Calcium Inflow-Dependent Protein Kinase C Activity Is Involved in the Modulation of Transmitter Release in the Neuromuscular Junction of the Adult Rat

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ABSTRACT Using intracellular recording, we studied how protein kinase C activity affected miniature endplate potentials (MEPPs) and evoked endplate potentials (EPPs) in the neuromuscular junctions of the levator auris longus muscle from adult rats. The protein kinase C activator phorbol 12-myristate 13-acetate (PMA, 10 nM) increased the quantal content by ~150% (P < 0.05). On the other hand, the quantal content decreased by ~40% (P < 0.05) for all the protein kinase C inhibitors tested (Calphostin-C, 10 μM; Chelerythrine, 1 μM; Staurosporine, 200 nM). These changes in acetylcholine release were maintained at plateau for 1 to 7 h. Moreover, none of the protein kinase C activators or inhibitors used could modify the spontaneous MEPP mean size (P > 0.05). We reduced the calcium influx in nerve terminals using the P/Q-type channel blocker ω-Aga-IVA (100 nM) or with 5 mM magnesium in physiological solution. In neither situation was the quantal content modified by PMA or by CaC. However, when high Ca²⁺ (5 mM) was added to a preparation that was previously blocked with ω-Aga-IVA, PMA and CaC had their full effect. We conclude that under physiological conditions PKC is dependent on the calcium inflow during evoked activity and works near the maximum rate at normal external calcium concentration. Synapse 57:76–84, 2005.

INTRODUCTION

A variety of extracellular factors and intracellular signal transduction cascades are involved in regulating synaptic function by protein phosphorylation. For instance, an extensive literature documents the regulation of ligand-gated ion channels (Nelson et al., 2003; Swope et al., 1999) and transmitter release (Byrne and Kandel, 1996; Nishizuka, 1992; Tanaka and Nishizuka, 1994) by serine threonine kinases, protein kinase C (PKC), and protein kinase A (PKA).

Synaptic activity depends on the influx of calcium from the external media via presynaptic voltage-dependent calcium channels (VDCCs) (Katz and Miledi, 1970). A presynaptic calcium-dependent “conventional” PKC isoform (cPKC) may contribute to the modulation of transmitter release. Activation of this enzyme in neurons is associated with the modulation of ion channels, desensitization of receptors, and enhancement of transmitter exocytosis (Tanaka and Nishizuka, 1994). All these processes are involved in the modulation of activity and efficacy in a variety of synapses. However, even in the paradigmatic frog neuromuscular junction (NMJ) the link between PKC activity and acetylcholine (ACh) release is not fully resolved. PKC inhibition seems not to affect tonic release or release increased by phorbol esters like phorbol 12-myristate 13-acetate (PMA, the most common PKC activator in experimental designs) (Searl and Silinsky, 1998). Moreover, PMA seems to have some additional direct effect on release in the frog (Searl and Silinsky, 1998) and hippocampal cultured cells (Waters and Smith, 2000).

On the other hand, in relation with the neurotransmission-related targets of PKC activity, the involvement of previously inactive, PKC-recruited L-type VDCC has been suggested both in frog (this channel...
is the functional one in amphibian motor nerve terminals (MNTs) (Arenson and Evans, 2001) and hippocampal neurons (L channel is not linked to release in these cells) (Waters and Smith, 2000). It has also been reported that masked L-type VDCC do not normally couple to release in the mouse (Urbano et al., 2002). In this study, therefore, we investigated the relationship between activity-dependent presynaptic calcium inflow, VDCCs, PKC activity, and transmitter release in the NMJs of the adult rat.

The main finding of this study is that PKC is involved in the modulation of neurotransmitter release at the rat levator auris longus (LAL) NMJ. The PKC action is dependent on the calcium inflow through the P/Q-type VDCC during evoked activity and works near the maximum rate at normal external calcium concentration.

**MATERIALS AND METHODS**

**Animals**

Experiments were performed on the LAL muscle of adult Sprague-Dawley rats (30–40 days postnatal; Crì́ffà, Barcelona, Spain). The rats were cared for in accordance with the guidelines of the European Community’s Council Directive of November 1986 (86/609/EEC) for the humane treatment of laboratory animals. The animals were anesthetized with 2% tribromoethanol (0.15 ml /10 g body weight, IP).

