Effect of L-Type Calcium Channel Blockers on Activity of Newly Formed Synapses in Mice

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 2, pp. 128-131, February, 2007
Original article submitted June 28, 2006

Verapamil (5 μM), nifedipine (10 μM), and ryanodine (10 μM) potentiated rhythmic activity of newly formed synapses, while apamin produced no effect on this potentiation. Ryanodine (1 μM) suppressed synaptic activity, and this effect can be prevented with nifedipine. It was hypothesized that in newly formed synapses Ca^{2+} entry through L-type channels triggers the release of stored Ca^{2+}, which inhibits secretion of the neurotransmitter.

Key Words: *newly formed synapses; verapamil; nifedipine; ryanodine*

The mechanisms underlying the formation of new synapses are currently in the focus of modern neurophysiology. Progress in this field is largely determined by the use of new neuromuscular synapses formed on skeletal muscle fibers during their reinnervation [3]. Transection or crushing of motor axons in the peripheral nerve is followed by their regeneration with the development of growth cones growing towards the muscle, where new motor synapses are formed. Calcium ions play an important role in the function of the growth cone in motor and other axons. Ca^{2+} entry via L-type calcium channels and/or the release of stored Ca^{2+} via ryanodine receptors (RR) into the cytoplasm elevate $[Ca^{2+}]_i$, which promote the growth of filopodia in the terminals of sprouting axons [8,9,11]. However, there are scanty and contradictory data on the role of these mechanisms of elevation of internal calcium concentration in the regulation of axon terminal activity during their stabilization and formation of new motor synapses [7,12].

Our aim was to study synaptic activity of newly formed motor synapses in a skeletal muscle of mouse shin at the early stages of its re-innervation. Selective agonists and antagonists of L-type calcium channels and RR were used for evaluation of their role in secretion of neurotransmitter in the developing motor synapses.

MATERIALS AND METHODS

Experiments were carried out on isolated mouse neuromuscular preparation of *n. peroneus communis — m. extensor digitorum longus* (mEDL). Twelve days before the experiment *n. peroneus communis* in albino random-bred mice was crushed immediately near the right edge of the lateral head of the gastrocnemius muscle. The crushing was made with ophthalmic forceps that had fine plane branches protected with plastic tips under ether narcosis. The length of the crushed portion of the nerve was about 1 mm. Twelve days after crushing, the animal was decapitated, mEDL was isolated with the nerve and placed into a chamber with normal Laily solution. Miniature end-plate potentials (MEPP, no less than 100 peaks in each synapse) and end-plate potentials (EPP, no less than 50 spikes in each synapse) were recorded intracellularly with glass microelectrodes (resistance 5-10 MΩ) filled with 2.5 M KCl. To record EPP, the muscle contractions were preliminary blocked with d-tubocurarine (dTC), thereafter the nerve was stimulated with rhythmic stimuli (repetition rate 20 Hz, pulse

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duration 0.20-0.25 msec, interval between records 1-2 min).

The records were processed with Axotape and MiniAnalysis software. The supplementary calculations were performed with Microsoft Excel and Statistica software. The data were processed statistically using Mann—Whitney non-parametric *U* test for independent samples and Wilcoxon test for linked samples. Normal Laily solution containing (in mM) 135 NaCl, 1.0 $MgCl_2$, 4.0 KCl, 0.9 NaH_2PO_4, 2.0 $CaCl_2$, 11.0 glucose, and 16.0 $NaHCO_3$ and oxygenated with 96% O_2 and 4% CO_2 (pH 7.2-7.4) and modified Laily solution containing (in mM) 7.8 NaCl, 1.0 $MgCl_2$, 20.0 KCl, 0.9 NaH_2PO_4, 2.0 $CaCl_2$, 11.0 glucose, 16.0 $NaHCO_3$, 61.7 sucrose, 55.6 Na_2SO_4 were used.

The chemicals dTC, verapamil, nifedipine, ryanodine, and apamin were from Sigma and Calbiochem.

