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Protein Kinase C Facilitation of Acetylcholine Release at the Rat Neuromuscular Junction

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Abstract

Protein kinase C (PKC) is a Ca^{2+}-dependent enzyme involved in synaptic transmission, which can be experimentally activated by the phorbol ester, phorbol 12-myristate-13-acetate (TPA). We studied the effects of TPA application on acetylcholine (ACh) release at the rat neuromuscular junction by means of the focal recording technique; possible effects of TPA at the postsynaptic site had been ruled out in preliminary studies. In extracellular solutions containing 2 mM Ca^{2+} and at the stimulation frequency of 0.1 Hz, TPA increased endplate current (EPC) amplitude. In non-stimulated preparations spontaneous current frequency was increased at a similar rate. The similar time course of TPA action on evoked and spontaneous currents suggests that an increased presynaptic Ca^{2+} efficacy can be considered to be the probable mechanism of action. The interactions of PKC with ACh release were further investigated. In 0.1 mM Ca^{2+} extracellular solutions, TPA enhanced evoked currents only at stimulation frequencies (e.g. 40 Hz) that were themselves capable of inducing facilitation. This facilitation is classically associated with presynaptic Ca^{2+} accumulation, indicating that PKC interacts synergistically with Ca^{2+} to facilitate ACh release. In particular, since mean quantum size and release probability remained almost unchanged during TPA facilitation, it was concluded that PKC acted by enlarging the immediately available store. Interestingly, TPA also increased the presynaptic currents that were observed to be largely brought about by Ca^{2+}-dependent K^+ currents: evidence was obtained to suggest that increases in these currents provide negative feedback against excess release activation rather than being an expression of enhanced Ca^{2+} influx.

Introduction

The phasic release of neurotransmitters is ultimately due to modifications of Ca^{2+} availability at specific presynaptic sites (Katz and Miledi, 1968; Charlton *et al.*, 1982); nevertheless the mechanisms modulating neurosecretion remain largely unknown. Great effort has recently been devoted to clarifying the involvement of second messenger pathways interacting with Ca^{2+} during the neurosecretion process. Considerable evidence from different cell types supports a role for protein kinase C (PKC), a Ca^{2+}/phospholipid-dependent enzyme (Kikkawa and Nishizuka, 1986; Kikkawa *et al.*, 1989; Rana and Hokin, 1990).

PKC represents one major branch of the phosphoinositide system (Nishizuka, 1986). Ca^{2+} in the low micromolar range (0.1–10 μM) is known to promote translocation of PKC into the plasma membrane [but differences between PKC isozymes in their Ca^{2+} sensitivity do exist (Nishizuka, 1988; Huang, 1989)]. After translocation PKC can be experimentally activated by the phorbol ester phorbol 12-myristate-13-acetate (TPA) (Castagna *et al.*, 1982) and by diacylglycerol analogues (Nishizuka, 1984). These PKC activators mimic the role of the physiological cofactor 1,2-diacylglycerol. PKC is in turn believed to act on the Ca^{2+}-dependent secretory process either by increasing Ca^{2+} affinity at some specific binding sites or by increasing presynaptic Ca^{2+} availability.

At the neuromuscular junction, TPA has been reported to increase evoked and spontaneous acetylcholine (ACh) release both in the frog (Publicover, 1985; Eusebi *et al.*, 1986; Haimann *et al.*, 1987; Shapira *et al.*, 1987; Branisteanu *et al.*, 1988; Caratsch *et al.*, 1988) and in the mouse (Murphy and Smith, 1987). PKC activators also facilitate ACh release in the nervous system of invertebrates (Fossier *et al.*, 1990). Enhanced transmitter release to PKC activators at some central synapses has been related to long-term modifications of synaptic efficacy (Malenka *et al.*, 1986). However, some differences among reported results, and the evidence that some PKC effects can originate postsynaptically (e.g. Caratsch *et al.*, 1986; Eusebi *et al.*, 1987; Malenka *et al.*, 1989), complicate our understanding of the interactions between Ca^{2+}, PKC and the mechanisms of quantal release. Such interactions are considered in this paper, with particular attention to the presynaptic current (Hubbard and Schmidt, 1963; Brigant and Mallart, 1982) and the quantum content of endplate currents (EPCs) (Boyd and Martin, 1956; Elmqvist and Quastel, 1965; Bennett and Lavidis, 1989), two main aspects of the neurosecretion process.

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Materials and methods

Hemidiaphragms of male Sprague–Dawley rats (weight 50–100 g) were dissected and pinned to the bottom of a Perspex chamber. Preparations were submerged in Krebs solution which was continuously bubbled with 95% CO_2–5% O_2 (pH 7.4) at room temperature (19–21°C).

The Krebs solution had the following composition (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 13 $NaHCO_3$, 11 glucose, 2 $CaCl_2$ (normal Ca^{2+} solution). In some experiments Ca^{2+} was 0.1 mM and Mg^{2+} 10 mM, and NaCl was reduced to 124 mM (low Ca^{2+} solution). Phorbol 12-myristate-13-acetate, 4-α-phorbol-12,13-didecanoate (4α-PDD) and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were dissolved in 50% dimethyl sulphoxide (DMSO). d-Tubocurarine (d-TC) and tetraethylammonium (TEA) were dissolved in Krebs solution. ACh chloride was dissolved in distilled water (all drugs from Sigma). Stock solutions of the drugs were stored at −20°C.

Stock solutions were thawed before use, and known amounts of the drugs were diluted in a few hundreds of microlitres of the appropriate Krebs solution. Drug solutions were added to the bath (2.5 ml at the beginning of the experiment) by fast ejection from a pipette: afterwards the bath was left static. Unless otherwise stated, the effects of TPA, OAG and 4α-PDD were considered to occur in the presence of the solvent DMSO at a final bath concentration of 0.1–1%.