**Electrophysiological recordings**

The LAL muscle with its nerve supply was excised and dissected on a Sylgard-coated Petri dish containing normal Ringer’s solution (in mM): NaCl, 137; KCl, 5; CaCl₂, 2; MgSO₄, 1; NaHCO₃, 12; Na₂HPO₄, 1; and glucose 11, continuously bubbled with 95% O₂/5% CO₂. The preparation was then transferred to a recording chamber of 1.5 ml. Experiments were performed at room temperature (22–25°C). The bath temperature was monitored during experiments (23.4°C ± 1.7, Digital Thermometer TMP 812, Letica, Barcelona, Spain).

Spontaneous miniature endplate potentials (MEPPs) and evoked endplate potentials (EPPs) were recorded intracellularly with conventional glass microelectrodes filled with 3M KCl (resistance: 20–40 MO). Recording electrodes were connected to an amplifier (Tecktronics, AMS02) and a distant Ag-AgCl electrode connected to the bath solution via an agar bridge (agar 3.5% in 137 mM NaCl) was used as reference. The signals were digitized (DIGIDATA 1322A Interface, Axon Instruments, Burlingame, CA), stored, and computer-analyzed. The software Axoscope 9.0 (Axon Instruments) was used for data acquisition and analysis.

We analyzed concentration–response curves in which MEPPs (frequency and amplitude) and quantal content of the EPPs are plotted as a function of phorbol 12-myristate 13-acetate (PMA), Calphostin C (CaC), Chelerythrine (CEL), and Staurosporine (STP) concentrations. Our most important consideration was to discard the postsynaptic effect of the drugs, so when needed we chose the drug concentration that did not change the size of the MEPPs.

During EPP recordings, muscle contraction was prevented by d-tubocurarine (dTTC; 0.7–1.2 μM). In some experiments (see Fig. 4) we used a high-Mg²⁺ solution (5 mM), so dTTC was not needed. This salt and the others were of analytical grade and purchased from Sigma (St. Louis, MO). After a muscle fiber had been impaled the nerve was continuously stimulated (70 stimuli at 0.5 Hz) using two platinum electrodes coupled to a pulse generator associated with a stimulus isolation unit. We recorded the last 50 EPPs. Records were rejected if the membrane potential (Vm) was <−45 mV or if this fell by more than 5 mV during the recording period. No correction for nonlinear summation was made, as dTTC concentration was adjusted to obtain EPPs of less than 4 mV (McLachlan and Martin, 1981). The high Mg²⁺ solution prevented muscle contraction and the EPPs obtained were less than 4 mV. The mean quantal content (mᵥ) of the evoked response was evaluated by the coefficient of variation method, calculated as:

\[ mᵥ = \frac{(V_{EPP})^2}{(S_{EPP})^2 - (S_{noise})^2}, \]

where \( V_{EPP} \) is the mean amplitude of the EPP, and \( S_{EPP} \) and \( S_{noise} \) are the standard deviation of the recorded EPP amplitudes and the standard deviation of the noise, respectively (Hubbard, 1969). The mean amplitude per fiber (EPPs and MEPPs) was calculated and corrected by assuming a membrane potential of ~80 mV. The frequency with which MEPPs appeared was counted over 1-min periods.

We studied a minimum of 15 fibers per muscle and usually a minimum of five muscles in each type of experiment. In the single-fiber experiments (time course of the effect of drugs on EPPs of the same fiber permanently impaled), the drug/s were added to the...
bathing solution and the EPPs were recorded as previously described every 15 min for a minimum of 60 min.

**Statistical procedure**

Values are expressed as means ± SEM. Percentage change was defined as: [final value divided by the initial value minus one] times 100. We used a one-way analysis of variance (ANOVA) to evaluate differences between groups and the Bonferroni test for multiple comparisons. When differences were evaluated only between two groups, we used two-tailed Welch’s *t*-test (for unpaired values and not assuming equal variances). Differences were considered significant at *P* < 0.05.

**Chemicals**

Working solutions of the drugs that modulate PKC activity: PMA, 10 nM; CaC, 10 μM; Chelerythrine, 1 μM; Staurosporine, 200 nM.

