Down Regulation of Protein Kinase C in Neuronal Cells: Effects on Neurotransmitter Release

Heinrich J. G. Matthies, H. Clive Palfrey, Lane D. Hirning, and Richard J. Miller

Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637

We investigated the effects of phorbol esters on protein kinase C (PKC) activity and on neurotransmitter release from cultured neuronal cells. Both differentiated and undifferentiated PC12 pheochromocytoma cells contained high levels of protein PKC. Under normal conditions all the enzyme activity was found in the cytoplasm. Addition of the phorbol esters phorbol 12-myristate-13-acetate (TPA) or phorbol 12,13-dibutyrate (PDBu) caused a rapid translocation of PKC from the cytoplasm to the particulate fraction. Continued culture of cells with these phorbol esters resulted in the decline of total PKC activity. After 10–20 hr of culture, both membrane and cytoplasmic PKC activity had declined to background levels. cAMP-dependent and Ca^{2+} /calmodulin-dependent protein kinase activities were only slightly affected by chronic phorbol ester treatment. Addition of active phorbol esters to PC12 cells produced an enhancement of the depolarization-induced release of ^3H -norepinephrine. Following chronic phorbol ester treatment, the ability of these substances to enhance evoked catecholamine release was lost. Furthermore, depolarizing stimuli released considerably less ^3H -norepinephrine than in control untreated cells. Phorbol esters also enhanced depolarization-induced ^3H -norepinephrine release from primary cultures of rat sympathetic neurons. Chronic treatment of these neurons with phorbol esters also resulted in the loss of their ability to enhance transmitter release and in a large reduction in the extent of depolarization-evoked transmitter release. Chronic phorbol ester treatment also resulted in the disappearance of PKC from sympathetic neurons, but had little effect on cAMP-dependent or Ca^{2+} /calmodulin-dependent kinase activities. These results demonstrate that PKC-deficient neurons can be prepared. The data also demonstrate that depolarization-induced neurotransmitter release is mediated by both protein kinase C-dependent and independent pathways.

Protein kinase C (PKC) is found in high concentrations in the nervous system (Kikkawa et al., 1982; Nishizuka, 1986). As in other tissues, activation of PKC in neuronal tissues, using drugs

such as phorbol esters, results in a very large number of effects. These include changes in neuronal development (Pahlman et al., 1981; Akerman et al., 1984; Montz et al., 1985), the modification of ion channels (Baraban et al., 1985; De Riemer et al., 1985b; Alkon et al., 1986; Di Virgilio et al., 1986; Madison et al., 1986; Malenka et al., 1986a, b; Strong et al., 1986) and receptors (Francel et al., 1986; Liles et al., 1986), alterations in neurotransmitter synthesis (Wang et al., 1986) and release (Pozzan et al., 1984; Harris et al., 1985; Publicover, 1985; Zurgil and Zisapel, 1985; Eusebi et al., 1986), and changes in synaptic plasticity (Akers et al., 1986; Malenka et al., 1986a). Such effects of phorbol esters suggest a role for PKC in the normal control of these processes. Clearly activation of PKC with phorbol esters has been of central importance in implicating the enzyme in these various cellular events.

In order to further define the roles of PKC, it would be useful to be able to inhibit the enzyme *in situ* in a routine manner. There have been several attempts to produce specific PKC antagonists (Hidaka et al., 1984; Kawamoto and Hidaka, 1984). However, although it can be demonstrated that a number of agents will block PKC, they are of low potency and poor specificity (Mazzei et al., 1982; Wooten and Wrenn, 1984; De Riemer et al., 1985a). For example, PKC can be inhibited by phenothiazines, but the same drugs also block Ca^{2+} /calmodulin-dependent protein kinase activity at least as well (De Riemer et al., 1985a).

An alternative approach to this problem would be the preparation of PKC-deficient cells. This has been realized in the case of a number of non-neuronal tissues by chronic phorbol ester treatment. PKC normally resides in the cytoplasm (Nishizuka, 1986). When activated by diacylglycerol (DAG) or a phorbol ester, the enzyme rapidly translocates to the cell membrane to which it binds with high affinity (Kraft and Anderson, 1983; Palfrey and Waseem, 1985; Wolf et al., 1985; Guy et al., 1986; Liles et al., 1986; McArdle and Conn, 1986). Extended treatment with phorbol esters leads to a decline in total PKC activity, eventually to undetectable levels (Yamasaki et al., 1980; Jaken et al., 1981; Solanki et al., 1981; Yamasaki et al., 1982; Collins and Rozengurt, 1984; Rodriguez-Pena and Rozengurt, 1984; Ashendel, 1985; Ballester and Rosen, 1985; Wickremasinghe et al., 1985; Fabbro et al., 1986; Glynn et al., 1986; Muldoon et al., 1987). In order to further pursue our investigations into the roles of PKC in neuronal function, we have analyzed the effects of chronic phorbol ester treatment on PKC activity in a nerve-related clonal cell line (PC12) and in primary cultures of rat sympathetic neurons. Our results indicate that PKC-deficient neurons can be conveniently prepared, and also suggest a role for PKC in the normal control of stimulus–secretion coupling.