Focal current recordings (Del Castillo and Katz, 1956; Liley, 1956; Hubbard and Schmidt, 1963; Katz and Miledi, 1965; Wernig, 1976; Brigant and Mallart, 1982) were performed using thick-walled fire-polished microelectrodes filled with Krebs solution. The use of a relatively large microelectrode tip (5–15 μM external diameter) improved the recording stability and signal-to-noise ratio. The microelectrode was gently apposed onto the endplate region, and extracellular currents were recorded through a low-noise amplifier (Tanzi *et al.*, 1987). Inward currents appeared as negative signals, and outward currents as positive signals. Maintaining the same recording configuration, extracellular currents were occasionally measured (with reversed polarity) using a patch-clamp amplifier (LIST EPC-7) with a comparable signal-to-noise ratio (Forda *et al.*, 1982). Recorded signals were both fed to an IBM-AT personal computer (100 μs sampling interval) and stored in FM on magnetic tape (6 kHz cut-off frequency).

In normal Ca^{2+} solutions EPCs were reduced by 2–5 μM d-TC to avoid muscle contraction, and simultaneous recordings of evoked pre- and postsynaptic currents were obtained. 30 μM d-TC was used to suppress EPCs, leaving only the presynaptic currents. The phrenic nerve was stimulated at a frequency of 0.1 Hz (0.2 ms, 5–20 V pulses); this frequency proved neither to facilitate nor to depress the EPCs in preliminary trials (Hubbard, 1963). Since presynaptic currents usually measured a few tens of μV in amplitude, they were averaged on-line and monitored on a computer display. The same computer program computed presynaptic current areas by summing squared data points, and also measured EPC peak amplitudes. The depression method (Elmqvist and Guastel, 1965) was applied to short EPC trains in order to evaluate changes in fractional ACh release. The focal recording technique was also used to record spontaneous currents (miniature endplate currents, MEPCs) in the absence of nerve stimulation (non-curarized preparations).

In some experiments EPCs were recorded in low Ca^{2+} solutions at the basal stimulation frequency of 1 Hz; in such cases no obvious EPC facilitation or depression was found in preliminary studies. To study EPC facilitation, trains of stimuli were applied at frequencies up to 40 Hz. It should be noted that in low Ca^{2+} solution presynaptic currents were too small to be reliably detected.

Intracellular recordings were performed by conventional techniques, by means of 3 M KCl filled microelectrodes (40 MΩ impedance). Muscle cell input resistance was monitored through a bridge circuit.

ACh iontophoresis was performed through microelectrodes filled with 1 M ACh chloride (20–40 MΩ impedance). 20-nA pulses lasting 2 ms were superimposed on a constant braking current of −5 nA. Intracellular recordings of iontophoretic responses were performed in the endplate region [spontaneous endplate potentials (miniature endplate potentials, MEPPs) were clearly detectable and rose to peak in <0.8 ms]. Then, postsynaptic depolarizations of as much as 15 mV could be obtained through careful positioning of the iontophoretic microelectrode. The iontophoretic sensitivity (given an iontophoretic mobility for ACh of 0.1) was 0.015–0.05 mV/pC. Pulses lasting 2 ms were employed in order to avoid desensitization of the response, which in fact was observed during ACh pulses of much longer duration. 100 ACh pulses per s were delivered in order to achieve high ACh receptor activation. To exclude the occurrence of electrical artefacts, iontophoretic potentials were blocked at the end of each experiment through 10 μM d-TC applications.

Data are reported as mean ± standard deviation, and the number of observations is in brackets. Two sample Student's *t*-tests were used for statistical comparison of the means.

Results

Preliminary observations

In preparations maintained in normal Ca^{2+} solutions (2–10 μM d-TC) and stimulated at 0.1 Hz, evoked responses typically consisted of pre- and postsynaptic currents (Hubbard and Schmidt, 1963). As shown in Figures 1, 2 and 3, presynaptic signals were bi/triphasic, and they resembled those observed in different portions of the presynaptic terminal at the mouse neuromuscular junction (Brigant and Mallart, 1982; Mallart, 1985; Penner and Dreyer, 1986; Tabti *et al.*, 1989). Some presynaptic currents mostly moved upwards and others mostly moved downwards, their shape varying with electrode positioning.

Because of its relatively large tip, the extracellular electrode might be able to collect currents from more than one endplate. To verify this possibility, simultaneous intra- and extracellular recordings were performed in preliminary studies (n = 4, not shown). We observed that spontaneous potentials (intracellularly recorded MEPPs) were always paired with spontaneous currents (extracellularly recorded MEPCs). Some MEPCs, however, were unpaired, so that MEPC frequency exceeded MEPP frequency by 2–3 times. Since the mammalian endplate is a single and compact structure, our observation indicated that the extracellular electrode was collecting MEPCs from more than one endplate, and that synchronous activation of neighbouring neuromuscular junctions could contribute to the generation of the EPCs and presynaptic currents. Extracellular electrodes much smaller than ours could indeed record MEPCs from a single endplate in the rat diaphragm (Liley, 1956), and perhaps even from a fraction of the endplate area (Hubbard and Schmidt, 1963). It should be noted that different intracellular/extracellular relationships for spontaneous events have been observed at the frog endplate (Del Castillo and Katz, 1956; Wernig, 1976).

