PHYSIOLOGY

Facilitation of Neurotransmitter Release in Mouse Motor Synapses in Different Modes of Protein Kinase C Activation

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 4, pp. 400-404, April, 2012
Original article submitted June 15, 2010

Protein kinase C blocker chelerythrine prevented the increase in quantal content of single and rhythmic evoked end-plate potentials after disinhibition of L-type Ca^{2+}-channels with paxillin. Phorbol ester increased quantal content of single end-plate potentials and changed rhythmic activity of mouse motor synapses. The effects of phorbol ester were to a great extent neutralized by L-type Ca^{2+}-channel blocker nitrendipine and were completely abolished by K^+-channel blocker 4-aminopyridine. Thus, we discovered different facilitations of transmission after protein kinase C activation with calcium current through L-type channels and with phorbol ester.

Key Words: *protein kinase C; L-type calcium channels; phorbol ester; 4-aminopyridine; neuromuscular synapse*

Protein kinase C (PKC) belongs to the group of serine-threonine kinases involved in modulation of synapse functioning [14]. Expression of PKC isoforms activated with participation of diacylglycerol and calcium ions was observed in nerve endings in mouse skeletal muscles [4]. Specific ways of presynaptic PKC activation followed by regulatory influences on neurotransmitter secretion in motor synapses remain unclear.

The objective of this study was to detect and compare changes in neurotransmitter secretion in PKC activation by calcium ions entering the ending through different types of voltage-gated Ca^{2+}-channels and by using diacylglycerol analogue, phorbol ester.

MATERIALS AND METHODS

Experiments were carried out on "dissected" isolated neuromuscular preparations of the diaphragm muscle (*m. diaphragma–n. phrenicus*) at 20°C [2,6]. 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) 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. Miniature end-plate potentials (MEPP) and evoked end-plate potentials were intracellularly recorded via microelectrodes filled with 2.5 M KCl (15 MΩ electrode tip resistance). 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 MiniAnalysis software (Synaptosoft). In control, MEPPs and EPPs were recorded at least in 5 different synapses, then the test compounds were added to the perfusion solution in a certain order and synaptic activity was recorded from different synapses for 1-1.5 h. At least 3 neuromuscular preparations were used in each experimental series.

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In single nerve stimulation regimen (suprathreshold impulses 0.1 msec in duration and 0.3 Hz in frequency), in addition to assessment of mean MEPP and EPP amplitudes, EPP quantal spectrum was estimated as the ratio of the mean corrected EPP amplitude to the mean MEPP [2,6]. In the study of rhythmic synaptic activity, the intensity of initial transmission facilitation period and plateau level in EPP burst were also calculated in addition to EPP amplitude assessment. To this end, EPP amplitudes in the burst were presented in relative units (in % from the first EPP in burst). Significance of differences between the samples was assessed by Student's *t* test and Mann–Whitney test at 0.05 significance level.

RESULTS

In I experimental series we found that PKC blocker chelerythrine (4 μM) applied for 30-60 min had no effect on the mean membrane potential (MP) of dissected muscle fibers, which was -38.0±1.6 mV (*n*=25) in control and -39.5±1.8 mV with chelerythrine (*n*=55), and also had no effect on mean MEPP amplitude (1.07±0.05 mV in control and 1.03±0.06 mV for chelerythrine).

Under conditions of evoked activity, presynaptic action potential in mouse motor nerve endings is known to be associated with calcium influx into the nerve ending through voltage-gated P/Q-type Ca^{2+}-channels [12]. Changes in quantal content of single EPPs before and after chelerythrine application on the muscle were investigated to test the possibility of this Ca^{2+}-current involvement in PKC activation and its participation in acetylcholine secretion. We found that mean EPP amplitude (22.67±0.10 mV in control) and quantal content of single evoked EPPs (22.7±1.06 in control, *n*=25) were not significantly changed under the influence of chelerythrine and were 22.32±0.63 mV and 20.6±0.76, respectively (*n*=55).