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Correspondence should be addressed to Professor Richard J. Miller, Department of Pharmacological and Physiological Sciences, University of Chicago, 947 E. 58th Street, Chicago, IL 60637.

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Figure 1. Time course of the effect of phorbol ester on particulate and cytosolic PKC activity. Cells were incubated with $1 \mu\text{M}$ TPA for the times indicated. The results are means of duplicate assays. $\square$, Cytosolic PKC activity. $\blacksquare$, Particulate PKC activity. PKC was assayed as in Materials and Methods. Control PKC-specific activity was 88 pmol phosphate incorporated per milligram of protein per minute (particulate) and 467.5 pmol phosphate incorporated per milligram of protein per minute (cytosol).

Figure 2. Effect of chronic phorbol ester treatment on the activities of various kinases in PC12 cells. Cells were cultured with $1 \mu\text{M}$ TPA for 24 hr. Enzymes were assayed as in Materials and Methods. *Part*, particulate; *Cytos*, cytosolic; *Syn*, assay using Synapsin I as exogenous substrate. $\blacksquare$, Control activity. $\square$, TPA-treated.

Materials and Methods

Materials. ^3H -Norepinephrine (43.9 Ci/mmol) and ^3H -phorbol-12,13-dibutyrate (15.8 Ci/mmol) were obtained from New England Nuclear, $\gamma^{32}\text{P}$ -ATP from Amersham. Phosphocellulose paper was from Whatman. Phorbol esters, norepinephrine, pargyline, ascorbate, histone H1 and f2b were purchased through Sigma Chemical Co. Synapsin I was purified from bovine brain as previously described (Palfrey et al., 1983). All other chemicals were obtained from local chemical suppliers and were of reagent grade.

Cell culture. PC12 rat pheochromocytoma cells were cultured as described (Greene and Tischler, 1976; Toll, 1982) with slight modifications. PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (vol/vol) heat-inactivated horse serum (Hazelton KC Biological), 5% (vol/vol) fetal bovine serum (Hazelton KC Biological), penicillin-streptomycin (100 U/ml and 100 $\mu\text{g}/\text{ml}$, respectively; Gibco), and glutamine (2 mM; Gibco). For release studies, cells were subcultured onto 12-well tissue culture plates (Costar) which were coated with rat tail collagen (Bornstein, 1958). Cells were differentiated by growing them in the presence of 1 mM dibutyryl cAMP (Sigma) and 50 ng/ml of NGF (7S NGF; Sigma) for about 2 weeks (Heidemann et al., 1985; Kongsamut and Miller, 1986). For phosphorylation experiments, cells were grown on 100 mm collagen-coated plates.

Sympathetic neurons were cultured from superior cervical ganglia (SCG) taken from 1–3-day-old rats (Perney et al., 1986). Cells were dissociated from ganglia essentially as described in Yamamoto et al. (1981), except that a mixture of collagenase (0.1 U/ml)/dispase (0.8 U/ml) was used instead of collagenase alone. The sympathetic neural cells from 2–3 ganglia were plated in 0.5 ml on 16 mm collagen-coated wells. The plating media and culture conditions were the same as those used in other studies (Hawrot and Patterson, 1979). Exposure to Ara C (Perney et al., 1986) resulted in preparations that were essentially free of fibroblasts and other non-neuronal cells.

^3H -norepinephrine release assays. PC12 release assays were performed according to previously published methods (Kongsamut and Miller, 1986). Briefly, cells were loaded with 0.5 ml of media containing 6 μM ^3H -norepinephrine (NE; sp. act., 1.65 $\mu\text{Ci}/\text{ml}$) for 1–2 hr at 37°C in a HEPES-buffered assay medium (Shalaby et al., 1984). Cells were then washed using 8 successive rinses with 0.5 ml of buffer in a shaking water bath at 37°C . Release was then quantitated by replacing the medium in the wells every 4 min and collecting the entire 0.5 ml for liquid-scintillation counting. Release was elicited in the third fraction by 70 mM K^+ . Na^+ and K^+ replaced each other reciprocally. Drugs were included as indicated. Cells were then extracted with 0.2% SDS to determine the radioactivity remaining in the cells. Release was expressed as a percentage of radioactivity present in the cells prior to the K^+ -induced release. Basal release varied from 1 to 3%, K^+ -induced release from 12 to 30%, and phorbol ester-induced release from 15 to 45% of the total labeled pool.