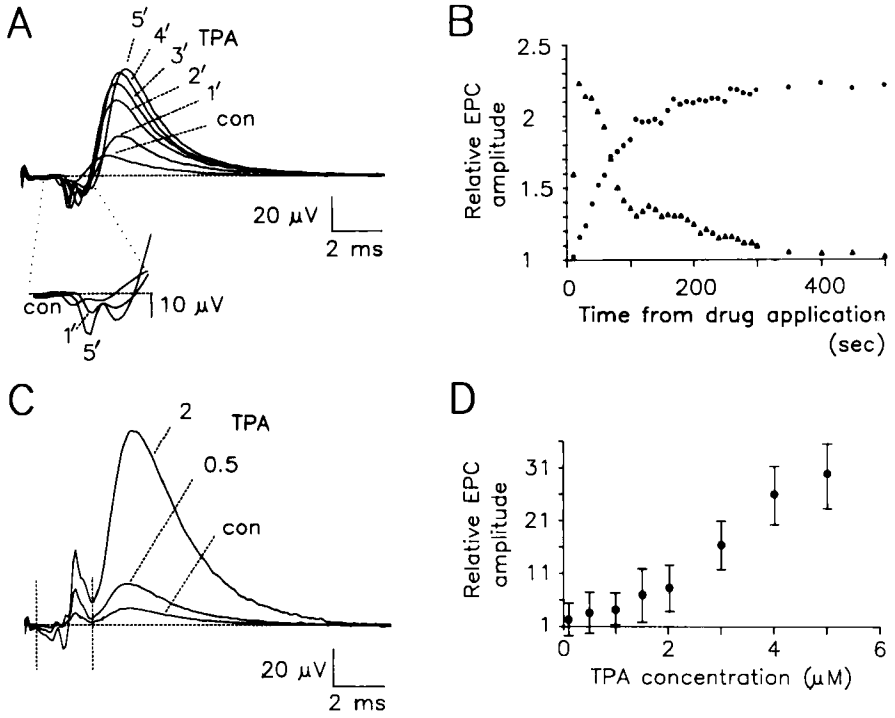

FIG. 1. Effects of TPA on pre- and postsynaptic currents in normal Ca^{2+} solutions. Experimental traces in A and C are the average of five responses. con, control. EPSC amplitudes in B and D are relative to control. (A) Pre- and postsynaptic currents 1–5 min after 0.5 μM TPA application. Three presynaptic currents are magnified in the inset. (B) Normalized EPC amplitude after 0.5 μM TPA application (circles). The effect of 10 μM OAG (triangles) is shown for comparison (OAG data points are scaled to one-fourth of their original size). (C) Pre- and postsynaptic currents 10 min after each of two sequential TPA applications; final TPA concentrations were 0.5 and 2 μM. Vertical dotted lines delimit the boundaries of presynaptic currents used to measure the underlying area. (D) Dose dependence of EPC amplitude increase (means ± SD from a minimum of three experiments).

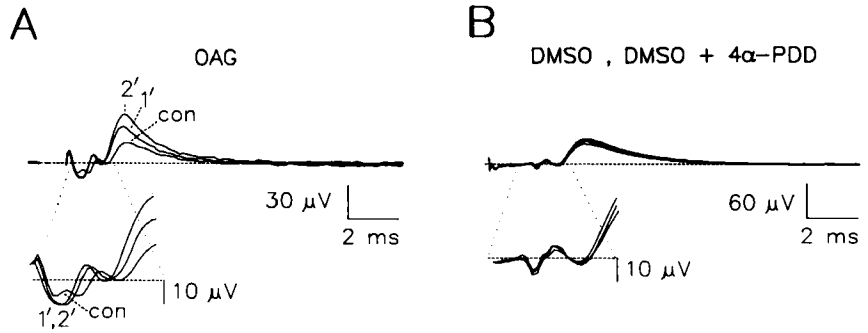

FIG. 2. Effects of OAG, 4α-PDD and DMSO in normal Ca^{2+} solutions. Experimental traces are the averages of five responses. Presynaptic currents are magnified in insets. (A) 10 μM OAG (con, control). (B) From lower to upper trace: control, 1% DMSO, 1% DMSO + 3 μM 4α-PDD.

Postsynaptic effects of TPA

Since some effects of TPA on postsynaptic ACh receptors have been reported at the frog endplate (Caratsch *et al.*, 1986; Eusebi *et al.*, 1987), we tested postsynaptic sensitivity by ACh iontophoresis (n = 4). The amplitude of iontophoretic potentials, 10 min after 1.5 μM TPA application, was 0.98 times that before application. The amplitude between responses at the 1st and 30th s of 100-Hz pulse trains was 1 in control and 1.01 after TPA application. The postsynaptic sensitivity to ACh was therefore unaffected by TPA. Muscle cell resting potential was -68.6 ± 5.5 mV and membrane input resistance was 8.47 ± 2.68 MΩ (n = 25). In the same cells 10 min after TPA application, resting potential was -68.2 ± 6 mV and membrane input resistance was 9.15 ± 2.09 MΩ (n = 25).

Effects of TPA at normal Ca^{2+} concentrations

Effects on pre- and postsynaptic currents

Figure 1A shows that both EPCs and presynaptic currents apparently increased after 0.5 μM TPA application. Similar results were obtained in 18 experiments performed at TPA concentrations from 0.1 to 5 μM.

In three cases the EPC increase led to muscle contraction. TPA effects settled at their maximum in about 5 min (Fig. 1A, B), and were not reversed by repeated washing (n = 5). In three experiments endplate potentials were recorded intracellularly and increased similarly to the EPCs after 1.5 μM TPA application (data not shown).

