction," *J. Neurosci. Res.*, **3**, 441–450 (1978).
26. E. Khaziev, D. Samigullin, N. Zhilyakov, et al., "Acetylcholine-induced inhibition of presynaptic calcium signals and transmitter release in the frog neuromuscular junction," *Front. Physiol.*, **7**, 621 (2016), doi: 10.3389/fphys.2016.00621.
27. A. N. Tsentsevitsky, D. V. Samigullin, L. F. Nurullin, et al., "Presynaptic voltage-dependent calcium channels at the frog neuromuscular junction," in: *Frogs: Genetic Diversity, Neural Development and Ecological Implications*, Nova Science Publishers Inc., New York (2014), Chpt. 5, pp. 179–194, ISBN: 978-1-63117-626-5.
28. R. Roncarati, M. Di Chio, A. Sava, et al., "Presynaptic localization of the small conductance calcium-activated potassium channel SK3 at the neuromuscular junction," *Neuroscience*, **104**, No. 1, 253–262 (2001).
29. E. A. Bukcharaeva, K. C. Kim, J. Moravec, et al., "Noradrenaline synchronizes evoked quantal release at frog neuromuscular junctions," *J. Physiol.*, **517**, No. 3, 879–888 (1999).
30. Z. P. Pang and T. C. Südhof, "Cell biology of $Ca^{2+}$-triggered exocytosis," *Curr. Opin. Cell Biol.*, **22**, No. 4, 496–505 (2010), doi: 10.1016/j.ceb.2010.05.001.

31. R. Robitaille, E. M. Adler, and M. P. Charlton, "Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses," *Neuron*, **5**, 773–779 (1990).
32. J. M. Pattillo, B. Yazejian, D. A. DiGregorio, et al., "Contribution of presynaptic calcium-activated potassium currents to transmitter release regulation in cultured *Xenopus* nerve-muscle synapses," *Neuroscience*, **102**, 229–240 (2001).
33. M. Dittrich, A. E.Homan, and S. D.Meriney, "Presynaptic mechanisms controlling calcium-triggered transmitter release at the neuromuscular junction," *Curr. Opin. Physiol.*, **4**, 15–24 (2018), doi: 10.1016/j.cophys.2018.03.004.