**Calcium channel blockers**

Nitrendipine (Research Biochemicals, Natick, MA) was made up as a 50-mM stock solution in DMSO, stored at 4 °C, and protected from light. Experiments in the presence of Nitrendipine were carried out in the absence of direct illumination. The toxins ω-Conotoxin GVIA (ω-CgTX-GVIA) and ω-Agatoxin IVA (ω-Aga-IVA) were purchased from Research Biochemicals. Controls and toxin-treated muscles were assayed in the presence of 0.01% BSA (Sigma). Working solutions: Nitrendipine, 1 μM; ω-Aga-IVA, 100 nM; ω-CgTX-GVIA, 1 μM.

The final DMSO concentration in control and drug-treated preparations (Nitrendipine, PMA, CaC, Chelerythrine, and Staurosporine) was 0.1% (v/v). In control experiments this concentration of DMSO did not affect any of the studied parameters (data not shown).

**RESULTS**

**PKC activity and transmitter release in NMJs**

In Figure 1, which shows rough concentration–response curves, MEPP frequency and quantal content of the EPPs (Fig. 1A), and MEPP amplitude (Fig. 1B) are plotted as a function of phorbol 12-myristate 13-acetate (PMA, a PKC activator) and Calphostin-C (CaC, a PKC inhibitor) concentrations (after 1 h incubation). We tried to determine a baseline concentration of PMA and CaC to use in this study. In no case was a change in the spontaneous MEPP amplitude observed, so a significant postsynaptic modification of the post-junctional AChRs by these drugs at the time-scale (see Fig. 2) and concentrations used (Fig. 1B) in the present experiments is not likely. Specifically, at the end of the longest intracellular recording experiments with PMA or CaC (about 7–9 h, although usually the presence of these drugs in the experiments is less than 4 h), the mean size of the MEPPs changed by less than 5% (*P* > 0.05, Fig. 2). Furthermore, in our conditions incubation with PMA or CaC did not change muscle cell membrane potential in any case (% of variation: 6.98 ± 2.60% for PMA and 2.81 ± 4.16% for Calphostin C; in both cases *P* > 0.05 after 7 h of incubation).

Figure 1A also shows that PMA and CaC both increased MEPP frequency, although with different potency. PMA increased MEPP frequency in a relatively stable manner (about 150–220%) in the 10–100 nM range but micromolar concentrations accelerated MEPPs further. However, only the highest CaC concentration tested (25 μM) increased MEPP frequency significantly (roughly 80%). This may indicate the existence of a PKC-independent effect of one of these drugs on spontaneous release. When required in this study, therefore, we used the lowest concentration of these drugs to reproduce a consistent effect on evoked ACh release (PMA, 10 nM; CaC, 10 μM). At these concentrations only PMA also affected MEPP frequency (Figs. 1A, 2).
CALCIUM-DEPENDENT PKC ACTIVITY IN ACh RELEASE

Fig. 2. Time course of the PMA and CaC effect on quantal content and MEPP frequency and amplitude. Values are mean ± SEM. For each kind of experiment n = 5 muscles and a minimum of 15 fibers per muscle. *P < 0.05 vs. control (time 0). MEPP frequency and quantal content were not modified for up to 7 h of normal Ringer’s incubation (% change: 16.42 ± 5.25 and 14.58 ± 9.69, respectively; in both cases: P > 0.05, n = 3 muscles, minimum 15 fibers per muscle). The top of the figure also shows representative superimposed traces of recorded EPPs and MEPPs before (0 min) and after 60 min of incubation with PMA (left) or CaC (right). Stimulation artifacts are reduced for clarity. Vertical bars: 2 mV for EPPs and 20 ms for MEPPs. Horizontal bars: 2 ms for EPPs and 20 ms for MEPPs.

Figure 2 shows the time courses of the PMA and CaC effects on spontaneous and evoked ACh release. We can see that the time courses of PMA and CaC actions on quantal content are similar (although in opposite directions). We found that PMA strongly stimulated evoked ACh secretion (largely doubling it, ~150%), whereas CaC reduced it (by about a half, ~45%). We observed no significant changes in threshold (% change: 4.30 ± 1.12% induced by CaC and 6.28 ± 1.43% by PMA) or latency of the EPPs (% change: 3.89 ± 0.7% induced by CaC and 4.03 ± 0.47% by PMA; in all cases, P > 0.05). The changes in evoked transmitter release induced by PMA and CaC began 15–30 min after the drug was applied to the bath. Moreover, the changes could not be reversed after washing out with normal Ringer’s perfusion for 1 h, at least after the first hour of incubation with the corresponding drug (after PMA incubation: 12.78 ± 1.67% of recovery in normal Ringer’s; after CaC incubation: 13.08 ± 2.1% of recovery; in both cases P > 0.05). At the top of Figure 2 we can also see superimposed raw data of EPPs and MEPPs after 1 h of incubation with PMA or CaC.