Sympathetic neuron release assays were performed as outlined in Perney et al. (1986), that is, essentially as for PC12 cells with minor modifications. Sympathetic cells on day 5 or 6 after plating were used. Incubation with 2 μM ^3H -NE lasted 1 hr and was followed by 4 washes over a 10 min period and then 4 more 5 min rinses. The next 5 changes of buffer were then collected and counted. The third solution contained 70 mM K^+ . Drugs were added as indicated. Cells were extracted and release was quantitated as for PC12 cells.

In vitro phosphorylation. PC12 cells were grown on 100 mm collagen-coated plates and SCG sympathetic cells on 60 mm collagen-coated plates for 5–6 d. After the appropriate pretreatment with phorbol esters, cells were washed 3 times with Tris-buffered saline and then scraped with a rubber policeman. Cells were pelleted ($1000 \times g$ for 5 min), resuspended in at least a 10-fold excess of homogenization buffer [25 mM Tris, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02 mM leupeptin], sonicated briefly (10 sec; setting 1–2, 50% output Branson 200) and centrifuged at low speed to remove nuclei and debris. To generate cytosolic and particulate fractions, the low-speed supernatant was spun at $120,000 \times g$ for 30 min in an ultracentrifuge. The pellet (particulate fraction) was resuspended in homogenization buffer (typically one-half the initial homogenate volume) by being passed 3–4 times through a 27-gauge needle or by brief sonication. Protein determinations (Biorad assay) were made. Both fractions were then diluted with 2% NP40 to give a final concentration of 0.2% NP40. Samples were now incubated for at least 30 min at 4°C . This protein solution was then again diluted approximately 10-fold to give 25–50 $\mu\text{g}/\text{ml}$ in the final assay. PKC activity was assessed as in Palfrey and Waseem (1985). Samples, 2.5–5 μg , were assayed in a final volume of 100 μl , containing 50 mM Tris-HCl, pH 7.4, 5 mM MgSO_4 , 1 mM EGTA or 0.5 mM Ca^{2+} , 50 $\mu\text{g}/\text{ml}$ phosphatidyl serine, 0.2 mg/ml histone H1, and 50 μM $\gamma^{32}\text{P}$ -ATP (100–500 mCi/mmol). Ca^{2+} /calmodulin-dependent protein kinase assays were carried out in a final volume of 100 μl , containing 50 mM Tris, pH 7.4, 1 mM EGTA or 0.5 mM Ca^{2+} plus 0.02 mg/ml calmodulin, 0.2 mg/ml Synapsin I, and 50 μM $\gamma^{32}\text{P}$ -ATP (Palfrey et al., 1983). Peptide mapping of phosphorylated Synapsin I revealed that >90% of the Ca^{2+} /calmodulin-stimulated incorporation occurred in a fragment of M_r 35,000, indicating that we were measuring primarily CaM-kinase II activity. cAMP-dependent protein kinase was assayed according to the method of Walter et al. (1977). Assays were performed in a final volume of 100 μl , containing 50 mM Tris, pH 7.5, 10 mM MgSO_4 , 1 mM EGTA, 0.2 mg/ml histone f2b, 20 μM 8Br-cAMP, and 50 μM $\gamma^{32}\text{P}$ -ATP. In samples lacking 8Br-cAMP, 5 $\mu\text{g}/\text{ml}$ Walsh inhibitor was added as indicated. All assay mixtures were preincubated for 1 min at 30°C and initiated by addition of ATP. The reactions for PKC and calcium/calmodulin-dependent kinase were terminated by the addition of an SDS sample buffer after 1 min. cAMP-dependent protein kinase assays were terminated by the addition of a 50 mM EDTA/5 mM ATP stop solution after 2–5 min. In phosphorylation assays stopped by SDS-containing buffers,

Figure 3. Dose-response curve illustrating the acute effects of PDBu on depolarization-induced release of ^3H -NE from PC12 cells. Cells were pretreated with PDBu for 8 min and then for a further 4 min during stimulation with 70 mM K^+ . Some cells were also treated with the inactive phorbol ester 4 α PDD (1 μM). $\square$, PDBu; $\blacksquare$, 4 α PDD (1 μM). Points represent the means of 4 incubations.

phosphorylation of substrate was assessed using SDS-7.5% PAGE, cutting out the appropriate substrate bands, followed by liquid-scintillation counting. Samples stopped by EDTA/ATP were blotted on to 1.2×2.1 cm phosphocellulose paper squares (Whatman P81) and washed with water for 20 min prior to counting. Results are expressed in activity units (pmol/vol/min), where the volume referred to is the "equivalent volume" of cytosol or membrane (so that the values may be quantitatively compared). In our experiments these volumes were equivalent to 2.5–5 μg protein for both cytosol and membranes.

