PHYSIOLOGY

Facilitation of Acetylcholine Secretion in Mouse Motor Synapses Caused by Calcium Release from Depots upon Activation of L-Type Calcium Channels

A. E. Gaydukov, S. N. Melnikova, and O. P. Balezina

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 148, No. 8, pp. 124-128, August, 2009
Original article submitted February 11, 2009

Pharmacological disinhibition of L-type Ca^{2+} channels by two ways (with agonist S(-) BAY K 8644 and iberiotoxin, a Ca^{2+}-activated BK-type K^+-channel blocker) increases quantal content of evoked end-plate potentials, which was completely prevented by ryanodine (2 μM) blockade of ryanodine receptors. We conclude that increased quantal secretion of the transmitter induced by L-type Ca^{2+} channel functioning requires activation of ryanodine receptors and calcium release from depots in motor terminals in mice.

Key Words: *rhythmic activity; quantal composition; ryanodine receptors; L-type calcium channels; iberiotoxin*

Acetylcholine secretion in mouse motor synapses is primarily determined by calcium entry through voltage-dependent P/Q-type Ca^{2+} channels [8,9,12]. Recently, latent "slow" L-type Ca^{2+} channels were discovered; upon activation these channels conduct Ca^{2+} current and induce the increase in quantal content of end-plate evoked potentials [3,7,13,14]. In most excitable and non-excitable cells, calcium entry through L-type channels is associated with ryanodine receptor activation and calcium release from depots [1,4,15]. Ca^{2+}-dependent release of stored calcium was shown by us and by other authors in motor terminals under conditions of slow L-type Ca^{2+} channel inactivation [2,10,11]. The possibility of activation this calcium release system by inward Ca^{2+} current through L-type Ca^{2+}-channels and subsequent changes in transmitter secretion remain little studied.

Department of Human and Animal Physiology, Biological Faculty, M. V. Lomonosov Moscow State University, Russia. ***Address for correspondence:*** gaydukov@gmail.com. A. E. Gaidukov

Here we studied functional coupling of L-type Ca^{2+}-channels, induction of calcium release from intraterminal Ca^{2+}-depots, and transmitter release in motor nerve terminals in mice.

MATERIALS AND METHODS

The experiments were carried out on "dissected" neuromuscular preparations *m. diaphragma—n. phrenicus* isolated from mice, at room temperature (20°C) [7]. Left half of the diaphragm muscle was isolated with a segment of the phrenic nerve and placed into a 3-ml chamber perfused with oxygenated Lilly solution (pH 7.2-7.4: 95% O_2 and 5% CO_2) containing (in mM): 135 NaCl, 4 KCl, 0.9 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 16.3 $NaHCO_3$, and 11 glucose. Synaptic potentials were intracellularly recorded via glass microelectrodes filled with 2.5 M KCl (15 MΩ electrode resistance). Individual end-plate potentials (EPP) were recording during stimulation of the phrenic nerve with 0.1-msec suprathreshold pulses delivered at a rate of 0.3 Hz. For

0007-4888/09/1482-0163

investigation of rhythmic synaptic activity, the nerve was stimulated with short pulse bursts (50 stimuli, 50 Hz). The signals were recorded using an Axoclamp-2B amplifier (Axon Instruments), inputted to a computer hard drive disk through DigiLine digitizer with Digiscope interface, and then processed using Mini-Analysis software (Synaptosoft). At least three neuromuscular preparations were used in each experimental series. Data analysis included standardizing the averaged miniature end-plate potentials (MEPP) and EPP values by the membrane potential -77 mV followed by correction of EPP amplitudes for summation nonlinearity [7]. Quantal spectrum of EPP was estimated as the ratio of the mean corrected EPP amplitude to the mean MEPP amplitude. Significance of differences was assessed by Student's *t* test and Mann—Whitney test. Level of significance of differences was set at 0.05 (*n* is the number of synapses investigated).