Interestingly, in previous studies we and other authors have demonstrated a time- and dose-dependent downregulation of PKC activity by PMA doses ≥100 nM (Girard and Kuo, 1990; Lanuza et al., 2000, 2002). In previous in vitro experiments (Lanuza et al., 2000) we found that 100 nM PMA produced a downregulation of the PKC theta isoform after 4 h of treatment in cultured myotubes, while 10 nM PMA had no such regulatory effect. In our present study, neither the PMA dose (10 nM) nor the time of incubation (generally less than 4 h) allowed downregulation of PKC activity. However, in some experiments of long duration (~9 h), some points of the dose–response curve were performed at concentrations ≥100 nM PMA (Fig. 1) but no downregulation (of the quantal content increase) was observed. This seems to indicate that some of the PMA effect may be PKC-independent because there was no downregulation of the ACh release mechanism at the highest doses of PMA. To verify the specificity of the PMA effect, we performed experiments with 4 α-phorbol 12,13-didecanoate, which is a biologically inactive phorbol. Incubation with 10 and 100 nM concentrations of this reagent did not modify the control values of the transmitter release parameters (data not shown). Additionally, to evaluate the specificity of the CaC-induced PKC inhibition we assayed the selective PKC inhibitor Chelerythrine (CEL, 1 μM) under the same conditions as for CaC and found a quantal content reduction of 46.36 ± 1.65% (P < 0.005) after 1 h of incubation. The respective effects of CaC and CEL on quantal content were not different (P > 0.05). Concentration–response curves in which MEPPs (frequency and amplitude) are plotted as a function of the drug concentration indicate that these spontaneous release parameters were not affected by CEL concentrations below 1 μM (data not shown). Moreover, Staurosporine (STP, 200 nM, a non-selective PKC inhibitor that acts at the ATP binding site on PKC) can almost exactly reproduce the CaC and CEL effect on quantal content by reducing it by 43.54 ± 1.35% (P < 0.005).

In some experiments we performed a pretreatment with PMA (10 nM, 1 h) before a second incubation with CaC (10 μM, an additional hour) and vice versa (Fig. 3). Figure 3A shows raw data of the changes in EPP size when a PMA- or CaC-preincubated muscle was then incubated with the other drug. Figure 3B shows representative examples of the time course of the changes in quantal content in the same muscle fibers during the consecutive incubation with the two PKC modulators. In these examples an ~130% increase in quantal content produced by PMA was then reduced to half (~48%) by CaC. Also, an ~38% CaC-induced reduction in quantal content was almost reversed by an ~127% increase produced by the con-
secutive action of PMA. Figure 3C shows that after preincubation with PMA (increase in transmitter release of ~150%), CaC made (in time course and magnitude) its inhibitory effect as normal (a mean quantal content reduction of 38.71 ± 6.90%; P < 0.05). This reduction is not different from that observed with CaC incubation alone (P > 0.05). After preincubation with CaC (40% release reduction), PMA action was not fully prevented. PMA increases release but not as effectively as if no there were no CaC preincubation. We observed a mean quantal content increase of 107 ± 12%, which was different from the quantal content after CaC pre-incubation (P < 0.05) but also different from the quantal content increase observed with PMA incubation alone (P < 0.05). Therefore, only some of the PMA effect on release could be prevented by preincubation with CaC. We also found that after preincubation with CEL (which reduces quantal content), CaC could not make its blocking effect on release (% of CaC-induced quantal content change: 2.49 ± 5.25%, P > 0.05; see also Fig. 3C).