Results

Effects of chronic phorbol ester treatment on protein kinase C activity in PC12 cells

We began our studies by using the rat pheochromocytoma cell line PC12. These cells contained high concentrations of PKC activity (Fig. 1). This activity was normally associated with the cell cytoplasmic fraction, but a small amount of histone kinase activity was associated with the particulate fraction under control conditions. Cells differentiated with NGF contained 5–10-fold higher specific activity than those found in undifferentiated cells. In differentiated PC12 cells, virtually all the enzyme activity was also found in the cytoplasm. We next examined the effect of phorbol ester treatment on the distribution of PKC activity in undifferentiated and differentiated PC12 cells. Following the addition of phorbol 12-myristate-13-acetate (TPA) or phorbol-12,13-dibutyrate (PDBu) to the incubation medium, there was a rapid and complete translocation of PKC activity from the cytoplasm to the particulate fraction (Fig. 1). This occurred in both differentiated and undifferentiated PC12 cells. In the continued presence of phorbol esters, PKC activity associated with the particulate fraction declined, such that after several hours it had essentially returned to control levels. While particulate PKC activity was declining, cytoplasmic PKC activity remained low, indicating that PKC was not merely returning to the cytoplasm from the membrane. Long-term treatment of both differentiated and undifferentiated PC12 cells led to a similar depletion of PKC activity. Phorbol esters that are ineffective as activators of PKC (e.g., 4 α phorbol-12,13-didecanoate; 4 α PDD) did not cause enzyme translocation or down regulation. Similar results were obtained when another

Figure 4. Time course of the effect of TPA (1 μM) on the evoked release by ^3H -NE from PC12 cells. Cells were incubated with TPA (1 μM) for the times indicated. TPA was now removed by extensive washings (60 min with TPA-free incubation medium, then 30 min with TPA-free medium containing 1% fatty acid-free BSA; see Fig. 6). Cells were now challenged with 70 mM K^+ -containing medium with ($\square$) or without ($\blacksquare$) TPA (100 nM). Points represent the means of 4 incubations.

exogenous substrate, Synapsin I, was used instead of histone (Fig. 2). We also examined the effect of chronic phorbol ester treatment on cAMP-dependent and Ca^{2+} /calmodulin-dependent protein kinase activities. Under conditions in which PKC activity was entirely depleted from PC12 cells, there was little change in the activities of these other 2 protein kinases (Fig. 2), which indicated specificity in the effects of phorbol esters on protein phosphorylation systems.

Effects of chronic phorbol ester treatment on evoked catecholamine release from PC12 cells

We have previously identified 2 effects of PKC activation in PC12 cells (Harris et al., 1985). The first of these is the inhibition of voltage-sensitive Ca^{2+} influx. This observation has been confirmed in other laboratories (Di Virgilio et al., 1986; Messing et al., 1986) and has also been demonstrated in sensory neurons (Rane and Dunlap, 1986). The second action is the enhancement of voltage-dependent catecholamine release. Similar enhancement of evoked transmitter release by phorbol esters has also been reported in other systems (Pozzan et al., 1984; Harris et al., 1985; Publicover, 1985; Zurgil and Zisapel, 1985; Eusebi et al., 1986). We used this action of phorbol esters in PC12 cells as a bioassay to assess the functional consequences of PKC down regulation. Figure 3 illustrates how acute phorbol ester treatment enhanced the depolarization-induced release of ^3H -NE from PC12 cells by about 30%. At very high concentrations of phorbol ester ($>1 \mu\text{M}$), an inhibitory effect was observed. Phorbol esters that are inactive as stimulators of PKC did not enhance release (see also Harris et al., 1985). We next treated PC12 cells with active phorbol esters (TPA or PDBu) for increasing periods of time. Cells were then washed very thoroughly to remove any drug (see below) and challenged with 70 mM K^+ or a 70 mM K^+ -phorbol ester combination. As the period of phorbol ester treatment increased, there were 2 major effects (Fig. 4). First, the ability of phorbol esters to enhance the evoked release of ^3H -NE was lost; and second, the ability of a 70 mM K^+ depolarizing stimulus alone to release ^3H -NE steadily declined over the treatment period. Ultimately, 70 mM K^+ released only about half as much ^3H -NE in treated cells as in the control. The actual