RESULTS

Experimental series I was focused on the analysis of the role of L-type Ca^{2+}-channels in transmitter secretion. It is known that selective L-type Ca^{2+}-channel blockers did not affect secretion in intact motor synapses, and that during recording of presynaptic action potential, no calcium current through L-type Ca^{2+}-channel was observed [8,9,12,13]. Selective agonist S(-) BAY K 8644 (1 μM) was used for L-type Ca^{2+}-channel activation. No significant changes in muscle fiber resting potential and in amplitude-time characteristics of MEPP were noted during BAY application; however, the mean amplitude of individual evoked EPP significantly increased due to an increase in EPP quantal content to 20.9±1.6 (*n*=15) *vs*. 14.8±0.7 (*n*=22) in the control, *i.e.* to 141% from the control value ($p<0.05$; Fig. 1).

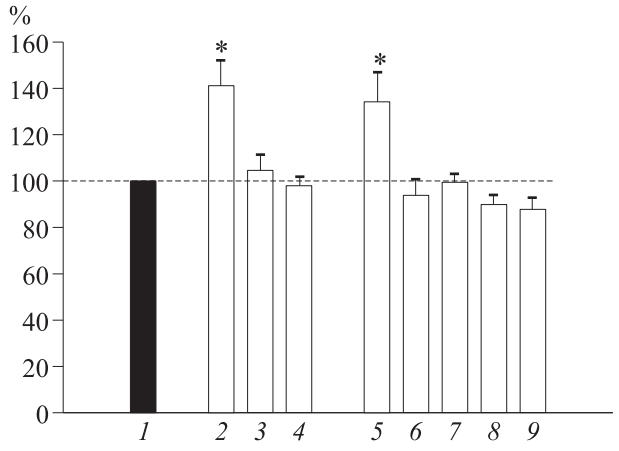

Fig. 1. Quantal content of individual evoked EPP (standardized by control (*1*) taken for 100%): after application of 1 μM S(-) BAY K 8644 (*2*), 2 μM ryanodine (*3*, *8*), 1 μM S(-) BAY K 8644 against the background of 2 μM ryanodine (*4*), 100 nM iberiotoxin (*5*), 10 nM nifedipine (*6*), 100 nM iberiotoxin against the background of 10 nM nifedipine (*7*), iberiotoxin 100 nM against the background of 2 μM ryanodine (*9*). *$p<0.05$ compared to the control.

Then we studied whether Ca^{2+}-dependent calcium release from depots participates in intensification of transmitter release observed during BAY application. For this purpose, changes in MEPP and EPP characteristic during application of ryanodine, a selective ryanodine receptor (RYR) modulator, were analyzed. Ryanodine depending on the concentration can either activate (<1 μM), or inhibit (>1 μM) RYR-depot [1]. Ryanodine (2 μM) by itself had no significant effect on quantal composition of EPP (Fig. 1). It can be hypothesized that during stimulation with individual neural impulses (0.3 Hz), calcium release from depots does not contribute to Ca^{2+}-dependent evoked secretion of the transmitter [1,2]. We found that during application of 2 μM ryanodine, BAY lost its ability to increase quantal composition of EPP: 17.4±0.7 (*n*=16) *vs.* 17.7±0.9 in the control (*n*=17, $p>0.05$; Fig. 1).

Our findings suggest that slow Ca^{2+}-flow into a terminal under the effect of S(-) BAY K 8644 increases Ca^{2+}-dependent acetylcholine secretion, but this effect requires activation of RYR and calcium release from depots.

In the next experimental series we used another approach for L-type Ca^{2+}-channels activation. This "disinhibition" recently was shown to take place under the action of Ca^{2+}-activated BK-type K^+-channel blockers leading to deceleration of the repolarization phase of the action potential, providing conditions for activation of slow L-type Ca^{2+}-channels [6,7]. BK-channel blocker iberiotoxin (100 nM) produced no significant changes in resting potential and amplitude-time characteristics of MEPP. Evoked transmitter release was affected by iberiotoxin in the same way as by BAY: mean EPP amplitude increased from 29.1±1.3 mV in the control group (*n*=18) to 42.2±6.3 mV after application of iberiotoxin ($p<0.05$; *n*=20), what was associated with increase in EPP quantal content: from 16.1±0.6 in control group to 21.61±2.10 ($p<0.05$), *i.e.* by 34% (Fig. 1).

