Involvement of Basal and Calcium-Activated Protein Kinase C in Neurotransmitter Secretion in Mouse Motor Synapses

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Blocker of presynaptic protein kinase C isoforms, GF109203X, reduced quantal content of single and rhythmic evoked end-plate potentials. The increase in quantal content of single potentials under the effect of 4- aminopyridine was neutralized by 75% under the effect of L-type Ca^{2+}-channel blocker nitrendipine and completely returned to the control level after protein kinase C inhibition with chelerythrine. Neither nitrendipine, nor GF109203X affected the potentiating effect of tetraethylammonium on quantal content of end-plate potentials. Thus, we discovered basal activity of presynaptic protein kinase C under normal conditions that is aimed at the maintenance of quantal content of evoked release. It has been concluded that 4-aminipyridine, but not tetraethylammonium, triggers Ca^{2+} entry into the terminal, which activates protein kinase C and enhanced the evoked acetylcholine release.

Key Words: *protein kinase C; L-type calcium channels; 4-animopyridine; tetraethylammonium; neuromuscular synapse*

The presence of a number of catalytically active protein kinase C (PKC) isoforms in the terminals of neuromuscular junctions in mice has been proven by biochemical methods [5]. However, the role of basal and Ca^{2+}-modulated PKC activity in the regulation of acethylcholine (ACh) secretion remains poorly studied. We and other authors have demonstrated that inhibition of synaptic PKC with chelerythrine, a wide-spectrum PKC blocker, had no effects on ACh secretion [2,9]. However, analysis with the use of more selective inhibitors of presynaptic PKC isoforms is required to conclude that basal PKC activity does not participate in ACh release, which had never been done before.

We have previously demonstrated that Ca^{2+} influx into the terminal via L-type Ca-channels induced by blockage of BK-type Ca-dependent K-channels results in selective Ca-dependent PKC activation and PKC-mediated facilitation of ACh secretion that can be completely abolished by PKC inhibitors [2]. It remains unclear, whether enhanced Ca^{2+} influx in case of blockage of voltage-dependent K-channels may result in selective activation of PKC and its participation in facilitation of ACh release.

The objectives of the study were: 1) to compare the effects of chelerythrine and GF109203X, a selective blocker of presynaptic PKC isoforms on neuromuscular synapse in mice; 2) to evaluate the possibility of Ca-dependent PKC activation and its role in facilitation of the transmission against the background of voltage-gated K-channel blockage with 4-aminopyridine (4-AP) and tetraethylammonium (TEA).

MATERIALS AND METHODS

Neurotransmitter release in motor synapses was studied on isolated "dissected" neuromuscular preparations of the diaphragm muscle (*m. diaphragma — n. phrenicus*) at 20°C [1]. The 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 (95% O_2, 5% CO_2) Lilly solution (pH 7.2-7.4) that contained (in mM): 135 NaCl, 4 KCl, 0.9 NaH_2PO_4,

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2 $CaCl_2$, 1 $MgCl_2$, 16.3 $NaHCO_3$, and 11 glucose. Simultaneous recording of miniature end-plate potentials (MEPP) and evoked end-plate potentials (EPP) was carried out using intracellular glass microelectrodes. The signals were recorded using an Axoclamp-2B amplifier (Axon Instruments) and inputted to a computer hard drive disk through DigiLine digitizer with Digiscope interface. The data were processed using MiniAnalysis software (Synaptosoft).

At least three neuromuscular preparations were used in each experimental series; under control conditions, MEPPs and EPPs were recorded at least in 5 different synapses and then the test compounds were added to the perfusion solution in a certain order and synaptic activity was recorded from different synapses. In addition to assessment of mean MEPP and EPP amplitudes, quantal spectrum of EPP was estimated (as the ratio of the mean corrected EPP amplitude to the mean MEPP) [1].

Significance of differences between samples was assessed by the Student *t* test and Mann-Whitney *U* test at significance level of 0.05 (*n* is the number of studied synapses).