Figure 5. Dose-response curves illustrating the effects of chronic PDBu treatment on 70 mM K⁺-induced ³H-NE release from PC12 cells. Cells were treated with PDBu at the concentrations indicated for 24 hr. The phorbol ester was then washed out (see Figs. 4, 6). Cells were then challenged with 70 mM K⁺ with or without PDBu. ■, 70 mM K⁺. □, 70 mM K⁺ + PDBu (1 μM). Some cells were treated with 4αPDD (1 μM) rather than PDBu for 24 hr and then stimulated with 70 mM K⁺ with (△) or without (▲) PDBu (1 μM). A further group of cells was treated with TPA (1 μM) rather than PDBu for 24 hr and then stimulated with 70 mM K⁺ with (◆) or without (◇) TPA (100 nM). Points are means of 4 incubations.

magnitude of this decrease varied somewhat from experiment to experiment, but could be as great as 60–70% (e.g., Fig. 5). The ability of chronic phorbol ester treatment to decrease transmitter release was dose-dependent, occurring over the same concentration range as the ability to down regulate PKC (Fig. 5).

We ran several control experiments to investigate issues that could possibly complicate the interpretation of these data. The first concerned the ability to wash out the phorbol ester. Thus, following chronic phorbol ester treatment we wished to remove the drug, which would yield a preparation of PKC-deficient cells that was not drug-contaminated. We investigated this by examining the washout of ³H-PDBu after treatment of cells with concentrations similar to those used for down regulation. Figure 6 shows that virtually all the PDBu could be removed by washing. This was also demonstrated using a “functional” assay (Table 1). Cells were challenged with 70 mM K⁺ to produce ³H-NE release. Following a wash period, a second challenge with 70 mM K⁺ produced a second phase of ³H-NE release. Phorbol

Figure 6. Washout of PDBu from PC12 cells. Cells were labeled with ³H-PDBu (1 μM) for 2 hr, rinsed 3 times, and then washed with PDBu-free incubation medium containing 1% fatty acid-free BSA for the times indicated.

esters added with either the first or second 70 mM K⁺ challenge enhanced release, as compared to control. If phorbol ester were added with the first 70 mM K⁺ challenge and the cells were then thoroughly washed, subsequent phorbol ester addition could still clearly augment the second 70 mM K⁺ challenge. This would presumably not be the case if the drug remained in the cells following the first challenge, as the cells would already be “augmented.”

We and others have also previously shown that upon differentiation with NGF, PC12 cells release less ³H-NE than do undifferentiated cells (Kongsamut and Miller, 1986). Furthermore, release becomes insensitive to blockade by dihydropyridine drugs, such as nitrendipine. It therefore seemed possible that chronic treatment with phorbol esters was initiating differentiation of the PC12 cells and that this could explain the lower evoked ³H-NE release. However, as can be seen in Figure 7, following chronic phorbol ester treatment, the release of ³H-NE from PC12 cells was still blocked by nitrendipine (1 μM). This is clearly different from the situation that occurs upon cell differentiation (Kongsamut and Miller, 1986). In conclusion, these experiments demonstrated that down regulation of PKC following chronic phorbol ester treatment is associated with a loss of the ability of phorbol esters to enhance catecholamine release, and with a reduction in the ability of depolarization to evoke neurotransmitter release.

The decrease in depolarization-induced release of ³H-NE observed following chronic phorbol ester treatment could be due to down regulation of PKC and the consequent lack of its participation in exocytosis. It is also conceivable, however, that chronic drug treatment leads to a long-term inhibition of voltage-dependent Ca²⁺ influx (Harris et al., 1985; Di Virgilio et al., 1986; Messing et al., 1986). In order to determine if an intracellular site of action was involved, we also examined the effect of phorbol ester treatment on the release of ³H-NE induced by the Ca²⁺ ionophore ionomycin (Fig. 8). Nitrendipine was included as well to block any Ca²⁺ influx through Ca²⁺ channels. Ionomycin stimulated ³H-NE release both in the presence and absence of extracellular Ca²⁺, although release was considerably greater in the presence of Ca²⁺. In either case, ionomycin-induced release was enhanced by phorbol ester. Following chronic phorbol ester treatment and washout, readdition of phorbol