To find out whether Ca^{2+} flow through L-type Ca^{2+}-channels is involved into realization of potentiating action of iberiotoxin on evoked transmitter secretion, the effect of 100 nM iberiotoxin under conditions of preliminary L-type Ca^{2+}-channel block with nifedipine (10 μM) was investigated. Nifedipine by itself had no effect on individual EPP quantal content: 20.4±1.0 (*n*=18) in the control and 18.6±1.3 (*n*=17) after nifedipine. At the same time, blockade of L-type Ca^{2+}-channels with nifedipine abolished the potentiating effect of iberiotoxin on quantal content of EPP (Fig. 1). It means that BK-channels are intended for limiting L-type Ca^{2+}-channel activity and their involvement

into induction of transmitter secretion after individual (0.3 Hz) nerve stimulation.

At the next stage of the study it was necessary to find out, whether activation of L-type Ca^{2+}-channels under conditions of BK-channel blockade and slow Ca^{2+} flow into terminal are sufficient for intensification of transmitter secretion, or it requires RYR activation and calcium release (similarly to the effect of S(-) BAY K 8644). Ryanodine in a concentration blocking presynaptic RYR (2 μM) prevented the increase in individual EPP quantal content after iberiotoxin application (Fig. 1): EPP quantal content in the control was 16.2±0.9 (n=15), after application of ryanodine 14.5±0.6 (n=15), and after application of iberiotoxin against the background of ryanodine 14.2±0.8 (n=16). Thus, the potentiating effect of BK-channel blocker on quantal content of individual EPP is mediated not so much by Ca^{2+} entry through L-type Ca^{2+}-channels, but by subsequent RYR activation and calcium release from depots.

The increase in individual EPP quantal content by more than 30% due to Ca^{2+}-dependent calcium release from depot during generation of individual presynaptic action potential did not answer the question whether this system can steady maintain enhanced release of the transmitter during rhythmic burst activity, the main working regimen in motor synapses. To this end, changes in EPP amplitude during the course of rhythmic burst were studied under conditions of L-type calcium channel "disinhibition" caused by blockade of BK-channels with iberiotoxin. Stimulation of the phrenic nerve with bursts of 50 pulses at 50-Hz frequency produces specific changes in EPP amplitude in mouse neuromuscular preparation during the burst: facilitation, *i.e.* short-term increase in EPP amplitude in the beginning of the burst (up to 110.0±1.5% amplitude of the first EPP in the burst; n=18) was followed by a decrease in EPP amplitude (depression) to a stable decreased level, EPP plateau (83.7±3.5% amplitude of the first EPP in a burst; Fig. 2). In the beginning of the burst we observed a significant increase in both the absolute (by 10 mV on average) and relative EPP amplitudes (by 10-12% compared to the first EPP) compared to the control (p<0.05; n=25) during both facilitation and depression. Absolute EPP amplitude remained increased by 25-30% during the "plateau" phase. Preliminary application of L-type Ca^{2+}-channel blocker nifedipine (10 μM) or verapamil (5 μM) produced no significant changes in EPP amplitude over the burst in comparison with the control, but completely prevented potentiating effect of iberiotoxin on acetylcholine secretion (Fig. 2). These data suggest that L-type Ca^{2+}-channels are normally inactive under conditions of individual and rhythmic mode of motor synapse functioning. In case of their demasking (for example, after blockage of BK-potassium channels), Ca^{2+} entry through L-type channels not only increases the quantal content of individual EPP, but also maintains steady absolute EPP increment during the whole burst. Moreover, after blockage of L-type Ca^{2+}-channels facilitation during the bursts markedly increased. Thus, we demonstrated that activated L-type Ca^{2+}-channels effectively facilitate transmission in rhythmic EPP burst.