Registration of short rhythmic EPP bursts generated at a frequency of 50 Hz showed typical changes in EPP amplitude in the course of the burst: transient increase in amplitude and quantal EPP content (initial facilitation) followed by depression of transmission characterized by a decrease in EPP amplitude. At the end of the burst EPP amplitude was stable (so called plateau) but lower than the amplitude of the first EPP. Chelerythrine application for 40-60 min induced no significant changes in burst pattern.

Alongside with P/Q-type Ca^{2+}-channels, slow voltage-gated L-type Ca^{2+}-channels were discovered in motor nerve endings. Under normal conditions, they are in a latent state and not involved in regulation of neurotransmitter secretion in intact mature synapses [2,13]. However, under certain conditions, for example against the background of blockade of BK-type calcium-gated potassium channels, disinhibition of L-type Ca^{2+}-cannels occurs and results in a significant increase in evoked neurotransmitter release [2,13]. The possibility of PKC activation and influence on secretion under conditions of calcium influx into the nerve ending via L-type channels remained poorly studied.

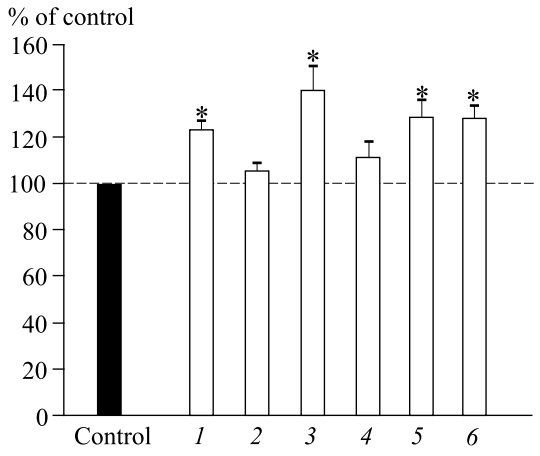

Fig. 1. Quantal content of single evoked EPPs: under the effect of paxillin 5 μM (*1*); chelerythrine (4 μM) against the background of paxillin 5 μM (*2*); PMA 10 nM (*3*); nitrendipine 1 μM against the background of PMA (*4*); 4-aminopyridin 100 μM (*5*); PMA 10 nM against the background of 4-aminopyridine (*6*). Here and in Fig. 2, 3: **p*<0.05 in comparison with the control.

To elucidate this issue, the next series was carried out by application of selective BL-channel blocker paxillin (5 μM) on isolated diaphragm preparation. Thereafter, effects of PKC blocker chelerythrine were tested against the background of paxillin influence. Resting potential was not affected by paxillin (5 μM) or chelerythrine (4 μM) against the background of

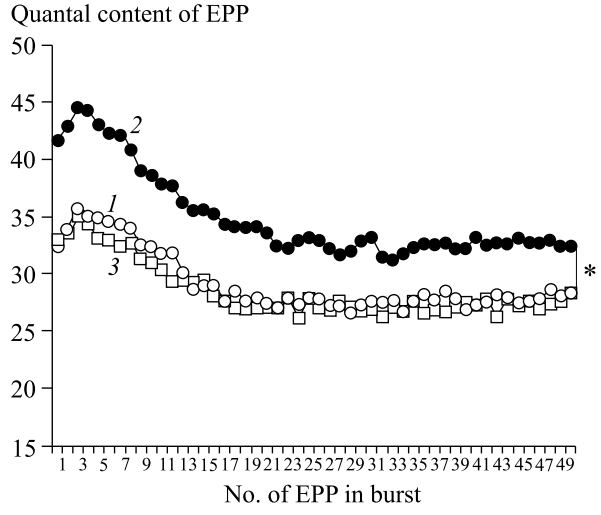

Fig. 2. Changes in EPP burst pattern in rhythmic synaptic activity with 50 Hz frequency. *1*) control; *2*) paxillin (5 μM); *3*) chelerythrine (4 μM) against the background of paxillin 5 μM.