RESULTS

In series I, spontaneous activity of intact and newly formed neuromuscular synapses of mEDL was examined. To this end, changes in the mean discharge rate of MEPP were analyzed in normal Laily solution and against the background of potassium depolarization induced by elevation of $[K^+]_e$ to 8 and 20 mM. It was found that after depolarization of a newly formed terminal caused by 8 mM $[K^+]_e$, the mean discharge rate of MEPP was 0.48±0.05 Hz, which was significantly higher than the mean discharge rate in normal Laily solution (0.29±0.02 Hz, $p<0.05$). Elevation of $[K^+]_e$ to 20 mM increased the mean discharge rate in newly formed synapses to 1.10±0.14 Hz, which significantly surpassed the control value (by 3.8 times). In intact mature synapses, the mean discharge rate of MEPP in the control was 1.29±0.12 Hz, while after increasing external potassium concentration to 8 and 20 mM it increased to 2.90±0.31 and 52.8±5.3 Hz, respectively.

According to our previous studies and published data, 8 mM $[K^+]_e$ activates L-type Ca^{2+}-channels [10], while 20 mM $[K^+]_e$ activates P/Q-type Ca^{2+}-channels [3]. In experimental animals, depolarization of the synapses with 8-10 KCl produced no elevation of the mean discharge rate of MEPP comparable to that in the synapses with intact mEDL. This suggests that the number of L- and P/Q-type channels in newly formed synapses is lower than in nerve terminal of mature synapse or that calcium entry via L-type Ca^{2+}-channels does not stimulate spontaneous release of the neurotransmitter.

In series II we compared evoked activity in intact and newly formed synapses. Individual EPP of newly formed synapses significantly differed from EPP in intact synapses: they had 2.8-fold longer latency, 2.5-fold lower amplitude, 1.5-2.0-fold longer duration, and lower quantal content. For example, the mean amplitude of EPP in intact synapses was 3.28±0.37 mV, the rise time and half-decay time were 2.10±0.08 and 1.81±0.13 msec, respectively, and the quantal content was 30±4. In newly formed synapses, the mean amplitude of EPP was 1.31±0.10 mV, rise time and half-decay time were 2.31±0.11 and 2.89±0.15 msec, respectively, and the quantal content was 17±2 (32 synapses were examined).

Then we compared changes in the amplitude of EPP during a rhythmic burst consisting of 50 EPP evoked at the rate of 20 Hz. In intact synapses, initial facilitation was followed by EPP decay and a plateau phase. In newly formed synapses, long-term facilitation of the transmission was followed by a plateau level surpassing the initial amplitude at the end of the burst.

In the next series we examined the effect of blockade of L-type Ca^{2+}-channels on the pattern of the burst. It was found that blockers with different chemical structure (5 μM verapamil and 10 μM nifedipine) significantly increased the amplitude of EPP during the plateau phase by 29% and 20%, respectively (Fig. 1). In contrast to pronounced effects on re-innervated muscle, application of verapamil to intact mEDL produced no significant changes in the pattern of the burst. The plateau level in this burst was 80.5% in the experiment and 82.7% in the control.

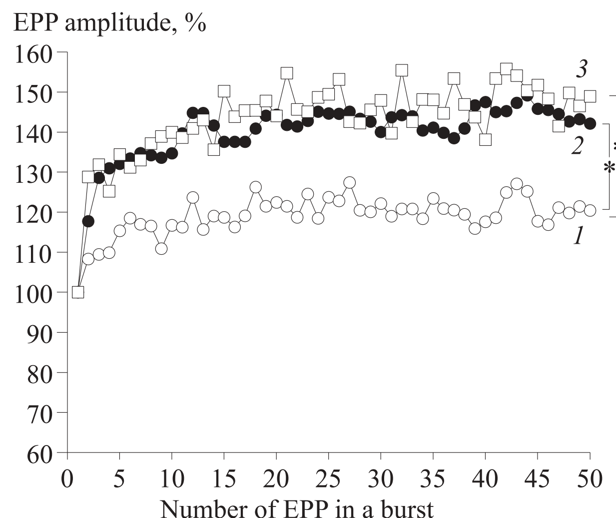

Fig. 1. Effect of L-type Ca^{2+}-channel blockers on EPP amplitude in newly formed synapses during a burst evoked at 20 Hz repetition rate. *1*) control; *2*) verapamil (5 μM), *3*) nifedipine (10 μM). Here and in Figs. 2, 3: EPP amplitude is shown in percentage of the first spike. $p<0.001$.

