Mechanism of Inhibition of Acetylcholine Secretion in Newly Formed Mouse Synapses Involving Ca^{2+}-Dependent Kinases and Voltage-Gated K^+-Channels

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Nifedipine, a blocker of L-type Ca^{2+}-channels, increased quantal content of endplate potentials in newly formed nerve-muscle synapses, while R 24571 (calmodulin inhibitor) and KN 62 (inhibitor of calmodulin-dependent kinase II) did not change it. KN 62 suppressed the increase in quantal content of endplate potentials evoked by nifedipine. Similar to nifedipine, chelerythrine and bisindolylmaleimide I (blockers of protein kinase C) increased quantal content of endplate potentials. In the presence of chelerythrine, nifedipine lost its ability to facilitate secretion of neurotransmitter. 4-Aminopyridine, a blocker of voltage-gated potassium channels, increased quantal content of endplate potentials. In the presence of chelerythrine, 4-aminopyridine induced no additional increase in the quantal content of endplate potentials. In its turn, chelerythrine induced no extra facilitation of secretion in the presence of 4-aminopyridine. It is hypothesized that Ca^{2+}-dependent inhibition of secretion results from suppression of calmodulin-dependent kinase II and activation of protein kinase C, which potentiates the work of voltage-gated K^+-channels.

Key Words: *newly formed synapse; calmodulin; calmodulin-dependent kinase II; protein kinase C; voltage-gated K^+-channels*

During innervations of skeletal muscles in early ontogeny or their reinnervation in mature age, excessive synapses are formed in muscle fibers to be shortly eliminated leaving a single endplate in a fiber. The mechanism of elimination of excessive synapses includes the initial inhibition of acetylcholine (Ach) secretion followed by the "silent synapse" stage culminated by elimination of these synapses [15]. Suppression of Ach secretion in terminals to be eliminated is a Ca^{2+}-dependent process triggered by calcium ions entering the presynaptic terminal via L-type Ca^{2+}-channels [11]. We found that Ca^{2+}-dependent inhibition of Ach secretion relates only to evoked release of the neurotransmitter, but produces no effect on spontaneous secretion [1]. Moreover, Ca^{2+}-dependent inhibition of induced Ach secretion of does not result from activation of Ca^{2+}-dependent K^+-channels of BK or SK types. We hypothesized that this inhibitory Ca^{2+}-signal is targeted to Ca^{2+}-dependent enzymes in the terminals such as calmodulin, calmodulin-dependent kinase II (CaM KII), and protein kinase C (PKC) capable of regulating activity of K^+-channels in terminals like other substrates [12,14]. To test this hypothesis, this work analyzes the presynaptic effects of calmodulin inhibitors, CaM KII, and PKC in relation to blockade of L-type Ca^{2+}- and K^+-channels.

MATERIALS AND METHODS

Experiments were carried out on "dissected" nerve-muscle preparation of *m. extensor digitorum longus* (*m. EDL*) and *n. peroneus communis* isolated from

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mouse on day 11 after mechanical compression of the nerve. The nerve was crushed with ophthalmic forceps with thin flat plastic-protected branches. The length of the crushed segment of the nerve was about 1 mm. On post-surgery day 11, the mice were decapitated, *m. EDL* was isolated with its nerve and placed into a chamber with normal Lilly solution containing (in mM): 135 NaCl, 1.0 $MgCl_2$, 4 KCl, 0.9 NaH_2PO_4, 2.0 $CaCl_2$, 11 glucose, 16 $NaHCO_3$ (pH 7.2-7.4) aerated with carbogen (96% O_2 and 4% CO_2). Miniature endplate potentials (mEPP, no less than 100 in each synapse) and endplate potentials (EPP, ≥50) were recorded intracellularly with glass microelectrodes (tip resistance 5-10 MΩ) filled with 2.5 M KCl solution. For recording individual EPP, the nerve was stimulated with 0.20-0.25 msec pulses applied at a repetition rate of 0.5 Hz. The membrane potential was -50 mV on the average and did not change under the effect of the examined substances. If the membrane potential varied by more than 5 mV during recording, this record was discarded. Quantal content of EPP was calculated as the ratio of the mean corrected EPP amplitude to the mean amplitude of mEPP. In each series, no less than 3 muscles were examined. The amplitude and quantal content of EPP measured prior to application of the examined agents were taken as the control values.

