PHYSIOLOGY

Kiss-and-Run Quantal Secretion in Frog Nerve-Muscle Synapse

A. L. Zefirov, M. M. Abdrakhmanov, and P. N. Grigor'ev

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Electrophysiological and optical methods were used to study exo- and endocytosis of synaptic vesicles underlying secretion of the neurotransmitter from motor nerve terminals in frog sternocutaneous muscle. Increase in extracellular concentration of K^+ or sucrose produced similar increase in the frequency of miniature endplate currents recorded by extracellular microelectrode. Fluorescent microscopy revealed bright spots in nerve terminal during stimulation of secretion with high-potassium solutions in the presence of endocytosis marker FMI-43. These spots corresponded to clusters of synaptic vesicles that passed through the cycles of exo- and endocytosis. Subsequent high-potassium stimulation of exocytosis in normal Ringer solution led to disappearance of marker spots, while in hyperosmotic saline the spots were preserved. No spots were seen after stimulation of neurotransmitter secretion with sucrose in the presence of FM1-43. It is concluded that quantal secretion of the neurotransmitter in frog motor nerve endings can be realized via both complete exocytosis of synaptic vesicles with subsequent endocytosis and kiss-and-run mechanism with the formation of a temporary pore.

Key Words: *transmitter secretion; exocytosis; endocytosis; K+ ions; sucrose; FM1-43; kiss-and-run*

According to the quantum-vesicular theory, transmitter in the nerve-muscle synapse is secreted in certain portions (quanta) at the specific sites (active zones) of nerve endings (NE), where synaptic vesicles with quanta of the transmitter are located [2,3,6,8-11,14, 15]. The classical theories consider that secretion of neurotransmitter is based on complete exocytosis including incorporation of vesicular membrane into the presynaptic plasmalemma, followed by endocytosis, formation of new vesicles, accumulation of the transmitter, and transport of loaded vesicles to the active zone. This cycle can be repeated many times [3,9,14]. However, an alternative mechanism of secretion was proposed for central synapses and endocrine cells. According to this mechanism secretion of a quantum of transmitter or hormone occurs through temporary pores formed between vesicular membrane and plasmalemma. After closing of the pore, the vesicle is refilled with the transmitter and is ready to secrete it again. This mechanism does not include classical endocytosis and was jokingly termed as "kiss-and-run" [6,12,13].

The intensity of synaptic secretion is usually assessed by electrophysiological (microelectrode) techniques via analysis of quantal composition of evoked postsynaptic currents or the frequency of spontaneous miniature endplate currents (MEPC) [3,14]. Recently, a novel technique was developed to study exo- and endocytosis. This technique employs specific membrane-targeted fluorescent markers, which bind reversibly to presynaptic membrane, are incorporated into membrane of newly formed synaptic vesicles during

Department of Normal Physiology, State Medical University, Kazan.
Address for correspondence: zefirov@kmu-mf.kcn.ru. Zefirov A. L.

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endocytosis, and can be detected by fluorescence. One of the most popular and adequate vesicular markers is FM1-43 [1,5,7,10,12].

Since the processes of exo- and endocytosis are interrelated in NE containing thousands of vesicles stimulation of exocytosis is an obligatory condition in these studies [5]. Exocytosis is usually stimulated with chemicals markedly increasing evoked or spontaneous quantal secretion of the transmitter [1,5,7,10,12]. In this case, the intensity of endocytosis is assessed by the intensity of fluorescence after stimulation of secretion in the presence of FM1-43 (marker loading). The dynamics of fluorescence decay in preloaded NE (in the absence of the marker in the medium) characterizes exocytosis of synaptic vesicles ("unloading" of the marker).

Here we studied the mechanisms of exo- and endocytosis underlying transmitter secretion from frog motor NE.

MATERIALS AND METHODS

The experiments were carried out on isolated nerve-muscle preparations of sternocutaneous muscle of *Rana ridibunda* in November-March.