Calcium ions and VDCC involvement in the PMA- and CaC-induced neurotransmission changes in the adult LAL muscle

We studied the involvement of calcium ions and VDCCs in the PMA- and CaC-induced changes in transmitter release (Fig. 4). We performed experiments in which we incubated with PMA or CaC in the presence of high Ca²⁺ (Fig. 4A). In preliminary experiments (see also Santafe et al., 2003) we observed that in the presence of 5 mM Ca²⁺, the mean EPP amplitude was higher than in normal Ringer’s (% enhancement: 124.90 ± 3.11%, P < 0.05, n = 5 muscles, minimum 15 fibers per muscle). Because of the increased electrochemical gradient for this ion, this indicates that there were increases in calcium inflow and membrane input resistance. Under these conditions, however, CaC and PMA carried out their transmitter release modifications as normal (% of CaC-induced release reduction: 35.07 ± 1.70%; % of PMA-induced release enhancement: 163.07 ± 22.26%; in both cases, P > 0.05 with respect to the corresponding control, n = 3 muscles, minimum 15 fibers per muscle). In another set of experiments we incubated with PMA or CaC in high magnesium (5 mM) media. We found that in this condition PMA could not increase quantal content (% variation with respect to the control: 1.99 ± 10.30%, P > 0.05) and CaC could not inhibit it (% variation with respect to the control: 2.49 ± 5.25%, P > 0.05) and CaC could not inhibit it (% variation with respect to the control: 2.49 ± 5.25%, P > 0.05) and CaC could not inhibit it (% variation with respect to the control: 2.49 ± 5.25%, P > 0.05) and CaC could not inhibit it (% variation with respect to the control: 2.49 ± 5.25%, P > 0.05) and CaC could not inhibit it (% variation with respect to the control: 2.49 ± 5.25%, P > 0.05).
PKC activity modulation. Values are mean ± SEM. In all bars n = 5 muscles and a minimum of 15 fibers per muscle. *P < 0.05 vs. the corresponding control.

Representative examples of raw data of the effect of CaC on EPPs in the presence of high calcium or high magnesium. Each trace represents an average of 10 EPPs recorded. Stimulation artifacts are reduced for clarity. The vertical bars indicate 2 mV and horizontal bars 2 ms.

In the adult LAL endplates, the P/Q-type channel blocker ω-Aga-IVA (100 nM) significantly reduced the EPP amplitude (~75%, P < 0.05), whereas the N-type blocker ω-CgTx-GVIA and the L-type blocker Nitrendipine did not significantly reduce transmitter release (see Santafé et al., 2001). Previous incubation with ω-Aga-IVA (for 1 h) prevented the transmitter-release action of PMA and CaC (% variation with respect to the control: 0.32 ± 3.59% for PMA, 2.25 ± 1.17% for CaC; all cases: P > 0.05; Fig. 4A). However, when these experiments were performed with ω- AAg-IVA preincubation in the presence of high Ca<sup>2+</sup> (5 mM), CaC reduced quantal content (37.17 ± 1.50%) as normal and PMA increased it (174.17 ± 20.80%; in both cases, P < 0.05 with respect to the corresponding control, n = 5 muscles, minimum 15 fibers per muscle). In summary, PKC activity seems only to be related ultimately to a certain amount of calcium entry. We performed several controls for the effect of ω-Aga-IVA in the presence of high Ca<sup>2+</sup>, ω-Aga-IVA can inhibit the size of EPP in 5 mM Ca<sup>2+</sup> (66.72 ± 3.38%, P < 0.05, n = 5 muscles, minimum 15 fibers per muscle) as in normal Ringer’s, so the P/Q-type channel was effectively blocked in the experiments with high calcium. However, when the high Ca<sup>2+</sup> was added to a preparation that was previously blocked with ω-Aga-IVA, release was enhanced (201.01 ± 34.01%, P < 0.05, n = 5 muscles, minimum 15 fibers per muscle). This shows that some external calcium can flow in spite the P/Q-type channel block and suggests that the CaC- and PMA-modulated PKC mechanisms actually depend only on the availability of sufficient internal calcium arriving from the extracellular site.

To gain insight into the possible target of the ACh-release-involved PKC activity, we investigated how preincubation with CaC or PMA would change the coupling of the P/Q-type VDCC to release. We also looked for the possible appearance of an N- or L-type VDCC in this situation.

We found that preincubation with PMA or CaC did not change the efficacy of the P/Q-type channel blocker ω-Aga-IVA for reducing transmitter release. After CaC incubation, ω-Aga-IVA reduced quantal content to 75 ± 6.8% and after PMA incubation it reduced it to 69.31 ± 5.77% (P > 0.05 with respect to the controls without preincubation; five muscles for each PKC modulator set of experiments). Altering PKC activity therefore did not change the P/Q-type VDCC sensitivity to the blocking action of this specific toxin, which indirectly suggests that the coupling of this channel to transmitter release was not affected. From these experiments, however, we cannot discard a PMA- or CaC-induced PKC modulation of the amount of calcium ions permeating the P/Q channel.