Table 1. Effect of washout on PDBu stimulation of NE release from PC12 cells

First release	³ H-NE released (%)	Wash (4 for 15 min)	Second release	³ H-NE released (%)
None	17.9	None	None	14.6
None	18.5	None	1 μM PDBu	21.23
1 μM PDBu	27.9	None	None	14.4
1 μM PDBu	28.5	None	1 μM PDBu	19.4

PC12 cells were stimulated to release ³H-NE using 70 mM K⁺ with or without PDBu, as indicated. Between the first and second release stimulations, cells were incubated 4 times for 15 min each in incubation buffer containing 1% fatty acid-free BSA.

Figure 7. Effect of nitrendipine on 70 mM K⁺-induced release of ³H-NE from PC12 cells before and after chronic phorbol ester treatment. Cells were challenged with 70 mM K⁺ following culture for 24 hr with (□) or without (■) TPA (1 μM). Nitrendipine (Nit; 1 μM) was included as indicated. Results are means of 4 separate incubations.

ester no longer enhanced ionomycin-induced release. Furthermore, absolute levels of ionomycin-induced release were reduced below control levels. Thus, the results obtained using ionomycin as a stimulus were comparable to those obtained using depolarization. The data imply that the reduction in the extent of 70 mM K⁺-induced release following chronic phorbol ester treatment is at least partially due to the involvement of PKC in some aspect of exocytosis rather than to the modulation of Ca²⁺ entry.

Effects of chronic phorbol ester treatment on cultured rat sympathetic neurons

We now wished to extend our observations to authentic neurons. We therefore used primary cultures of sympathetic neurons taken from 2-d-old rat pups. These cultures are essentially free of non-neuronal cells (see Materials and Methods). As we have previously demonstrated, depolarizing stimuli evoked release of ³H-NE from these cells (Perney et al., 1986). We have shown that this evoked release is very sensitive to blockade by Cd²⁺ or by removal of external Ca²⁺. As in the case of PC12 cells, we observed that phorbol esters enhanced the 70 mM K⁺-induced release of ³H-NE (Fig. 9). Chronic treatment with phorbol esters led to the loss of the phorbol ester-induced enhancement of 70 mM K⁺-evoked release. Moreover, a large reduction in the percentage of ³H-NE release evoked by 70 mM K⁺ alone was obtained (Fig. 10). These results again parallel those found with PC12 cells. We also examined the effect of chronic phorbol ester treatment on PKC activity in sympathetic neurons. Cytosol fractions from these neurons contained PKC of high specific activity. Following chronic phorbol ester treatment, enzyme activity decreased dramatically, essentially to background levels in both the particulate and cytoplasmic fractions (Fig. 11). Chronic drug treatment had little effect on the activities of cAMP-dependent or Ca²⁺/calmodulin-dependent protein kinases (Fig. 11). The decline in PKC was also reflected in a reduction of endogenous protein phosphorylation. Untreated sympathetic neurons exhibited a major endogenous *M*_r 66,000 substrate for Ca²⁺/phospholipid-stimulated phosphorylation. After chronic drug treatment, incorporation of ³²P into this substrate dramatically decreased, substantiating the results obtained using exogenous PKC substrates (data not shown).

Discussion

The effects of phorbol esters on a wide variety of neuronal processes have aroused a great deal of interest in the potential

Figure 8. Effect of phorbol esters on ionomycin (5 μg/ml)-stimulated ³H-NE release from PC12 cells. Cells were stimulated with either 70 mM K⁺ or ionomycin in medium containing normal Ca²⁺ (1.3 mM) or 0 Ca²⁺ + 50 μM EGTA. All ionomycin experiments also contained nitrendipine (1 μM) to block any Ca²⁺ entry via Ca²⁺ channels. ■, Control (1.3 Ca²⁺). □, Chronic (24 hr) treatment with TPA (1 μM) followed by washout of phorbol ester (see Fig. 4). ■, Control (0 Ca²⁺). □, Chronic (24 hr) treatment with TPA (1 μM) followed by washout of phorbol ester (see Fig. 4). Results are means of 4 separate incubations. Total ³H-NE accumulated was $1.70 \times 10^5 \pm 2.04 \times 10^4$ cpm/well for control cells and $1.71 \times 10^5 \pm 1.23 \times 10^4$ cpm/well in TPA-treated cells. Mean $\pm$ SEM for 16 wells.

roles of PKC in neuronal function. In order to provide further evidence for the participation of PKC in these various activities, it would be advantageous to be able to selectively inhibit the enzyme in intact cells. Ideally this would be achieved pharmacologically, by the use of a specific antagonist. However, no specific blocker of PKC is available at this time. Drugs such as trifluoperazine and polymyxin B do inhibit PKC, but only in concentrations at which they may interfere with many other cellular functions, including those of other kinases. In the present study, we have demonstrated that PKC-free neuronal cells may be prepared by chronic phorbol ester treatment. As far as can be judged for protein kinase activities, such treatment appears to be specific for PKC. Analysis of cAMP-dependent and Ca²⁺/calmodulin-dependent kinases following drug treatment indicated no major changes in the activities of these enzymes.