Figure 1C shows the effects of TPA at increasing concentrations in the same preparation: a new dose was applied after a steady response was attained. Pre- and postsynaptic currents increased additively. Data from sequential TPA applications in different experiments were used to obtain Figure 1D. It should be noted that TPA effects in presynaptic currents consisted of an amplitude increase and a broadening of the trace towards the right side (Figs 1 and 3). On the other hand, EPCs did not show apparent changes in their time courses.

Similarly to TPA, the other ACh activator, OAG (Castagna *et al.*, 1992), increased both pre- and postsynaptic currents (Fig. 2A). OAG was effective in the concentration range from 6 to 20 μM (n = 8). EPCs increased over 20–100 s and then recovered spontaneously towards their initial amplitude (Fig. 1B).

Figure 2B illustrates the effect of applying either the inactive TPA analogue 1 μM 4α-PDD (dissolved in 1% DMSO; Castagna *et al.*, 1982) or 1% DMSO only. In both cases the increase in EPC amplitude ranged from 1.1 to 1.2 times. The presynaptic currents were virtually unaffected.

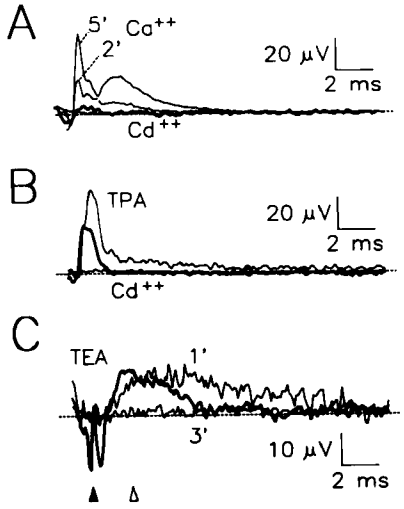

FIG. 3. Ca^{2+} dependence of the presynaptic currents. All experimental traces are the mean of five responses. Thicker lines indicate control traces in 2 mM Ca^{2+} solutions; pre- and postsynaptic current positions are indicated by a filled and open arrowhead respectively. (A) Effects of 10 mM Ca^{2+} 2 and 5 min after application. Further application of 1 mM Cd^{2+} blocked any evoked response. (B) Effects of 2 μM TPA were subsequently blocked by 1 mM Cd^{2+}. (C) Effects of 10 mM TEA 1 and 3 min after application. Note the block of the presynaptic currents and the transient broadening of the EPC trace 1 min after TEA application.

Ca^{2+} dependence of the presynaptic currents

Presynaptic currents are related to Ca^{2+} influx into the presynaptic terminal (Mallart, 1985). Since, as observed before, presynaptic currents were enhanced by TPA, it was of interest to investigate their relation with extracellular Ca^{2+}, and to test the effects of Cd^{2+}, a Ca^{2+} channel blocker. Recordings were performed at high d-TC concentration (10–30 μM) to better isolate the presynaptic currents from the EPCs.

Figure 3A shows that presynaptic currents were increased together with the EPCs by 10 mM Ca^{2+}. Subsequent application of 1 mM Cd^{2+} blocked any evoked response (n = 3). In other experiments (n = 5, an example is shown in Fig. 3B) TPA was applied; the presynaptic current increased, and was afterwards blocked by 1 mM Cd^{2+}. Thereafter, subsequent TPA applications (up to 5 μM) were ineffective. Ca^{2+} entry therefore appeared essential to the building up of the presynaptic currents.

Presynaptic Ca^{2+} currents studied at the mouse neuromuscular junction (Mallart, 1985; Tabti *et al.*, 1989) were, however, very small and were dominated by K^+ currents of much larger amplitude. We therefore tested the effect of TEA, which in millimolar concentrations has been shown to block quite specifically a presynaptic Ca^{2+}-dependent K^+ current [$I_{K(Ca)}$] in the mouse. As shown in Figure 3C, 10 mM TEA depressed the presynaptic current (n = 4). Presynaptic current depression was accompanied by a very transient increase and broadening of the EPCs (still partially evident after 1 min in Fig. 3C). EPCs subsequently reduced to a definitive block.

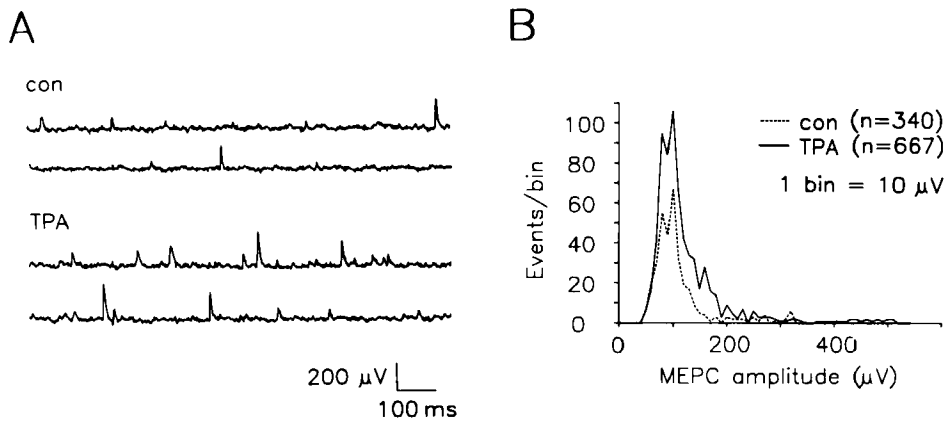

FIG. 4. (A) Spontaneous activity before (con) and 5 min after 1 μM TPA application (TPA). In this experiment mean MEPC frequencies were 2.7/s in control and 5.4/s after TPA. Background noise variance was 245 μV^2 before and 301 μV^2 after TPA application. (B) Amplitude histograms from MEPCs recorded in the same experiment as in A. Only MEPCs greater than 35 μV, i.e. at least twice the background noise standard deviation, were considered. Data samples lasted 120 s: bin width and the number of observations are indicated in the figure.