The preparation was perfused with a standard physiological solution (pH 7.2-7.4) containing (in mM): 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, and 2.4 $NaHCO_3$ at 20°C. In experiments with various external concentrations of K^+ ions (2.5-40.0 mM) osmotic equilibrium was maintained with NaCl. In other experiments, the osmotic balance was varied by adding sucrose (0-100 mM). One-quantum MEPC were recorded from visually controlled NE using extracellular glass micropipettes filled with 2 M NaCl (tip diameter ~1 μ, resistance 1-5 MΩ). The signals were amplified and digitized. The interval between two successive MEPC was measured and used to calculate the rate of MEPC (in Hz).

Fluorescent microscopy was performed using fluorescent marker FM1-43 (SynaptoGreen, Biotium) in a concentration of 6 μmol/liter. The experimental protocol is shown in Fig. 1. Fluorescence was observed under a MIKMED-2 universal microscope (LOMO). The optical setup for the analysis of FM1-43 fluorescene was described in details [1]. All recordings were made only from NE located at the surface of the muscle. To store the fluorescent images, we used a WAT-902H rapid black-and-white camera (Watec Co. Ltd.) coupled with PC via a frame grabber plate. Recordings were carried out at the rate of 25 frames per second. To improve signal-to-noise ratio, 20 most clearly focused frames were averaged. Fluorescence intensity was evaluated in relative units, in which the maximum pixel brightness (256) was taken for 1. The intensity of fluorescence of all pixels of each nerve terminal in NE was analyzed, and the data were averaged. To improve the quality of illustrations, the fluorescent images were inverted, so bright fluorescent spots are presented here by dark blots. The data were processed statistically using Student's *t* test.

RESULTS

For evaluation of spontaneous secretion of the transmitter in high-potassium or hyperosmotic media, the micropipette was first attached successively to 3-5 different synapses of the same muscle in standard Ringer solution, 50-100 MEPC were recorded, and their rate was determined in each synapse. Then the standard solution was promptly replaced with the test saline, and recordings were repeated in several synapses. The rates of MEPC were averaged in the control and for each tested concentration. The effects of hyperosmotic and high-potassium solutions were examined in individual series of experiments. Increasing potassium concentration from 2.5 to 40.0 mM (Fig. 2, *a*) increased the rate of MEPC from 0.23±0.03 (*n*=28) to 7.25± 0.90 Hz (*n*=21). Similarly, the increase in sucrose concentration from 0 to 100 mM (Fig. 2, *b*) increased MEPC rate from 0.21±0.03 (*n*=25) to 22.0± 2.8 Hz (*n*=18). It is noteworthy that 40 mM KCl and 30 mM sucrose induced approximately equal increase in MEPC rate. Thus, these concentrations of KCl and sucrose can be considered as equipotent in stimulation of synaptic secretion.

Incubation with FM1-43 in standard Ringer solution led to the appearance of a weak nonspecific fluorescence of NE (Fig. 3) resulting from marker binding to membranes of NE and Schwann cell [1,5]. The mean fluorescence intensity was 0.091±0.005 arb. units (*n*=32). Incubation of the muscle in marker-containing

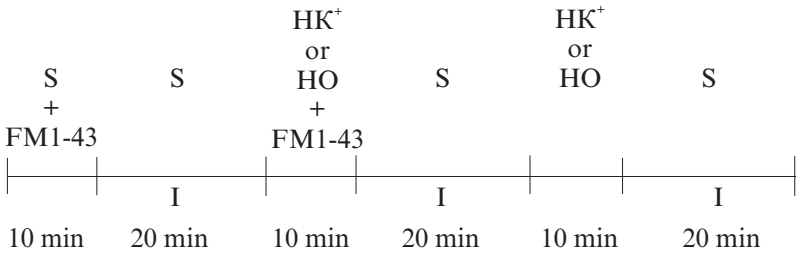

Fig. 1. Experiment profile. Here and in Fig. 3: S: standard Ringer solution; HK^+: high-potassium solution (40 mM KCl); HO: hyperosmotic solution (30 mM sucrose); I: imaging (fluorescence of nerve terminal).