The following chemicals were used in the study: nifedipine, 4-aminopyridine, chelerythrine, and KN 62 (Sigma); ryanodine (Biomol); bisindolylmaleimide I (BIM, Calbiochem), R 24571 (Serva).

The records were processes with Axotape and MiniAnalysis software. Calculations were performed with Microsoft Excel and Statistica software. The data were processed statistically using Mann–Whitney test and presented as *m*±*SEM*.

RESULTS

Preliminary experimental series showed that structurally different blockers of L-type Ca^{2+}-channels verapamil (5 μM) and nifedipine (10 μM) facilitate nerve-muscle transmission in newly formed synapses by increasing the amplitude and quantal content of EPP by 50% on the average. Specifically, nifedipine elevated the quantal content of EPP from 10±1 (control value) to 16±2 ($p<0.05$, $n=47$). This effect appeared immediately after application of the blockers and persisted for 40-60 min. Facilitating action of L-type Ca^{2+}-channel blockers was observed during a short period of the first 7 days of re-innervations, which agrees with the data on the period of elimination of the excessive cell-cell contacts in this model [15]. These data suggest that Ca^{2+} entry into nerve terminal via L-type Ca^{2+}-channels inhibits neurotarnsmitter release in newly-formed synapses.

However, it cannot be excluded that calcium ions entering the presynaptic terminal via L-type Ca^{2+}-channels during generation of presynaptic action potential are bound by calmodulin protein, which is involved in Ca-dependent inhibition of Ach secretion when activated by calcium ions. For example, recent studies showed that calmodulin participates in inactivation of P/Q-type Ca^{2+}-channels under conditions of excessive Ca^{2+} entry into terminal [2]. In case of similar functioning of calmodulin in newly formed synapses, its blockade should disinhibit synaptic activity and increase quantal content of EPP as it does during the action of nifedipine or verapamil.

The quantal content of EPP did not significantly change during 60-90 min incubation of the nerve-muscle preparation in a bathing solution containing R 24571 (2 μM), a selective blocker of calmodulin. In control, the quantal content was 12±1, while R 24571 insignificantly decreased it to 11±1 ($p>0.05$, $n=43$). This negligible (if any) effect shows that calmodulin is not involved in Ca-dependent inhibition of secretion.

It is widely known that not only calmodulin, but also CaM KII plays an important role in modulation of presynaptic processes [6]. Therefore, we examined synaptic activity under conditions of CaM KII blockade. Incubation of muscle in solution with selective CaM KII blocker KN 62 (3 μM) for 60-90 min had no effects on the amplitude and quantal content of EPP in newly formed synapses: the mean EPP quantal content in the control and in the presence of KN 62 was 12±1 and 11±1, respectively ($p>0.05$, $n=41$). However, when the nerve-muscle preparation was exposed to nifedipine (it elevated the quantal content of EPP from the control value of 12±1 to 21±1), the subsequent addition of KN 62 to the bathing solution decreased EPP value to the control level of 13±2 ($p<0.05$, $n=74$). These data suggest that CaM KII facilitating secretion of Ach and generation of single EPP is present in newly formed synapses, but its activity is suppressed by calcium ions entering the presynaptic terminal via L-type Ca^{2+}-channels and can be observed only during blockade of this calcium current.

The inhibitory effects of CaM KII blockers were documented in the terminals of mature motor synapses of drosophila [13]. It is a common knowledge that activation of this enzyme can be induced by calcium donated from various sources, including inflow of these ions via L-type Ca^{2+}-channels in the central synapses [9]. In presynaptic terminals, possible substrates of CaM KII include synapsin and cytoskeleton protein involved in mobilization of vesicles at the actives zones, modulation of ionic channels, and other processes to be studied in future [3,5,7]. Our data are the first indication of the presence of CaM KII in newly formed synapses and ability of this enzyme

to facilitate secretion of neurotransmitter only during blockade of L-type Ca^{2+}-channels. It should be stressed that in mature central synapses, the presynaptic CaM KII can bilaterally control secretion of neurotransmitter in dependence of calcium level, the degree of enzyme autophosphorylation, and coupling of secretion with Ca^{2+}-dependent phosphatases [4]. Further studies should describe the targets and specificity of CaM KII activity in newly formed synapses. Probably, our experiments revealed a novel mechanism of Ca^{2+}-dependent inhibition of Ach secretion in newly formed synapses via calcium inhibition of the potentiating action of CaM KII on nerve-muscle transmission.