It was concluded that a TEA-sensitive $I_{K(Ca)}$ accounted for the largest part of the recorded presynaptic current, and that it was controlled by Ca^{2+} currents too small to be detected. The importance of $I_{K(Ca)}$ seemed larger in this preparation than in the mouse.

Effects on MEPCs: frequency and amplitude

MEPCs were recorded in non-curarized preparations. As illustrated in Figure 4A, 1 μM TPA increased MEPC frequency, the mean increase being 1.7 ± 0.2 times ($P < 0.01$, $n = 5$) after 5 min. In the same traces, background noise variance increased by 15–20%, probably due to increased MEPC frequency at remote synapses. TPA concentrations as low as 0.1 μM ($n = 3$) were also effective. On the contrary, application of 1 μM 4α-PDD (in 1% DMSO; $n = 5$) or of 1% DMSO only ($n = 5$), did not significantly increase MEPC frequency; the increase was 1.07 ± 0.01 when both data groups were pooled, the difference from the control not being statistically significant ($P > 0.05$, $n = 10$).

In the same experiments, 1 μM TPA increased MEPC amplitude by 1.1–1.2 times (Fig. 4B). A similar increase occurred following the application of 1 μM 4α-PDD or of 1% DMSO only ($P < 0.05$, $n = 10$). Therefore it turned out that DMSO may increase MEPC amplitude by itself, and that neither TPA nor 4α-PDD are required (Geron and Meiri, 1985). Although this increase was irrelevant to the action of TPA on EPCs, it probably explains the effect of DMSO on EPC amplitude observed in Figure 2B.

1 μM TPA was also applied in three of the four experiments in which simultaneous intra- and extracellular recordings of spontaneous activity were performed (see above). 5 min after TPA application, MEPP frequency increased by 1.9 ± 0.2 times, and MEPC frequency by 1.8 ± 0.2 times, the difference not being statistically significant ($P > 0.2$, $n = 3$). In the same samples MEPP amplitude increased by 1.05 ± 0.1 times and MEPC amplitude by 1.11 ± 0.12 times, the difference not being statistically significant ($P > 0.2$, $n = 3$).

Time course of TPA effects

The time courses of TPA effects on evoked and spontaneous currents are compared in Figure 5. EPC peak amplitude increased exponentially at a rate scarcely influenced by TPA concentration (Fig. 5, top). The mean time constant was 114 ± 9.2 s ($n = 8$). A steady level was reached after about 5 min (see also Fig. 1A), and was maintained for several tens of minutes.

Due to their complex waveform, presynaptic currents were monitored by measuring the underlying area (see Fig. 1C). Presynaptic currents were in fact inherently variable between different recordings and changed their shape after TPA application. Presynaptic current areas increased exponentially following TPA application (middle), with a time constant of 112 ± 56 s ($n = 4$).

MEPC frequency was measured in subsequent periods of 60 s following TPA application (bottom). Although this subdivision using relatively long time-intervals may smooth its real kinetics, MEPC frequency appeared to increase exponentially; the time constant from the mean of five experiments was 136 ± 34 ms.

It should be noted that the intrinsic EPC amplitude variability, the selection of limits for presynaptic area calculation and the averaging of MEPC frequency over quite long time periods conferred different sensitivity to the parameters measured. Nevertheless the time courses of EPC amplitude, presynaptic current area and MEPC frequency after TPA application were similar. Parallel increases in MEPC frequency and EPCs were also reported at the frog neuromuscular junction (Shapira *et al.*, 1987).

Synaptic depression

The hypothesis that depletion of quanta from the immediately available store is responsible for EPC amplitude depression during repetitive stimulation (Elmqvist and Quastel, 1965) provided a way to compare ACh fractional release before and after TPA application. The comparison was possible since TPA had virtually no effect on MEPC amplitude and on ACh receptor sensitivity, and therefore the quantum size, q, remained unchanged. During four pulses delivered at the frequency of 40 Hz, fractional release was very similar before and after TPA application (Fig. 6). In Figure 6B EPC amplitudes are plotted against the sum of previous EPC amplitudes in the train. Linear regression in these plots (Fig. 6B) was highly significant in all the experiments ($r > 0.98$, $n = 10$). Plot slopes averaged 0.328 ± 0.099 ($n = 7$) before and 0.389 ± 0.059 ($n = 10$) 5–30 min after 1 μm TPA application, the difference being statistically non-significant ($P > 0.5$).