In newly formed synapses, opening of Ca^{2+}-channels and maintaining their open state with agonist Bay K-8644 led to a small but significant decrease of EPP plateau level in the bursts by 11.5%. In contrast, in intact synapses this agonist increased EPP amplitude by 20% during the plateau phase.

Our findings suggest that in newly formed synapses, calcium entry into the terminal via L-type Ca^{2+}-channels inhibits some processes underlying neurotransmitter release. It cannot be excluded that the entry of external Ca^{2+} activates K^{+}_{Ca}-channels, which hyperpolarize the membrane and decrease calcium current via potential-dependent Ca^{2+}-channels, thus decreasing neurotransmitter secretion. To test this hypothesis, we examined the effects of apamin, a specific blocker of low-conductance K^{+}_{Ca}-channels.

Apamin (5 μM) produced no effect on burst pattern, *i.e.*, K^{+}_{Ca}-channels were not activated during calcium entry via L-type Ca^{2+}-channels.

This negative result prompted us to analyze another hypothesis: calcium entry via L-type Ca^{2+}-channels indirectly inhibits secretion of neurotransmitter via activation of RR and release of stored calcium.

To test this hypothesis, the next series examined burst activity of newly formed synapses after RR blockade with 10^{-5} M ryanodine.

Ryanodine, a blocker of RR and release of stored calcium, produced an effect qualitatively similar to that of L-type Ca^{2+}-channel blockers: it markedly elevated the amplitude of EPP in a short burst with repetition rate of 20 Hz (Fig. 2). The initial facilitation and plateau level increased by 44% and 132%, respectively.

If the release of stored calcium inhibits secretion of acetylcholine, potentiation of this release should further inhibit synaptic transmission. To test this assumption, the synapses were treated with ryanodine in low (micromolar) concentration capable to maintain RR in the open state and therefore to augment the release of stored calcium ions. As suggested, this maneuver significantly moderated synaptic activity: the amplitude of EPP at the plateau dropped by 56% (Fig. 2).

If calcium entry via L-type Ca^{2+}-channels and the release of stored calcium are casually related, nifedipine (a blocker to L-type Ca^{2+}-channels) should prevent the inhibitory effect of ryanodine. In other words, by blocking both calcium entry and calcium release it should produce a disinhibitory effect manifesting in increased synaptic transmission.

In the final series of experiments, nifedipine (10 μM) was added to Laily solution containing ryanodine (1 μM). Nifedipine prevented the inhibitory effect of ryanodine and to some extent preserved its potency to facilitate neuromuscular transmission in newly formed synapses during burst activity (Fig. 3).

Thus, our experiments demonstrated that L-type Ca^{2+}-channel blockers significantly potentiated the release of neurotransmitter and facilitated the synaptic transmission in newly formed synapses of reinnervated mouse mEDL, although they produced no such effects in intact synapses. The specificity of the observed facilitating effect of Ca^{2+}-channel antagonists on transmission in newly formed synapses is confirmed by the fact that Ca^{2+}-channel agonist Bay K-8644 produced an opposite inhibitory effect. Our data agree with published reports on facilitating effects of L-type Ca^{2+}-channel bloc-