RESULTS

In experimental series I, we studied the presynaptic effects of GF109203X, a selective blocker of calcium-dependent PKC isoforms (α, βI, βII, and γ). Mean MEPP for dissected diaphragmal muscle fibers was -35.26±1.02 mV (*n*=39) and remained unchanged after 30-60-min exposure to 0.1 μM GF109203X (-33.61±1.12 mV; *n*=43). No significant changes in spontaneous ACh secretion were revealed: mean MEPP amplitude was 0.74±0.04 mV in the control and 0.74±0.06 mV against the background of GF109203X. Considering evoked ACh release, GF109203X significantly reduced the amplitude of the first EPP in the burst by 25% from 31.59±3.00 mV in the control to 23.62±1.99 mV (*n*=43, $p<0.05$). In parallel with the amplitude, EPP quantal content decreased from 44.06±3.51 in the control to 32.98±1.88 ($p<0.05$). Suppression of EPP quantal content under conditions of PKC blockade with GF109203X was stable throughout the rhythmic burst (Fig. 1). This suggests that basal PKC located in the nerve terminals is involved in the maintenance of evoked neurotransmitter secretion in synapses, since its blockade reduces the release of both single and rhythmic evoked EPP.

In experimental series II we studied possible involvement of Ca-activated PKC in stimulation of ACh secretion under conditions of blockade of voltage-gated K-channels with 4-AP or TEA.

Application of 4-AP (100 μM) on the muscle was associated with 2-fold increase in the amplitude of single evoked EPP from 28.07±1.81 mV in the control (*n*=33) to 54.96±5.60 mV (*n*=31, $p<0.05$) over 30-60 min. In parallel, a 2-fold increase in EPP quantal content was observed ($p<0.05$; Fig. 2). The mean MEPP amplitude was not affected by 4-AP. Against the background of the 2-fold increase in EPP quantal content induced by 4-AP ($p<0.05$), subsequent application of chelerythrine (4 μM) resulted in reduction of EPP quantal content to virtually control level over 30 min (*n*=36; Fig. 2, *a*). It is indicative of possible involvement of PKC activity in potentiation of ACh release induced by 4-AP. It was necessary to test whether L-type Ca-channels in terminals can be activated against the background of 4-AP treatment? To this end, the selective L-type Ca-channel blocker nitrendipine (1 μM) was applied against the background of 4-AP exposure. Nitrendipine *per se*, as it was shown previously, did not alter EPP amplitude and quantal content in intact nerve-muscle preparations [2]. At the same time, the

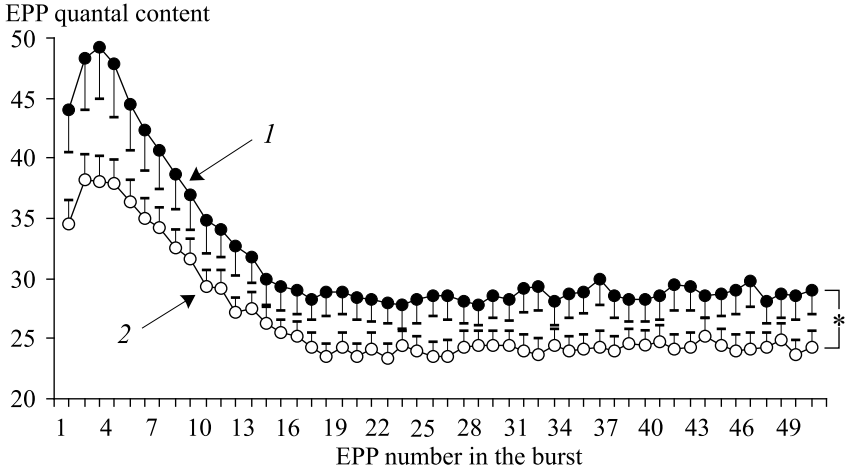

Fig. 1. Changes in EPP quantal content in a rhythmic burst with 50 Hz frequency in the control (*1*) and under the effect of GF109203X (0.1 μM, *2*). *$p<0.05$ in comparison with the control.

more than 2-fold increase in EPP amplitude and quantal content caused by 4-AP exposure appeared to be sensitive to the L-type Ca-channel blocker and was significantly reduced (by 70%) by subsequent 15-30-min exposure of the muscle to nitrendipine (Fig. 2, *b*). It suggests that 4-AP induces disinhibition of L-type Ca-channels and Ca influx in nerve terminals. The latter stimulates PKC that provides the increase in neurotransmitter release.