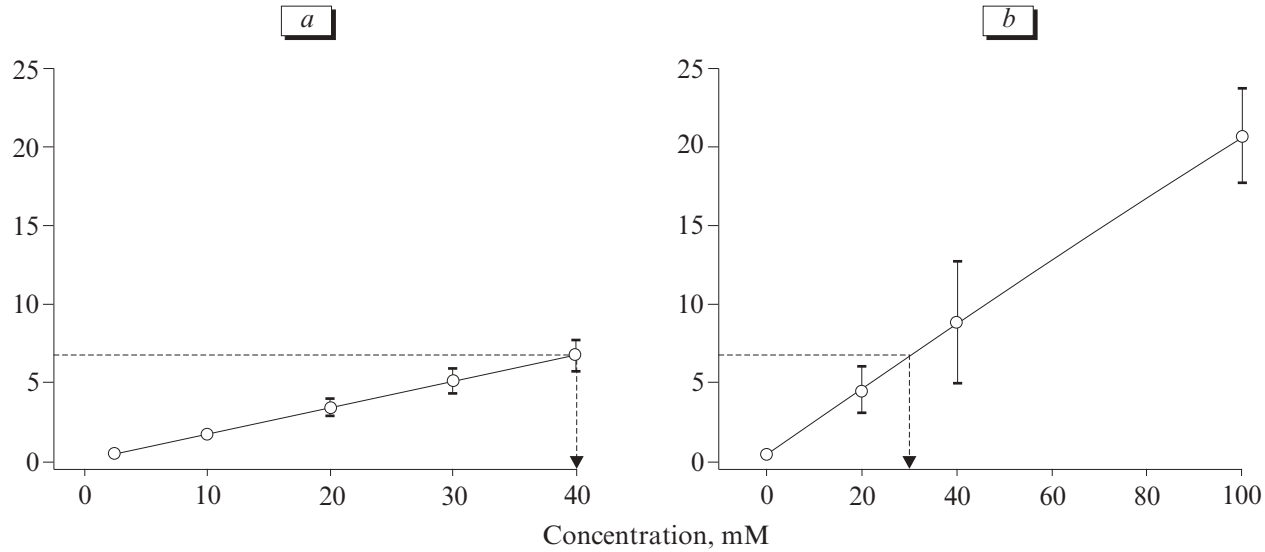

Fig. 2. Dose-response effects of intracellular KCl (*a*) and sucrose (*b*) on the frequency of miniature endplate currents (MEPC). Ordinate: MEPC frequency (Hz). The arrows mark concentrations inducing equal MEPC frequency (~7 Hz).

high-potassium solution revealed spatially separated bright sports in NE (Fig. 3, *a*, *b*). The mean intensity of NE fluorescence increased to 230% and became 0.21±0.01 arb. units (n=27). This fluorescence continued for several hours after placing the muscle in standard Ringer solution. Therefore, during stimulation of spontaneous secretion by high-potassium solution, the synaptic vesicles in NE participate in exo- and endocytosis, so the marker enters the synaptic vesicles and concentrates in the active zones [1].

The intensity of fluorescence of NE preloaded with FM1-43 markedly decreased and returned to initial values after stimulation of exocytose in high-potassium solution (Fig. 3, *a*). Indeed, after 10 min the