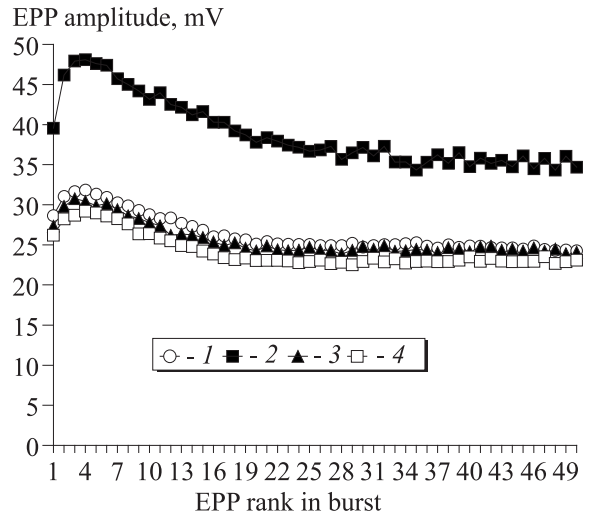

Fig. 2. Changes in EPP burst pattern during synaptic rhythmic activity with 50 Hz frequency: control (*1*), after iberiotoxin 100 nM (*2*), after verapamil 5 μM (*3*) and iberiotoxin 100 nM against the background of verapamil 5 μM. *p<0.05 compared to the control.

In the last experimental series we studied whether RYR activation and calcium release from depots par-

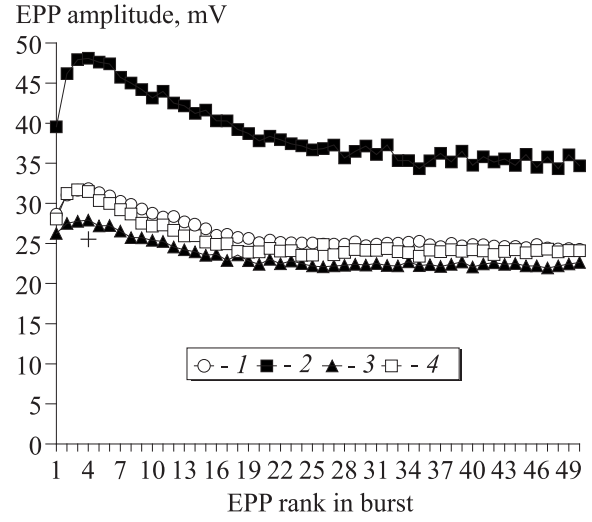

Fig. 3. Changes in EPP burst pattern during synaptic rhythmic activity with a frequency of 50 Hz: control (*1*), 100 nM iberiotoxin (*2*), 2 μM ryanodine (*3*), and 100 nM iberiotoxin against the background of 2 μM ryanodine (*4*). p<0.05 for 2, 3, and 4 compared to the control (1). +p<0.05 between 1 and 3 in the segment from 1st to 8th EPP.

ticipate in the maintenance of increased acetylcholine secretion during the whole burst under conditions of activated L-type Ca^{2+}-channels. To test this assumption, we studied the effects of ryanodine as a RYR blocker (2 μM) on the pattern of synapse burst activity and effects of ryanodine on facilitation of transmitter secretion produced by iberiotoxin during EPP burst. Ryanodine produced significant initial facilitation of the transmission in the burst by 6.7% compared to the control (n=32; Fig. 3), which attests to possible RYR activation and calcium release from depots during rhythmic activity in motor synapses. Moreover, in this case RYR are activated without participation of L-type Ca^{2+}-channels, probably due to Ca^{2+} entry though P/Q-type channels. If RYR are completely blocked with ryanodine, iberiotoxin losses ability to increase EPP amplitude during rhythmic burst (n=37; Fig. 3).