TEA (2 mM), similarly to 4-AP, induced a rapid 2-fold increase in EPP amplitude to 57.46±3.69 mV (n=19) in comparison with 28.77±3.43 mV in the control (n=19, p<0.05) and significantly increased EPP quantal content in comparison with the control (p<0.05; Fig. 3). Moreover, the effect of TEA (2 mM) on neuromuscular preparation was associated with significant

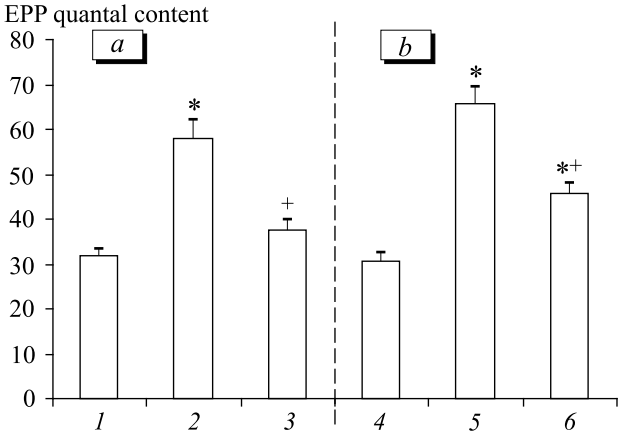

Fig. 2. Quantal content of single evoked EPP under the effect of 4-AP, chelerythrine, and nitrendipine. *a* and *b*: different experiments. *1*, *4*) control; *2*, *5*) 100 μM 4-AP; *3*) 4 μM chelerythrine against the background of 4-AP (100 μM); *6*) nitrendipine 1 μM against the background of 4-AP (100 μM). *p<0.05 in comparison with the control, +p<0.05 in comparison with 4-AP.

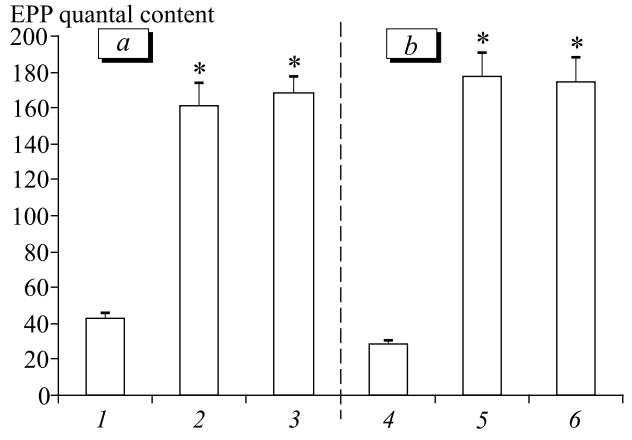

Fig. 3. Quantal content of single evoked EPP under the effect of TEA, nitrendipine, and GF109203X. *a* and *b*: different experiments. *1*, *4*) control; *2*, *5*) 2 mM TEA; *3*) 1 μM nitrendipine against the background of TEA (2 mM); *6*) 0.1 μM GF109203X against the background of TEA (2 mM). *p<0.05 in comparison with the control.

(p<0.05) reduction of MEPP amplitude to 0.38±0.03 mV *vs*. 0.66±0.06 mV in the control. It may be associated with known TEA ability to exert competitive inhibitory and channel-blocking effects on the muscle type of postsynaptic nicotinic receptors. Against the background of pronounced increase in EPP quantal content produced by TEA, subsequent application of nitrendipine (1 μM) to the muscle did not significantly changed EPP quantal content (Fig. 3, *a*). Therefore, no enhancement of Ca^{2+} influx into the terminal via L-type Ca-channels occurred under the effect of TEA.

The TEA-induced increase in EPP quantal content was not lowered by subsequent application of PKC blocker GF109203X (0.1 μM) on the nerve-muscle preparation. Thus, addition of 0.1 μM GF109203X induced no significant changes in MEPP amplitude (it was 0.20±0.03 and 0.23±0.02 mV against the background of GF109203X and TEA, respectively) and changes in evoked neurotransmitter secretion (EPP amplitude after exposure to PKC blocker against the background of TEA was 31.78±2.49 mV); no significant changes were observed in EPP quantal content (Fig. 3, *b*). Thus, in the case of muscle exposure to TEA, the increase in EPP quantal content appeared to be insensitive to both L-type Ca-channel blockade with nitrendipine and PKC inhibition with GF109203X (Fig. 3). This means that in contrast to 4-AP, TEA induced an increase in EPP quantal content that was not associated with Ca^{2+} influx activation via L-type Ca-channels and additional PKC activation.