L- and N-type VDCCs are not operative in the normal adult mammalian NMJs, as indicated by the fact that the respective specific blockers Nitrrendipine and ω-CgTx-GVIA do not affect evoked ACh release. As a control, we found that after preincubation with any one of these blockers, both PMA and CaC can make their respective stimulatory and inhibitory actions on ACh release as normal (data not shown). We also found that after preincubation with either of the two PKC modulators considered, neither the L-type blocker Nitrrendipine nor the N-type blocker ω-CgTx-GVIA were capable of revealing a previously masked or functionally suppressed L- or N-type VDCC component in the transmitter release machinery. This clearly indicates that these VDCC are not involved in PKC-induced transmitter release modulation in the adult mammalian muscles.

**DISCUSSION**

We show that presynaptic PKC is involved in the modulation of neurotransmission in the adult rat NMJ. Specifically, ACh release increases when PKC is activated by a phorbol ester. In normal conditions,
a CaC-inhibitable component of the ACh release operates tonically by increasing quantal content. The PKC effect is dependent on the calcium inflow through the P/Q-type VDCC during evoked activity and works near the maximum rate at the physiological level of external calcium concentration because in PMA-stimulated preparations, raising calcium does not increase transmitter output.

We found that at the concentrations used during the time scale of the present experiments the drugs did not modify the size or time course of the single quantum event, and therefore a postsynaptic effect is unlikely. Thus, the effects observed in neurotransmission are really presynaptic and due to changes in transmitter release. On the other hand, the PKC-modulated changes in release that we describe in this study are calcium-dependent and this points to the involvement of a “conventional” presynaptic (ePKC, calcium-activated) PKC isoform.

Presynaptic PKC can phosphorylate sodium channels in neurons, resulting in a rapid and persistent enhancement of presynaptic excitability (Catterall, 1999; Numann et al., 1994; West et al., 1991). On the other hand, synapsin PKC and PKA (both kinases overlapping their function in complex time-dependent and previous state-dependent interactions) can also modulate different potassium channels (by reducing their conductances), thus leading to spike broadening and enhancement of calcium influx (Byrne and Kandel, 1996). Therefore, both presynaptic sodium and potassium channel modulation by PKC may enhance transmitter release in the synapses involved. Our present results in the adult rat do not show any significant change in the threshold of excitability or in the conduction velocity (measured as the latency of the EPPs) of the intramuscular axons. This suggests that a PKC-induced excitability change on axons is not a significant component of the transmitter release changes we describe here. However, a modulation by PKC of the calcium-dependent potassium channels of the nerve terminals cannot be discarded.

In the frog NMJs, PMA increases spontaneous and evoked ACh release (Arenson and Evans, 2001; Redman et al., 1997; Searl and Silinsky, 1998; Shapira et al., 1987). This increase is prevented by the L-type VDCC blocker nifedipine (Arenson and Evans, 2001), which suggests that PKC activation can increase quantal release by opening quiescent L-type VDCC (the normal VDCC in frog MNTs) at resting potential. L-type VDCC also seems to be involved in phorbol ester-induced enhancement of spontaneous release in hippocampal neurons (Waters and Smith, 2000). However, in other experiments (Searl and Silinsky, 1998) PKC inhibitors fail to affect ACh release and fail to diminish phorbol ester-induced increases in transmitter release, which suggests that a protein of the exocytotic machinery is the target site for the stimulatory action of phorbol esters at frog motor nerve endings. In the adult rat we found that highly specific and selective PKC inhibitors (however, see Plomp and Molenaar, 1996, for the effect of nonspecific PKC inhibitors) can reduce evoked ACh release (which indicates a tonic modulation of release by PKC manifested upon PKC inhibition). We also found that pretreatment with CaC does not prevent PMA-induced increase in the quantal content of release. Reciprocally, pretreatment with PMA does not prevent CaC-reducing quantal content. The best interpretation of our results is that there may be a PKC-dependent (inhibited by CaC, CEL, or STP and stimulated by PMA) evoked release-facilitating mechanism. However, the fact that the PMA-induced increase in transmitter release is greater than the PKC-inhibitors release reduction may support the notion that there are two components in the phorbol ester action and that the PKC-independent component is the smaller of the two. The observation that no down-regulation of release is apparent at the highest doses of PMA supports the notion that there are two different mechanisms for the PMA action. The PKC-independent PMA-target may be Munc-13, as has been previously suggested by Searl and Silinsky (1998) (see also Betz et al., 1998).