In the next experimental series, we examined possible involvement of PKC, another Ca-dependent enzyme, in Ca-dependent inhibition of neurotransmitter secretion in reinnervated synapses. To this end, we applied selective inhibitors of PKC chelerythrine (4 μM) or BIM (1 μM) to the nerve-muscle preparation.

Incubation with chelerythrine increased the quantal content of EPP from 12±1 to 18±2 ($p<0.05$, $n=45$). The effect developed during 10-15 min after application of this inhibitor and persisted at a stable level during subsequent 40-60 min. BIM rapidly elevated the mean amplitude of single EPP and increased the quantal content of EPP from the control value of 12±2 to 20±2 ($p<0.05$, $n=48$). Similar to chelerythrine, BIM induced facilitating effect as early as 10-15 min after the start of incubation. At the same time, PKC blockers produced no effect on the amplitude and frequency of mEPP. The increase in the quantal content of EPP induced by either PKC blocker was comparable to the facilitation produced by nifedipine (Fig. 1). It is noteworthy that similar to nifedipine, the tested PKC blockers produced no effect on the frequency of spontaneous secretion of Ach.

If Ca-dependent activation of PKC is really caused by entry of Ca^{2+} ions into presynaptic terminal via L-type Ca^{2+}-channels, the increment of quantal content of EPP should not be changed by blockade of these channels, if PKC was previously inactivated. Indeed, while chelerythrine (a PKC inhibitor) significantly elevated the quantal content of EPP from 12±1 to 18±2 ($p<0.05$), the subsequent application of nifedipine produced no significant change in EEP quantal content, which remained at the level of 17±2 ($p>0.05$, $n=78$, Fig. 2). These data suggest that in newly formed synapses, PKC responds to Ca^{2+} ions entering the terminal via L-type Ca^{2+}-channels, and PKC arousal is coupled with Ca^{2+}-dependent inhibition of neurotransmission secretion. Taking into consideration the fact, that chelerythrine did not affect spontaneous secretion similar to nifedipine [1], we hypothesized that PKC can modify the parameters and kinetics of presynaptic action potential inducing evoked release of Ach, specifically

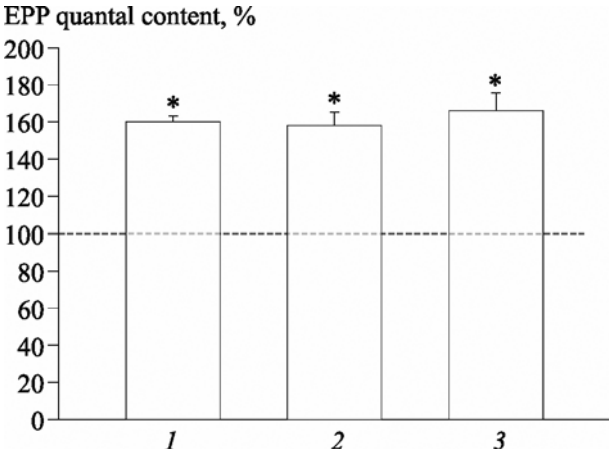

Fig. 1. Effect of L-type Ca^{2+}-channel blocker nifedipine (10 μM, *1*) and PKC blockers chelerythrine (4 μM, *2*) and BIM (1 μM, *3*) on quantal content of EPP in mice nerve-muscle preparation. The data are given in percentage to control (dash line). Here and in Figs. 2 and 3: *$p<0.05$ compared to the control.

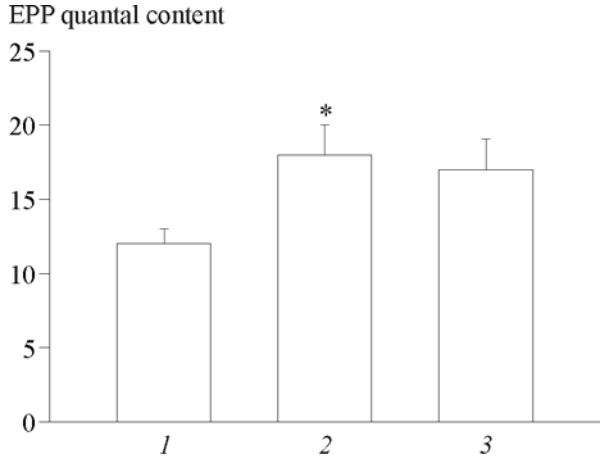

Fig. 2. Quantal content of EPP in control (*1*) and after application of chelerythrine (4 μM, *2*) or nifedipine (10 μM) added to chelerythrine (*3*). The data are given in absolute values.

those which determine the magnitude of the corresponding potassium current [13].