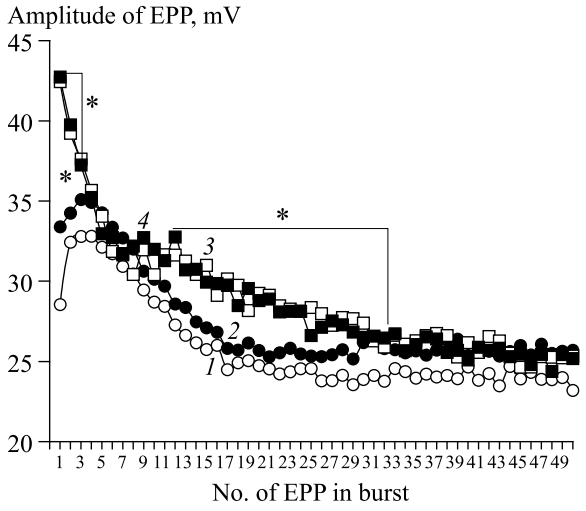

Fig. 3. Changes in EPP burst pattern in rhythmic synaptic activity with 50 Hz frequency. *1*) control; *2*) PMA (10 nM); *3*) 4-aminopyridine (100 μM); *4*) PMA 10 nM against the background of 4-aminopyridine 100 μM.

paxillin: the mean MP was -38±1.62 mV in the control (n=15), -39.25±2.12 mV after paxillin application (n=15), and 39.5±1.76 mV after paxillin application in the presence of chelerythrine (n=16). Paxillin added to bathing solution in a concentration of 5 μM for 30 min significantly (p<0.05) increased in amplitude (by 36.8%) and quantal content (by 23%) of single EPPs (Fig. 1). Mean amplitude and quantal EPP content in the control were 19.8±1.5 mV and 16.09±0.53, respectively; after application of paxillin the corresponding values were 26.88±2.25 mV and 19.77±0.64 for. The increase in quantal EPP content was also uniform against the background of paxillin and steadily persisted throughout the whole rhythmic EPP burst (Fig. 2).

PKC blocker chelerythrine applied on the muscle against the background of paxillin reduced quantal EPP content to the control values both for single EPPs and throughout the burst consisting of 50 EPPs (Fig. 1, 2). It means that PKC blockade eliminates paxillin facilitation effects on the neurotransmitter release. Therefore it suggests that L-type Ca^{2+}-channel disinhibition and calcium influx into nerve ending are associated with activation of PKC that is involved in facilitation of both single and rhythmically generated EPPs.

In the next experimental series, PKC was activated using phorbol ester, 4-beta-phorbol-12-myristate-13-acetate (PMA, 10 nM). PMA significantly increased quantal content of single EPPs by 25-40%. This increase was comparable with that observed during Ca^{2+}-dependent PKC activation (under conditions of L-type Ca^{2+}-channel disinhibition with paxillin; Fig. 1). However, the pattern of changes in neurotransmitter release during rhythmic burst in the presence of PMA differed from that observed with paxillin (Fig. 3).

Thus, during PKC activation with PMA, despite increased amplitude of the first EPP in the burst under the influence of PMA, synapse was unable to maintain increased release throughout the whole burst, as it was demonstrated in PKC activation using Ca^{2+}-current through L-type channels. Pronounced transmission depression neutralizing at the end of the burst initially increased EPP level was observed (Fig. 3). Such pattern of burst activity is quite comparable with that observed in 4-aminopyridin block of voltage-gated K^+- delayed rectifier channels (K_v-channels) in mammal motor synapses [9]. Therefore we assumed that enzyme inhibition of the voltage-gated K^+-channels of nerve ending may serve as a mechanism of PKC facilitation of neurotransmitter secretion (in case of its activation by PMA).

To test this hypothesis, we applied 4-aminopyridin on the muscle at the concentration 100 μM. It resulted in significant (by 28.5%) increase of mean values of quantal content of single EPPs: from 21.46±1.1 in the control to 27.57±1.6 for 4-aminopyridine (p<0.05; Fig. 1). PMA application (10 nM) against the background of 4-aminopyridine resulted in no further significant changes in EPP quantal content (27.58±1.1; Fig. 1).