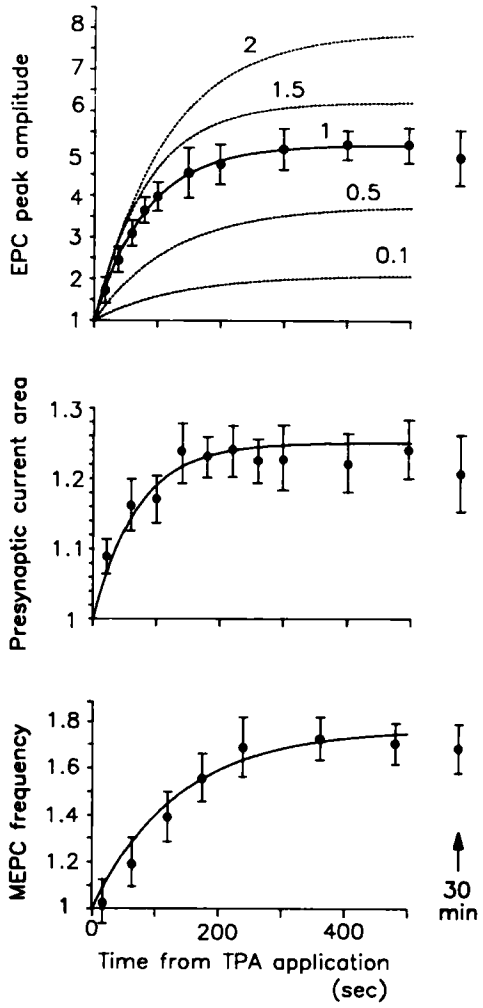

FIG. 5. Time course of TPA effects. Data points represent the normalized values, after 1 μM TPA application, of EPC amplitude (top, $n = 3$), presynaptic current areas (middle, $n = 3$) and MEPC frequency (bottom, $n = 5$). (EPC amplitude and presynaptic current areas were measured in the same experiments.) Continuous lines are exponential fittings to the points with time constants of 85, 82 and 136 s (from top to bottom respectively). Dotted lines in the top panel represent data fittings in single experiments performed with 0.1–2 μM TPA: from the lowest to the highest TPA concentration, time constants are 134, 123, 85, 95 and 123 s.

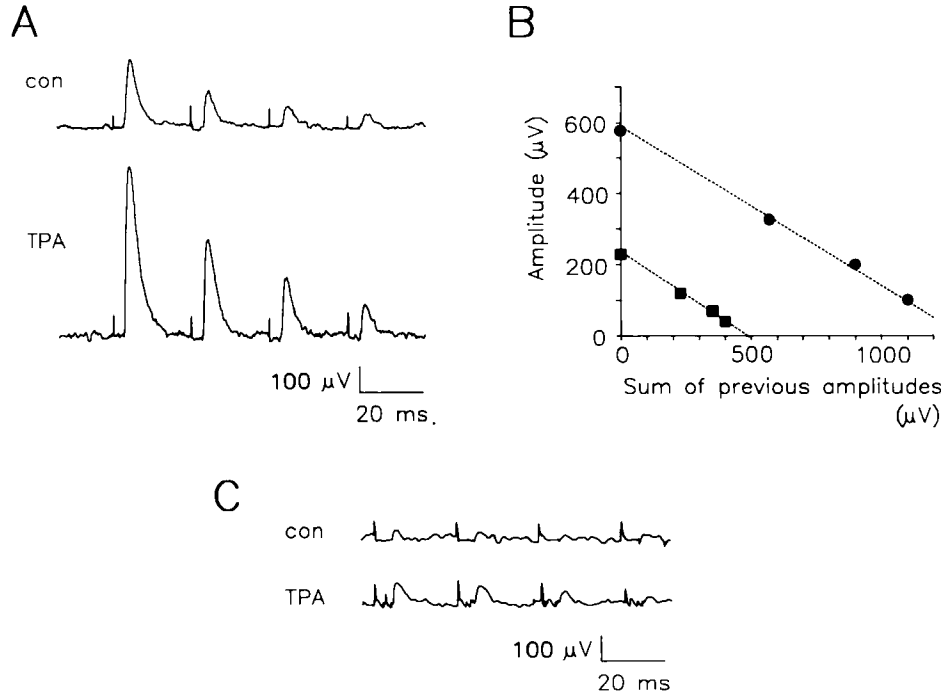

FIG. 6. Depression of EPC amplitude in normal Ca^{2+} during 40-Hz stimulation. (A) The first four EPCs in a train before (con) and 5 min after 1 μM TPA application (TPA). (B) Plot of EPC amplitudes in A versus the sum of previous EPC amplitudes (squares, control; circles, TPA). The slope of fitted lines corresponds to the release probabilities. $P = 0.45$ in control conditions and $P = 0.47$ after TPA. The linear regression is highly significant in both cases ($r = 0.98$). (C) EPCs after 200 ms of 40-Hz stimulation during the same experiment.

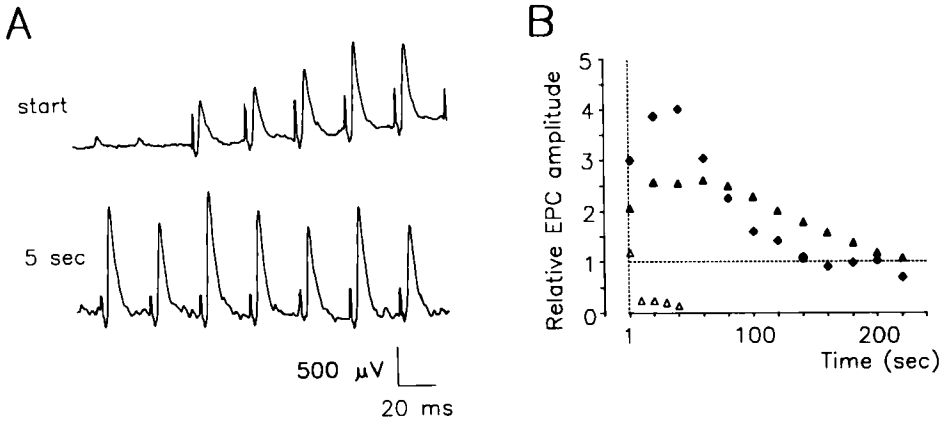

FIG. 7. Effects of TPA in low Ca^{2+} solution (0.1 mM Ca^{2+}, 11 mM Mg^{2+}). 1.5 μM TPA was applied; thereafter EPC facilitation was evidenced by 40-Hz trains of stimuli. (A) EPCs at the start and after 5 s in a train (two MEPCs are also observable before the start of a train). (B) EPC amplitudes are plotted for control (open triangles), 5 min after TPA application (filled triangles) and 120 min after washing with control solution (filled diamonds). Data points are averages of ten measurements. EPC amplitude is relative to the mean of the last five EPCs before the first impulse.