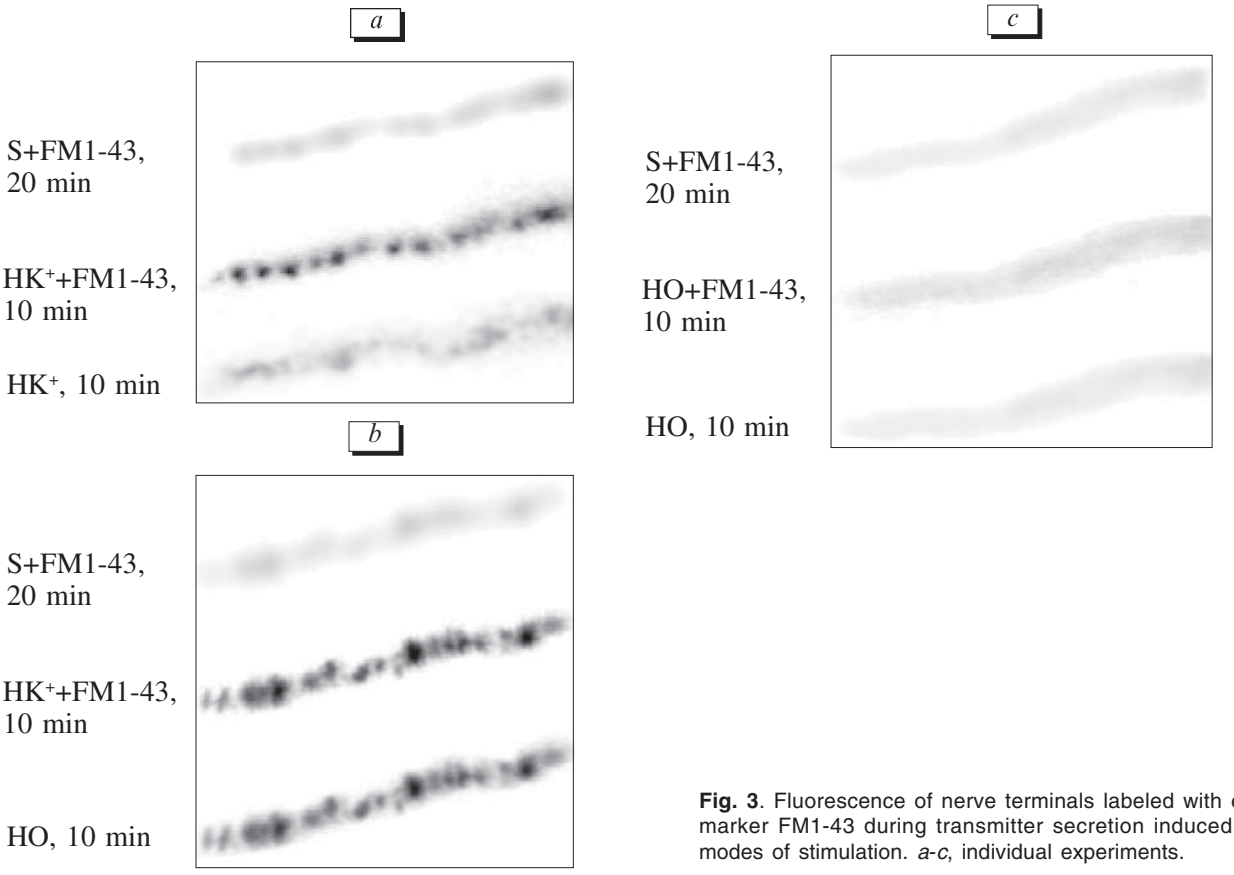

Fig. 3. Fluorescence of nerve terminals labeled with endocytosis marker FM1-43 during transmitter secretion induced by various modes of stimulation. *a*-*c*, individual experiments.

mean intensity of fluorescence was 0.10±0.01 arb. units (109±11% initial value, *n*=12). These data show that during stimulation of secretion with high-potassium solution, NE loses FM1-43 due to complete exocytosis of synaptic vesicles. Quite different events were observed during stimulation of secretion from FM1-43-preloaded NE with sucrose (Fig. 1, *b*). In this case, the intensity of fluorescence remained virtually unchanged: 0.20±0.01 arb. units (220±10% initial value, *n*=14) after 10 min. Therefore, sucrose did not induce unloading of FM1-43 marker from NE. At first glance, these data can be explained by lower rate of spontaneous secretion in hyperosmotic solution compared to that in high-potassium medium. However, the above electrophysiological data showed that the applied concentrations of sucrose and KCl induced approximately similar increase in MEPC rate (Fig. 1). Evidently, stimulation of transmitter secretion in hyperosmotic solutions was not accompanied by complete exocytosis of synaptic vesicles, but was realized via the formation of a transient pore connecting vesicle with the synaptic cleft. In this aggregate, the transmitter can diffuse across the pore, but the marker cannot.