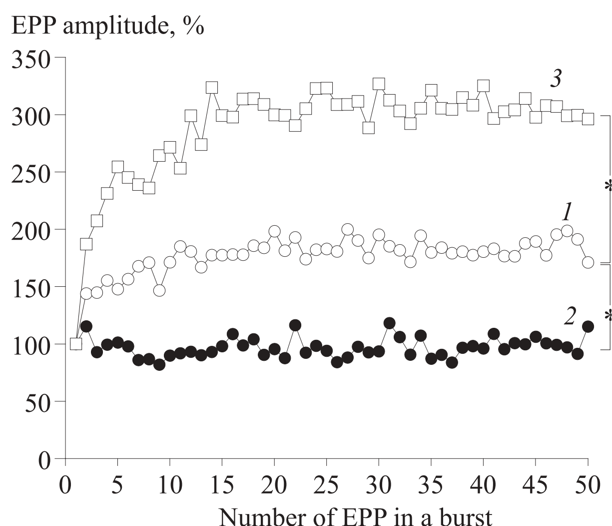

Fig. 2. Effect of ryanodine on burst activity in newly formed synapses of mEDL. *1*) control; *2*) ryanodine (1 μM), *3*) ryanodine (10 μM).

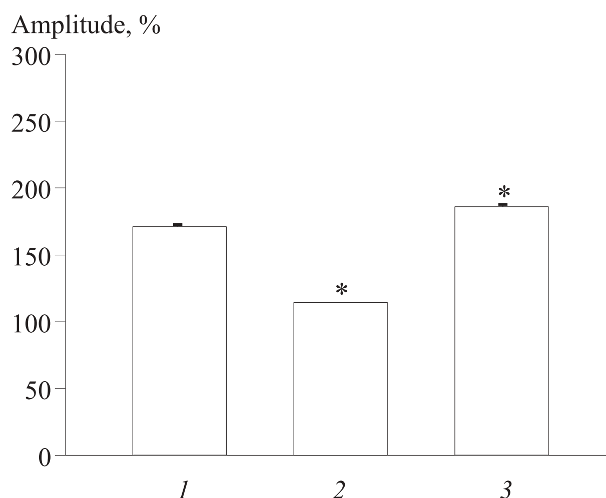

Fig. 3. Effect of ryanodine and nifedipine on plateau level of the bursts evoked in newly formed synapses of mEDL. *1*) control; *2*) ryanodine (1 μM), *3*) nifedipine (10 μM) applied against the background of ryanodine (1 μM).

kers on neuromuscular transmission in mammalian re-innervated synapses [13,15]. In mature synapses, inhibition of neurotransmitter secretion by calcium entry into the nerve terminal can be effected via activation of Ca^{2+}-dependent K^+-channels [1,2]. This mechanism was inefficient in newly formed synapses: involvement of high and medium conductance Ca^{2+}-dependent K^+-channels in the inhibitory effects of calcium entry was excluded [15]. We showed that low-conductance Ca^{2+}-dependent K^+-channels also produce no effect on burst activity in re-innervated synapses. There is an assumption that calcium entry via L-type Ca^{2+}-channels stimulates the release of some neuromodulator in the synaptic cleft of newly formed motor synapses [15]. Binding of this modulator to presynaptic receptors coupled with G-proteins triggers intracellular cascade leading to inactivation of P/Q-type Ca^{2+}-channels controlling secretion of the neurotransmitter. Blockade of G-protein prevented the facilitating effect of L-type Ca^{2+}-channel blockers on transmission [15].

We showed that calcium entry via L-type Ca^{2+}-channels leads to activation of RR and release of stored calcium, which inhibits secretion of neurotransmission. In fact, blockade of the release of stored calcium facilitated rhythmic burst activity in the synapses. Potentiation of the release of stored calcium after application of low dose of ryanodine significantly suppressed EPP during entire time course of the burst, and this inhibitory effect was abolished by nifedipine.

It cannot be excluded that the release of stored calcium in small newly formed terminals is accompanied by generalized elevation in $[Ca^{2+}]_i$ [8,11] capable to induce Ca^{2+}-dependent inactivation of P/Q-type Ca^{2+}-channels and inhibition of neurotransmitter secretion [5,6]. Further studies should clarify the mechanism actually triggered by the release of stored calcium and inhibiting secretion of neurotransmitter in newly formed synapses.

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