As shown in Figure 6C, EPCs recorded in the presence of TPA also remained larger than in control conditions when 40-Hz stimulation was prolonged for some tens of stimuli.

Effects of TPA in low Ca^{2+} solutions

In four experiments, Ca^{2+} concentration was lowered to 0.1 mM and Mg^{2+} was raised to 10 mM, and a basal 1-Hz stimulation frequency was adopted. In control conditions, stimuli delivered with a frequency of 10–40 Hz induced brief facilitation lasting a few seconds, which was followed by strong depression (Fig. 7B, open triangles).

In the same four experiments, subsequent application of 1.5 μM TPA did not produce any relevant effect on EPC amplitude at the basal 1-Hz stimulation frequency (not shown). However, a large facilitation took place when trains of stimuli were delivered at frequencies >10 Hz,

as shown in Figure 7A for a 40-Hz train. Facilitation progressed for several tens of seconds before beginning a slow decaying phase (Fig. 7B, filled triangles). The effect was reversed by restoring the basal 1-Hz frequency, and facilitation could be reproduced several times (for up to 3 h in one case). Prolonged washing with control solution was unable to abolish the effect of TPA (Fig. 7B, filled diamonds).

Discussion

In this paper we report that the PKC activator, TPA, increased both pre- and postsynaptic currents, as well as MEPC frequency, when applied to the rat neuromuscular junction at normal Ca^{2+} concentration. TPA was active in a range of concentrations comparable to that reported in preceding works (Publicover, 1985; Eusebi *et al.*, 1986; Haimann *et al.*, 1987; Murphy and Smith, 1987; Shapira *et al.*, 1987). We observed a direct relation between increases in the evoked responses and the different TPA dose applied during the experiments.

TPA effects were faster than in the frog (Shapira *et al.*, 1987), settling down at a stable level in about 5 min. The effects persisted after prolonged washing, presumably due to the very slow metabolism of TPA (Castagna *et al.*, 1982). The pre- and postsynaptic current increases induced by the PKC activator, OAG, were even faster, and were transient. This was probably due to the faster metabolism of OAG. This effect might contribute to the lower potency of OAG with respect to TPA observed in different experiments (Kaczmarek, 1987; Rana and Hokin, 1990).

The ineffectiveness of the phorbol ester 4α-PDD and the effectiveness of OAG in facilitating neurotransmission are consistent with specific activation of the C kinase by TPA. In addition, no changes in postsynaptic ACh receptor sensitivity were observed following TPA application during ACh iontophoresis, thus ruling out postsynaptic TPA effects during EPC recordings. Accordingly, MEPC amplitude remained virtually unchanged. It was therefore concluded that PKC activation occurred almost exclusively presynaptically, inducing facilitation of ACh release.

It should be noted that the action of TPA on EPC facilitation in low Ca^{2+} solutions was reversible and reproducible for several tens of minutes. This fact, together with the additive effect of sequential TPA applications in normal Ca^{2+} solutions, tends to exclude PKC self-activation or self-inactivation, as well as PKC subunit separation by proteases such as calpain, or the formation of long-lived, inserted forms of PKC. Since all of these PKC functional states would lead to uncontrolled enzyme activation (for critical review see Kikkawa *et al.*, 1989; Huang *et al.*, 1989), it is suggested that PKC could translocate dynamically inside and outside of the plasma membrane.

Ca^{2+}/PKC interactions

The TPA increase in evoked release and presynaptic currents, as well as in MEPC frequency, occurred in normal Ca^{2+} solutions. This indicates that PKC could be activated by resting extracellular Ca^{2+} concentrations. In low Ca^{2+} solutions, however, EPCs were facilitated by TPA only during stimulation at 10–40 Hz, i.e. at a frequency allowing Ca^{2+} accumulation in the presynaptic cytoplasm (Katz and Miledi, 1968; Charlton *et al.*, 1982; Zucker and Fogelson, 1986). It was therefore concluded that presynaptic Ca^{2+} concentration was critical in allowing PKC activation induced by TPA. Interestingly, in the proximity of releasing sites, free Ca^{2+} is thought to range from 100 nM at rest to >10 μM after Ca^{2+} channel opening (Zucker and Fogelson, 1986), i.e. at concentrations able to modulate the translocation of some PKC isoforms into the plasma membrane (Shearman *et al.*, 1988; Kikkawa *et al.*, 1989).

It appears, therefore, that PKC requires Ca^{2+} to be activated, providing a pathway synergistic with the final Ca^{2+}-dependent process, ACh release. This mechanism differs from that described during experiments on the frog, where PKC activation by TPA was supposed to be independent of presynaptic Ca^{2+} (Haimann *et al.*, 1987).

On the other hand, we found that the presynaptic current increase promoted by TPA was largely brought about by an $I_{K(Ca)}$. Evidence for presynaptic $I_{K(Ca)}$ enhancement by PKC activators has also been reported in *Aplysia* neurons (Fossier *et al.*, 1990). Since the physiological role of presynaptic K^+ currents is to interrupt Ca^{2+} influx through nerve terminal repolarization (Mallart, 1985), $I_{K(Ca)}$ enhancement may be considered to act as a negative feedback opposing excessive activation of the release process. In this way PKC would therefore be synergic with Ca^{2+}, while it should at the same time provide a path reducing the effectiveness of Ca^{2+}. This conclusion should be compared to the theoretical considerations and experimental evidence on complex Ca^{2+}–PKC interactions reported in different systems (Nishizuka, 1984, 1986, 1988; Kikkawa *et al.*, 1989).