This conclusion was corroborated in the next experimental series, in which we tried to load NE with FM1-43 by stimulating secretion with sucrose. The muscle was placed for 10 min in a solution containing sucrose and FM1-43. No bright sports appeared in NE (Fig. 3, *c*), and the mean intensity of fluorescence of NE was close to baseline value (0.11±0.01 arb. units).

These findings show that two modes of quantal secretion of the transmitter coexist in frog nerve-muscle synapse. The first mechanisms consists in complete exocytosis of synaptic vesicle followed by endocytosis, the second mechanism is realized via the formation of a transient pore between the vesicle and synaptic cleft ("kiss-and-run" mechanism). The first mechanism underlies activation of secretion in high-potassium solutions, while the second one is responsible for stimulation of secretion in hyperosmotic medium. However, it cannot be excluded that both modes belong to the same fundamental process, where the "kiss-and-run" mechanism is only the initial stage of vesicular fusion, which can be arrested in hyperosmotic medium. Probably, the choice between alternative modes of secretion depends on intracellular concentration of Ca^{2+} ions. It is noteworthy that stimulation of secretion in high-potassium solution is accompanied by elevation of internal concentration of Ca^{2+}, while stimulation of secretion in hyperosmotic medium is effected via a Ca-independent pathway [12].

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REFERENCES

1. A. L. Zefirov, P. N. Grigor'ev, A. M. Petrov, *et al.*, *Tsitologiya*, **45**, No. 12, 34-40 (2003).
2. A. L. Zefirov and G. F. Sitdikova, *Usp. Fiziol. Nauk*, **33**, No. 4, 1-33 (2002).
3. A. L. Zefirov and S. Yu. Cheranov, *Ibid.*, **31**, No. 3, 3-22 (2000).
4. J. K. Angleson and W. J. Betz, *J. Neurophysiol.*, **85**, No. 1, 287-294 (2001).
5. W. J. Betz and G. S. Bewick, *J. Physiol.*, **460**, 287-309 (1993).
6. B. Ceccarelli, W. P. Nurbult, and A. Mauro, *J. Cell Biol.*, **57**, No. 2, 499-524 (1973).
7. M. A. Cousin and P. J. Robinson, *J. Neurochem.*, **73**, No. 6, 2227-2239 (1999).
8. R. Couteaux and M. Pecot-Dechavassine, *C. R. Acad. Sci. Hebd. Seances. Acad. Sci. D.*, **271**, No. 25, 2346-2349 (1970).
9. J. E. Heuser and J. E. Reese, *J. Cell Biol.*, **57**, No. 2, 315-344 (1973).
10. G. T. Macleod, J.-B. Gan, and M. R. Bennet, *J. Neurophysiol.*, **82**, No. 3, 1133-1146 (1999).
11. K. Peper, F. Dreyer, C. Sandri, *et al.*, *Cell Tissue Res.*, **149**, No. 4, 437-455 (1974).
12. C. F. Stevens and J. H. Williams, *PNAS*, **97**, No. 23, 12,828-12,833 (2000).
13. F. Valtorta, J. Meldolesi, and R. Fesce, *Trends Cell. Biol.*, **11**, No. 8, 324-328 (2001).
14. W. van der Kloot and J. Molgo, *Physiol. Rev.*, **74**, No. 4, 899-991 (1994).
15. A. L. Zefirov, T. Benish, N. Fatkullin, *et al.*, *Nature*, **376**, 265-268 (1995).