Figure 9. Effect of acute phorbol ester treatment on the 70 mM K⁺-induced release of ³H-NE from cultures of rat sympathetic neurons. Assays were performed as in Materials and Methods. TPA at the concentrations indicated was added for 10 min prior to the 70 mM K⁺ challenge and also during 70 mM K⁺ stimulation. Points represent means $\pm$ SEM for 4 incubations.

Figure 10. Effect of chronic phorbol ester treatment on 70 mM K^+ -induced 3H -NE release from cultured rat sympathetic neurons. Cells were treated with TPA (1 μM) for the times indicated. The phorbol ester was then washed out (see Figs. 4, 6) and cells were challenged with 70 mM K^+ with (▨) or without (□) TPA (100 mM). Data represent the means $\pm$ SEM for 5 incubations.

In addition, both undifferentiated and differentiated PC12 cells and sympathetic neurons appeared perfectly normal and adhered quite normally to the substratum after chronic phorbol ester treatment. Furthermore, undifferentiated PC12 cells appeared to divide at a normal rate.

In most respects, our results for PKC activity resemble those already described for a number of non-neuronal cells when using similar concentrations of phorbol esters (Yamasaki et al., 1980; Jaken et al., 1981; Solanki et al., 1981; Yamasaki et al., 1982; Collins and Rozengurt, 1984; Rodriguez-Pena and Rozengurt, 1984; Ashendel, 1985; Ballester and Rosen, 1985; Palfrey and Waseem, 1985; Wickremasinghe et al., 1985; Fabbro et al., 1986; Glynn et al., 1986). In all of these cases, acute treatment of cells with phorbol esters or a DAG-generating agonist leads to the translocation of PKC from the cytoplasm to the particulate fraction (see also Kraft and Anderson 1983; Wolf et al., 1985; Guy et al., 1986; Liles et al., 1986; McArdle and Conn, 1986). This process occurs over a period of about 15 min. Further treatment with phorbol esters or agonists that activate the enzyme then leads to the depletion of total cellular PKC activity over a period of about 10–20 hr. This effect is seen in most but not all cell types (Ashendel, 1985). In previous studies, dividing cells, such as fibroblasts or tumor lines, have been used. It has been suggested that the ability to down regulate PKC might be associated with the state of differentiation of the cell, being apparent only in such relatively undifferentiated cases (discussed in Ashendel, 1985). However, we show here that the phenomenon is equally evident in nondividing sympathetic neurons and in differentiated PC12 cells, which divide very slowly. We have also observed similar down regulation of PKC in primary cultures of rat dorsal root ganglion neurons (unpublished observations).

The biochemical basis for the down regulation of PKC is unclear at this time, but probably involves proteolysis of the enzyme. Ballester and Rosen (1985) used immunoprecipitation of the enzyme to study the effect of chronic phorbol ester treatment on the fate of radiolabeled PKC in GH₃ pituitary tumor cells. They observed that phorbol ester treatment increased the rate of enzyme removal, presumably because of degradation, at least 20-fold. It is probable that the activation of PKC and its associated binding to membranes also trigger its degradation. Indeed, a brain protease has been described that cleaves PKC to a Ca^{2+} /phospholipid-independent form, designated "PKM"

Figure 11. Effect of chronic phorbol ester treatment on the activities of various protein kinases in rat sympathetic neurons. PKC was assayed in separate cytosolic and particulate fractions. PKA and Ca^{2+} /calmodulin-dependent kinase activity was assessed in total cell homogenates. ■, Control. □, TPA (1 μM) for 24 hr.

(Kishimoto et al., 1983). This protease is stimulated by Ca^{2+} , phospholipid, and DAG. The existence of PKM should be revealed as an increase in Ca^{2+} -independent histone kinase activity in subfractions. However, we did not observe such an increase. Thus, it is possible that PKC is degraded rapidly to an inactive form in neurons.