PKC may act by enhancing Ca^{2+} efficacy

The relative rate of growth of MEPC frequency and EPC amplitude following TPA application was 1.19 (Fig. 5). Since spontaneous release is dependent on the first power of extracellular Ca^{2+} concentration, while evoked release depends on the fourth power (Dodge and Rahamimoff, 1967), the increase of Ca^{2+} in the motor terminal is unlikely to be considered a primary effect of PKC activation. Higher Ca^{2+} efficacy is therefore more likely, as pointed out by Haimann *et al.* (1987) through different considerations.

Since the TPA-induced increase in $I_{K(Ca)}$ occurred at a similar rate to the TPA-induced increase in ACh release, the former process may also be due to higher Ca^{2+} efficacy. In a sense this conclusion is contrary to the expectation that PKC may enhance Ca^{2+} influx during terminal depolarization, as well as, consequently, presynaptic $I_{K(Ca)}$ and evoked release. The question is of interest, since PKC may regulate ion channels, and may therefore also regulate evoked release (Kaczmarek, 1987). In particular, enhanced Ca^{2+} influx is supported by presynaptic intracellular recordings in *Aplysia* neurons (Fossier *et al.*, 1990). On the other hand, Ca^{2+} influx may hypothetically be prolonged and/or enhanced through primary $I_{K(Ca)}$ inhibition. However, this hypothesis can also be ruled out, since presynaptic currents would decrease rather than increase, as was the case after TEA application; moreover, EPC enhancement by TEA was only transient, unlike that observed with TPA.

Modifications of statistical parameters of release

The mean quantum size, q, was monitored through the amplitude of MEPCs. TPA application increased q by about 1.2 times: this effect was almost completely attributable to the solvent DMSO (Geron and Meiri, 1985; Cherki-Vakil and Meiri, 1991). A similar q increase was reported after TPA application in the frog (Haimann *et al.*, 1987); in other experiments (Shapira *et al.*, 1987), however, no changes in q were found.

Since in practice q remained constant, the large EPC amplitude increase after TPA application reflected an increase (up to 20-fold) in m, the mean quantum content. A large m increase was also observed in low Ca^{2+}, where stimulation frequency played a critical role. The

frequency dependence of TPA action could explain why phorbol esters failed to facilitate ACh release in the mouse (reported stimulation frequency in low Ca^{2+} was 2 Hz; Murphy and Smith, 1987). More difficult to reconcile is the greater effectiveness of TPA in increasing m in low Ca^{2+} than in normal Ca^{2+}, observed during experiments carried out with low-frequency stimulation in the frog (Haimann *et al.*, 1987; Caratsch *et al.*, 1988). It would seem again that the mechanisms of action of PKC in the frog and in the rat are not identical.

Fractional ACh release during repetitive stimulation, and therefore the slope of depression plots, did not change significantly after TPA application. According to the hypothesis that neurotransmitter depletion is the main cause of EPC depression (Elmqvist and Quastel, 1965), the slope of depression plots may give an estimate of p, the probability of a single quantum being released. Although an overestimate of the p value by the depression method has been demonstrated (Christensen and Martin, 1970), it is reasonable to assume that release probability—as well as ACh fractional release—remained almost unchanged during TPA facilitation.

Since neither q nor p changed, the dramatic increase in m could be attributed to n, i.e. the number of quanta available for release (Wernig, 1975; McLachlan, 1978). It should be noted that an increase in n is a physical correlate of frequency-dependent facilitation in mammals (Bennett *et al.*, 1975; Bennett and Lavidis, 1989), and is believed to reflect the transition of synaptic vesicles from a 'reserve pool' to an 'available pool' (Martin, 1966). A greater number of quanta available for release with unchanged release probability is consistent with the observation that EPCs remained larger during prolonged 40-Hz stimulation with applied TPA (Fig. 6C). Analogously, EPC depression was greatly reduced by TPA during high-frequency stimulation in low Ca^{2+} (Fig. 7A). TPA has also been reported to reduce depression of the second of two paired stimuli in the frog (Shapira *et al.*, 1987).

Conclusions

PKC activation facilitated neuromuscular transmission in the rat by increasing the number of quanta available for ACh release several-fold. This suggests the recruitment of a surprisingly high number of new releasing sites (Bennett and Lavidis, 1989), and reveals a wide security factor for neuromuscular transmission. Release facilitation, which was probably mediated through an increased Ca^{2+} efficacy, was simultaneous with the enhancement of the presynaptic current. The latter was brought about by an $I_{K(Ca)}$, whose role seems to counterbalance the increased ACh release. It may be noted that the TPA-induced increase in the releasable ACh pool resembles the actions mediated by Ca^{2+}-calmodulin kinase-II, which promotes vesicle detachment from the cytoskeleton through synapsin I phosphorylation (Benfenati *et al.*, 1991; Browning and Dudek, 1992; Trifarò *et al.*, 1992). However, although a role for PKC in neuromuscular plasticity can be suggested, molecular pathways leading to PKC activation and mediating its effects remain to be studied.

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Abbreviations

ACh	acetylcholine
DMSO	dimethyl sulphoxide
d-TC	d-tubocurarine
EPC	endplate current
$I_{K(Ca)}$	Ca^{2+}-dependent K^+ current
m	quantum content
MEPC	miniature endplate current
MEPP	miniature endplate potential
n	quanta available for release
OAG	1-oleoyl-2-acetyl-sn-glycerol
p	probability of release
4α-PDD	4-α-phorbol-12,13-didecanoate
PKC	protein kinase C
q	quantum size
TEA	tetraethylammonium
TPA	phorbol 12-myristate-13-acetate

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