We found that both the PMA-induced increase in release and the CaC-induced decrease in release are fully dependent on the normal calcium entry by the P/Q-type channel during evoked synaptic activity. Calcium-dependent changes in PKC activity, therefore, seem to produce some tuning of the normal calcium-induced evoked neurotransmitter release. With regard to the functional role of this mechanism, calcium ions entering the terminals may directly activate PKC. However, we found that forcing a higher calcium entry does not improve the efficacy of PMA and CaC as, respectively, positive and negative PKC modulators. It is also possible that a sufficient calcium inflow is only the necessary condition for producing evoked ACh secretion, the PKC mechanism being linked to the transmitter release itself. A fine and complex control of depolarization and transmitter release at individual terminals is possible because of the presence of several metabotropic receptors that can modulate calcium entry and exocytosis. In cholinergic synapses there may be several mechanisms of neurotransmitter release modulation associated with several subtypes of presynaptic adenosine receptors (Song et al., 2000) and muscarinic autoreceptors (Allen, 1999; Caulfield, 1993; Minic et al., 2002; Santafe et al., 2003; Slutsky et al., 1999). In the adult NMJ, PKC may be linked to the M1- and/or M2-type mACHRs that are present in the nerve terminal membrane. Specifically, the autoreceptor M1 is involved in a calcium-dependent and P/Q-type VDCC-dependent positive feedback of the ACh
release (Santafe et al., 2003). Therefore, we are currently exploring the relationships between VDCC, metabotropic presynaptic receptors, serine-threonine kinases, and ACh release.

The target of PKC and the mediator in release modulation could be the calcium channels themselves, another component of the release machinery, or both. Interestingly, phorbol esters decrease the nerve ending calcium currents that promote evoked release in the frog (Redman et al., 1997) and the calcium current in dorsal root ganglion neurons (Shapira et al., 1987). This suggests that the corresponding VDCCs are effectively targets of the PMA-stimulated PKC activity, but, as previously suggested, it may also indicate the direct effect (potentiating ACh release) of phorbol esters on a component of the release machinery. Moreover, potentiation of evoked release in hippocampal cultures by phorbol esters was insensitive to VDCC antagonists (Stevens and Sullivan, 1998; Waters and Smith, 2000). This phorbol effect is interpreted as resulting from an increased number of vesicles in the readily releasable pool (Waters and Smith, 2000). In the mammalian NMJs we have studied here, the functionality of the P/Q-type VDCC and the normal inflow of calcium through them are necessary conditions for the drug-induced changes in PKC-modulated release. However, forcing some calcium entry because of a larger electrochemical gradient with high external calcium, indeed with the P/Q channel specifically blocked (although not at 100% with ω-Aga-IVA), is sufficient to allow the CaC- and PMA-induced PKC modulation of release. This shows the pivotal role of external calcium ions in controlling the PKC-mediated mechanism and emphasizes the possible involvement of the VDCCs as PKC targets.

In mammalian NMJs, functional L- and N-type channels are expressed during synaptogenesis (in development and regeneration), although L channels can be inactivated in the adult and recruited to conduct calcium in certain conditions (Urbano et al., 2002). We found that after preincubation with the PKC activity modulators, neither the L-type blocker Nifedipine nor the N-type blocker ω-CgTx-GVIA were capable of revealing a newly appearing, previously masked, or functionally suppressed L- or N-type VDCC component in the transmitter release machinery. This clearly indicates that these VDCC are not involved in the PKC-induced transmitter release modulation in the adult mammalian muscles. Moreover, the PKC modulators do not change the P/Q-type channel sensitivity to the blocking action of their specific toxin ω-Aga-IVA, which indirectly suggests that the coupling of this channel to transmitter release is not affected. However, a PMA- or CaC-induced PKC modulation of the number of calcium ions permeating the P/Q channel cannot be discarded.

The changes in release produced by drug manipulation of the PKC activity are established in minutes and are not washable by fresh Ringer for hours. This indicates a certain stability of the modulation produced in the target/s of the kinase and so opens up the possibility of a relatively long-lasting capacity of the PKC to modulate neurotransmission.

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