To test this hypothesis, the following experimental series examined the effects of blockade of voltage-gated K^+-channels and their relation to the effects produced by PKC blockers. The experiments showed that application of 4-aminopyridine (6 μM), a blocker of voltage-gated K^+-channels, induced a pronounced facilitation of nerve-muscle transmission. During 5-10 min, the mean values of quantal content of EPP increased from the control level of 19±2 to 30±2 ($p<0.05$, $n=54$). This effect persisted during entire length of the experiment (60-90 min). However, preliminary incubation of the muscle with chelerythrine completely prevented further increment of the quantal content of EPP induced by 4-aminopyridine (Fig. 3). The quantal content of EPP was 14±2 in the control, 20±2 ($p<0.05$)

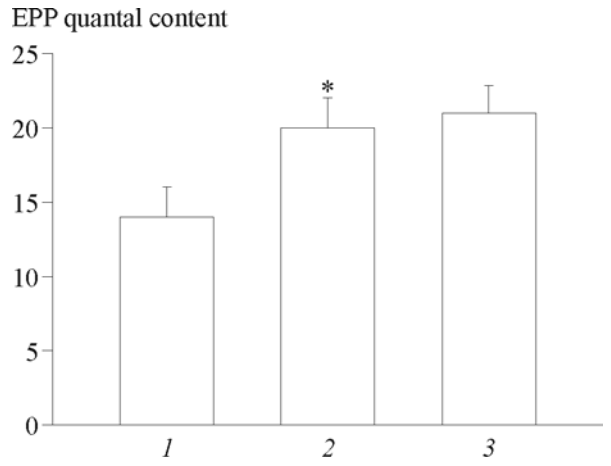

Fig. 3. Quantal content of EPP in control (*1*) and after application of chelerythrine (4 μM, *2*) or 4-aminopyridine (6 μM) added to chelerythrine (*3*). The data are given in absolute values.

under the action of chelerythrine ($p<0.05$), and 21±2 after addition of 4-aminopyridine to the bathing solution ($p>0.05$, $n=80$). Similarly, when both blockers were applied in the reverse order, the blockade of PKC with chelerythrine after application of 4-aminopyridine produced no additional increment in the amplitude and quantal content of EPP. In this case, the quantal content increased from the control level of 17±2 to 29±2 after application of 4-aminopyridine ($p<0.05$), although it remained at this increased level after the subsequent application of chelerythrine ($p>0.05$, $n=78$).

These data indicate functional coupling between Ca-dependent activity of PKC and elevation of potassium channel activity in the presynaptic terminal. Probably, a novel mechanism of Ca-dependent inhibition of Ach secretion is revealed, which increase potassium conductivity in the nerve terminal via PKC activation.

Recently, the data appeared on the effects of PKC inhibitors and activators on neurotransmitter release in mature and newly formed motor synapses. Specifically, PKC blockers facilitate the evoked secretion in the newly formed synapses developed during early ontogeny in mice [10]. The results of our examination of the effect of PKC blockers on Ach secretion in re-innervated synapses of mature mice agreed with p0ublished data [10]: structurally different PKC blockers elevated quantal content of EPP in the terminals of such synapses. Activation of PKC in the presynaptic terminals of reinnervated synapses by Ca^{2+} ions entering the terminals via L-type Ca^{2+}-channels is documented here for the first time. Similar phenomenon was described for chromaffin cells [8]. At present, several potential targets are described for presynaptic PKC, voltage-gated potassium channels included [12,14]. Our study shows that activity of presynaptic PKC can be coupled with potentiation of potassium channels.

On the whole, this study revealed a correlation between the presence or absence of Ca^{2+}-current entering the presynaptic terminals via L-type Ca^{2+}-channels and the effects of CaM KII and PKC blockers on evoked secretion of neurotransmitter. Our data substantiate the hypothesis that CaM KII and PKC are subjected to reciprocal influences from Ca^{2+} ions entering the presynaptic terminals via L-type Ca^{2+}-channels. These ions inhibit activity of CaM KII thereby moderating its ability to facilitate transmission. However, they also activate PKC and therefore enhance its ability to potentiate K^+-current and the work of presynaptic voltage-gated potassium channels. These previously unknown processes can underlie Ca-dependent inhibition of neurotransmitter release in newly formed synapses.

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