Thus, our experiments demonstrated the capacity of GF109203X, a selective inhibitor of PKC isoforms, including α- and βI-isoforms detected in motor terminals of rodents [5], to inhibit the amplitude and quantal content of EPP during evoked rhythmic ACh secretion in mouse motor synapses. This distinguishes GF109203X from other previously used PKC blockers chelerythrine and calphostin C that do not alter ACh secretion [2,9]. The differences can be attributed to more efficient inhibition of the PKC isoforms located in the terminals with GF109203X. As a result, we succeeded to demonstrate that the described catalytic activity of basal PKC is aimed at the maintenance of evoked ACh secretion under normal conditions.

We also were the first to show that apart from basal activity, additional activation of Ca-dependent PKC by the Ca influx into the terminal is possible, which can substantially enhance ACh secretion; however this occurs only when external Ca enters into the terminal via L-, but not P/Q-type Ca-channels.

It is believed that lengthening of the second phase of presynaptic action potential in mouse motor nerve terminals with blocked voltage-gated K-channels with selective blocker TEA creates prerequisites for more prolonged Ca influx into the terminal via P/Q-type Ca-

channels, the main Ca input, located at active zones [6,8]. In addition, Ca^{2+} itself (without intermediator Ca-dependent enzymes, *e.g.* PKC) enhances CA-dependent neurotransmitter release. Our findings are in line with these considerations, because substantial increase in EPP quantal content induced by TEA was not altered by PKC inhibition or L-type Ca-channel blockage with nitrendipine. Thus, no activation of Ca-dependent PKC occurred upon the TEA-induced increase in Ca^{2+} entry via P/Q-type Ca-channels. As for 4-AP, despite its long-term usage as voltage-gated K-channel blocker similar to TEA, the actual spectrum and the mechanisms of its action on neuromuscular and other synapses are still not completely clear [4,7,8]. We found that 4-AP-induced increase in EPP quantal content could be reduced by 75% in the presence of nitrendipine. This suggests that the 2-fold increase in EPP quantal content against the background of 4-AP application is not related to its effect as terminal K-channel blocker that increases calcium influx via P/Q-channels (as it was in the case with TEA), but is explained by facilitation of latent voltage-gated L-type Ca-channel activity and calcium influx through them. Indeed, a pool of latent L-type Ca-channels was described in motor terminals that after disinhibition under the effect of blockers of Ca-dependent BK-type K-channels provide slow L-type Ca inward current that facilitates ACh release [1,2]. In the present study we demonstrated for the first time that 4-AP in a concentration of 100 μM exhibits the same property, *i.e.* disinhibits L-type Ca-channels and therefore enhances ACh release. The assumption that 4-AP not only blocks K-channel, but also directly facilitates Ca influx into the terminals was previously proposed by a number of authors during the analysis of 4-AP effects in low micromolar concentrations (4-AP in micromolar concentrations weakly blocks K-channels in terminals, but appreciably facilitates ACh secretion) [8]. Finally, analogous action mechanism of 4-AP effect, facilitation of L-type Ca influx, was directly described during testing of the effects of 4-AP in a concentration of 500 μM on central neurons and neuromuscular synapses [10]. Coupling between facilitation of Ca^{2+} influx via L-type Ca-channels and activation of Ca-dependent PKC in the terminals appeared to be an important feature of 4-AP effects. Chelerythrine completely blocked the 2-fold increase in EPP quantal content induced by 4-AP. Thus, irrespective of whether disinhibition and activation of Ca-channels and L-type currents are due to BK-type K-channel block (as we revealed earlier) or facilitating effect of 4-AP on the L-type Ca-channels (as was shown in this study), it is associated with activation of Ca-dependent PKC that provides the increase in evoked ACh release.

Apart from well-established opinion considering PKC activation with diacylglycerol in combination with calcium released from Ca-depot through channels of IP_3-receptors, some cases were described when local increase in intracellular calcium was sufficient for selective activation of latent pools of Ca-dependent PKC. It was proven by PKC activation upon Ca^{2+} entry via NMDA-channels of neurons [3]. Simulation of Ca-dependent PKC by calcium entry exactly and only via L- (but not P/Q) type Ca-channels and resultant steady 2-fold increase on EPP quantal content also support this assumption. The exact mechanisms underlying selective activation of PKC by calcium entering into the terminals and its facilitating effect on neurotransmitter secretion and what are the targets of PKC activity in motor synapse terminals are to be investigated.

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