Our studies showed that different ways for activation (disinhibition) of L-type Ca^{2+}-channels provide similar effect, potentiation of transmitter secretion. Similar effects were also shown in individual EPP by other authors [3,7,13]. In our experiments, rhythmic burst activity of synapses was investigated apart from individual activity, L-type Ca^{2+}-channel disinhibition was shown to increase transmitter release during both generation of individual EPP and during whole rhythmic EPP burst. RYR activation occurred when L-type Ca^{2+}-channels operate. It can be hypothesized that RYR and calcium release from depots play a role of an effector for Ca^{2+}-signal amplification and stimulation of acetylcholine secretion.

A question arises, why RYR activation is necessary for amplification of transmitter secretion under conditions of disinhibition of slow activated L-type Ca^{2+}-channels? One may assume, that L-type Ca^{2+}-channels are located far from active zones, but are tightly associated with RYR, whose activation provides elevation of calcium concentration in the terminal, and this calcium can reach exocytosis sites. Functional coupling of RYR and L-type Ca^{2+}-channel was reported not only in muscle cells, but also in some neurons, where Ca^{2+} entry into the soma through L-type Ca^{2+}-channels triggers RYR activation and calcium release from depots [4,15]. Since direct physical coupling is well known for RYR and dihydropyridine receptors in skeletal muscle fibers, this type of interaction can not be excluded for RYR and L-type Ca^{2+}-channels. Physical coupling between RYR and Ca^{2+}-channels in neural terminals in magnocellular nucleus was previously reported and involvement of this system into facilitation of transmitter secretion was shown [5]. Final answer for the question concerning the type of interaction between RYR and L-type Ca^{2+}-channels in neuromuscular synapse requires further investigations. In any way, the observed coupling between presynaptic RYR and L-type Ca^{2+}-channels resulting in significant facilitation of transduction indicates the possible involvement of the tandem L-type Ca^{2+}-channel — RYR into regulation of transmitter secretion in neuronal terminals in mice.

This work was supported by Russian Foundation for Basic Researches (grant No. 07-04-00920).

REFERENCES

1. O. P. Balezina, *Uspekhi Fiziol. Nauk*, **33**, No. 3, 38-56 (2002).
2. O. P. Balezina and A. N. Bukiya, *Neirofiziologiya*, **35**, No. 2, 83-89 (2003).
3. W. D. Atchison and S. M. O'Leary, *Brain Res.,* **419**, Nos. 1-2, 315-319 (1987).
4. P. Chavis, L. Fagni, J. B. Lansman, and J. Bockaert, *Nature*, **382**, No. 6593, 719-722 (1996).
5. V. De Crescenzo, K. E. Fogarty, R. Lhuge, *et al.*, *J. Neurosci.*, **26**, No. 29, 7565-7574 (2006).
6. E. Faber and P. Sah, *Neuroscientinst*, **9**, No. 3, 181-194 (2003).
7. M. Flink and W. Atchinson, *J. Pharmacol. Exp. Ther.*, **305**, No. 2, 646-652 (2003).
8. F. Giovannini, E. Sher, R. Webster, *et al.*, *Br. J. Pharmacol*., **136**, No. 8, 1135-1145 (2002).
9. E. Katz, D. A. Protti, P. A. Ferro, *et al.*, *Ibid*, **121**, No. 8, 1531-1540 (1997).
10. M. Kubota, K. Natita, T. Murayama, *et al.*, *Cell Calcium*, **38**, No. 6, 557-567 (2005).
11. K. Narita, T. Akita, J. Hachisuka, *et al.*, *J. Gen. Physiol.*, **115**, No. 4, 519-532 (2000).
12. S. Nudler, *Ann. N. Y. Acad. Sci.*, **998**, 11-17 (2003).
13. F. Urbano and O. Uchitel, *Pflugers Arch*., **437**, No. 4, 523-528 (1999).
14. F. Urbano, R. S. Depetris, O. D. Uchitel, *et al.*, *Ibid*, **441**, No. 6, 824-831 (2001).
15. P. Welsby, M. Rowan, R. Anwyl, *et al.*, *Eur. J. Neurosci.*, **24**, No. 11, 3109-3118 (2006).