In the study of rhythmic synaptic activity, application of 100 μM 4-aminopyridine resulted in changes of the burst pattern similar to PMA effects: despite the level of the first EPPs in the burst of 50 signals increased almost by one third, the initial facilitation was absent and transmission depression developed immediately, so by the end of the burst, at the plateau phase, the amplitude decreased to control level. PMA 10 nM administration against the background of 4-aminopyridine resulted in no additional significant changes in burst activity pattern (Fig. 3). It means that under conditions of voltage-gated K^+-channel blockade with 4-aminopyridine, PKC activation with PMA cannot produce transmission facilitation. Therefore, it cannot be excluded that synaptic transmission facilitation, produced by PMA-activated PKC, is associated with inhibition of voltage-gated K^+-channels of nerve endings.

Facilitation of neurotransmitter secretion under PMA activation of PKC in motor synapses may be associated with the effects of this enzyme on calcium influx into the nerve ending via L-type Ca^{2+}-channels [3]. To examine this possibility, we investigated how the increase in EPP quantal content under PMA effect will be affected in the presence of selective blocker of L-type Ca^{2+}-channels nitrendipine (1 мкМ). Nitrendipine *per se* induced no changes in quantal content of single EPPs in intact synapses, but reduced significantly potentiation of quantal EPP content produced by PMA: from 41.09±3.20 (n=17) to 32.75±2.02 (n=15, p<0.05; Fig. 1).

Thus, under conditions of evoked activity of mouse motor synapses, when neurotransmitter release occurs due to calcium ion influx into the nerve ending via P/Q-type Ca^{2+}-channels [12], there were found no Ca^{2+}-dependent PKC activation, capable to exert effect on neurotransmitter release. That is consistent with the data of other authors, who observed no changes in single EPPs against the background of PKC blockers in neuromuscular synapses [3,11]. Alongside with that we were first who discovered that disinhibition of L-type Ca^{2+}-channels is associated with increase in quantal EPP content and transmission facilitation due to PKC activation, since it can be blocked by chelerythrine. It is important to note, that facilitation of transmission was also present in the period of burst activity. We also observed transmission facilitation in another way of PKC activation using PMA, as it was described also by other authors [3,11]. However, we showed for the first time that PMA-induced transmission facilitation in not maintained in the course of rhythmic activity and rapidly depletes as soon as at the end of short burst of 50 EPPs. Thus, different ways of PKC activation is associated with different types of PKC facilitating effects on synaptic transmission. It may be associated with differential involvement of different PKC isoforms or with the effect of one PKC isoform on different targets depending on the character of its activation. K+-channels are known to be PKC targets when it activated by calcium ions, entering through L-type channels in adrenal chromaffin cells and newly formed motor synapses [1,8]. Targets of Ca^{2+}-activated PKC in mature motor synapses are to be established. It is believed that in the case of PMA activation of PKC, transmission facilitation may be the consequence of direct PMA action on the Munc-13 proteins, or of the effects of activated PKC on some exocytosis proteins, particularly SNAP-25 and Munc-18 [7], or nerve ending Ca^{2+}-channels [3]. We also established that effects of activated PKC against the background of PMA may be directed towards stimulation of influx Ca^{2+}-current into the nerve ending sensitive to the nitrendipine blocking effect. In addition, nitrendipine only partially neutralized facilitating PMA effects on neurotransmitter release. In its turn, 4-aminopyridine, as we showed for the first time, was able for complete prevention of PMA facilitation effects on single and rhythmic neurotransmitter secretion in motor synapses. It suggests that voltage-gated K+-channels of nerve endings may appear as more important target of PKC facilitation effects on neurotransmitter secretion (when it's activated by PMA). Similar PKC ability to modulate function of presynaptic K^+-channels sensitive to 4-aminopyridine was described for central synapses [5].

Thus, activation of presynaptic PKC occurs under the influence of both PMA and calcium entering the nerve ending via L-type Ca^{2+}-channels and leads to different in character and intensity transmission facilitation. Discovered variations in facilitation may be stipulated by different ways of PKC activation and/or by different targets of its action in mouse motor merve endings.

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