One of the suggested roles for PKC in neuronal function is in the regulation of neurotransmitter release. In many cases, phorbol esters can be shown to enhance evoked transmitter release (Pozzan et al., 1984; Harris et al., 1985; Publicover, 1985; Zurgil and Zisapel, 1985; Eusebi et al., 1986). Furthermore, phorbol esters also augment the secretion of products from non-neuronal cells, such as mast cells and most endocrine and exocrine cells (Kojima et al., 1983; Katakami et al., 1984; Koenig et al., 1984; Yamatani et al., 1985; Ronning and Martin, 1986; Shaw and Hanson, 1986; Stutchfield et al., 1986; Takuma and Ichida, 1986; Hishikawa et al., 1985; Turgeon and Waring, 1986). Effects of phorbol esters on transmitter release from neurons do not appear to be due to an enhancement of voltage-sensitive Ca^{2+} influx (but see Wakade et al., 1986). Indeed, in vertebrate neurons, phorbol esters have been reported to block Ca^{2+} channels (Rane and Dunlap, 1986). It has been shown that in permeabilized adrenal medullary cells, phorbol esters allow secretion to occur at lower than normal concentrations of Ca^{2+} (Knight and Baker, 1983; Pocotte et al., 1985). This indicates that the site of phorbol ester-induced augmentation may be 1 or more of the proteins actually involved in vesicle secretion.

In PC12 cells, phorbol esters augment 70 mM K^+ -evoked 3H -NE release but have virtually no effect in 5 mM K^+ (Harris et al., 1985). We used this phorbol ester effect as a functional assay to monitor the consequences of PKC down regulation. Indeed, at a time corresponding to the commencement of PKC down regulation, the ability of phorbol esters to enhance release was lost, as we had expected. What was unexpected, however, was the observation that as PKC down regulation proceeded further, evoked release declined to levels well below those seen in control cells. The fact that phorbol esters acutely augment evoked catecholamine release and that PKC-deficient cells exhibit a reduced level of depolarization-induced release implies that PKC nor-

mally participates in the process of stimulus–secretion coupling. It could be supposed that the process of synaptic transmission was altogether too rapid to allow for such an intermediate step. Interestingly, however, depolarization of synaptosomes is associated with increased inositol phospholipid hydrolysis, leading to an increase in diacylglycerol (Kendall and Nahorski, 1985; Gusovsky et al., 1986) and to activation of PKC, as reflected in the Ca^{2+} -dependent stimulation of a specific PKC substrate (Wu et al., 1982). It can be seen from the results reported here that following complete depletion of PKC from PC12 cells or sympathetic neurons, substantial depolarization-evoked secretion still occurs. This PKC-independent process is presumably also regulated by Ca^{2+} and is conceivably mediated by activation of calmodulin and subsequent stimulation of calmodulin-dependent kinase(s) (Llinas et al., 1985). In addition, the PKC-dependent and independent pathways may affect different populations of secretory vesicles. Thus, the ability to release 1 population of vesicles might be lost following PKC down regulation. Certain other possible explanations appear less likely. For example, we may be observing the result of a shift in the affinity for Ca^{2+} of a key component of the release apparatus in PKC-deficient cells. Such a change would be the opposite of the phorbol ester-induced increase in affinity observed in permeabilized adrenal medullary cells (see above) (Knight and Baker, 1983; Pocotte et al., 1985). Also, chronic phorbol ester treatment could lead to a down regulation of Ca^{2+} channels. However, our results with ionomycin appear to mitigate against this hypothesis and indicate that the site of phorbol ester action in these studies is principally at a point beyond that of Ca^{2+} entry. Yet another possibility is that chronic phorbol ester treatment prevents the appropriate uptake and storage of ^3H -NE by the cells. Our observations indicate that chronically treated cells take up as much ^3H -NE as control cells (see Fig. 8 legend), but we do not know if this catecholamine is appropriately stored in secretory vesicles. We are currently measuring the absolute pool sizes of stored NE to ascertain if these are modified upon phorbol ester treatment.

We conclude that the most parsimonious interpretation of our data at this time is that PKC is an important element in the neurotransmitter release process. There are clearly similarities between the acute effects of phorbol esters reported here and elsewhere and the increase in transmitter release associated with long-term potentiation in the hippocampus (Malenka et al., 1986a) and in sympathetic ganglia (Briggs et al., 1985; Kuba and Kamamoto, 1986). Indeed, it has been hypothesized that PKC is important in the genesis of this type of synaptic plasticity. The ability to remove PKC from such neurons could be most helpful in the investigation of this phenomenon.

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