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Protein Kinase C Evokes Quantal Catecholamine Release from PC12 Cells via Activation of L-type Ca^{2+} Channels*

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Application of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to PC12 cells under resting conditions evoked quantal catecholamine secretion, as detected amperometrically. This effect was not mimicked by 4α-phorbol-12,13-didecanoate, another phorbol ester, which is inactive with respect to protein kinase C activation, and was prevented by the protein kinase C inhibitor bisindolylmaleimide. TPA also caused a rise of $[Ca^{2+}]_i$ in Fura-2-loaded PC12 cells, and again this was not mimicked by 4α-phorbol-12,13-didecanoate and could be blocked by bisindolylmaleimide. TPA-evoked secretion was entirely dependent on extracellular Ca^{2+} and was fully abolished by nifedipine, as were TPA-induced rises of $[Ca^{2+}]_i$. Resting membrane potential, monitored using perforated patch recordings, was unaffected by TPA. However, a small (6–8 mV) hyperpolarizing shift in the voltage dependence of Ca^{2+} channel currents (determined using whole-cell patch clamp recordings) was induced by TPA, and this could be fully prevented by nifedipine. In contrast to results with depolarizing stimuli, which evoke exocytosis because of Ca^{2+} influx through *N*-type channels in these cells, the present results indicate that protein kinase C activation leads directly to quantal catecholamine secretion in the absence of depolarizing stimuli via a selective shift in the activation of L-type Ca^{2+} channels.

Regulated release of transmitters and hormones from neurons and neuroendocrine cells classically involves rapid, local rises of $[Ca^{2+}]_i$ due to Ca^{2+} influx through voltage-gated Ca^{2+} channels. The entire process from Ca^{2+} influx to exocytosis is tightly controlled and can be modulated at numerous stages by receptors and protein kinases to allow a sophisticated degree of control of transmitter release. Protein kinase C (PKC)[1] is well known to modulate evoked transmitter release, and most reports indicate that it potentiates exocytosis, for example at hippocampal synapses (1, 2) and motor nerve terminals (3). At present, the site or mechanism of action of PKC to potentiate regulated exocytosis is not fully determined, but several suggestions have been put forward, including actions of PKC to phosphorylate membrane-bound exocytotic proteins (4), to delay repolarization following an action potential via inhibition of K^+ channels (as seen in cerebellar granule neurons (5)), or to potentiate Ca^{2+} influx through voltage-gated Ca^{2+} channels (6, 7). Additionally, PKC has been shown to enhance the readily releasable pool of vesicles (8) so that the probability of vesicle-plasma membrane fusion is increased (9).

Recent studies have provided the novel observation that PKC can promote exocytosis from excitable neuroendocrine cells in the absence of a rise of $[Ca^{2+}]_i$ (10, 11). Thus, in rat pituitary gonadotrophs, peptide hormone secretion could be activated by two separate pathways; the first pathway is Ca^{2+}-dependent and insensitive to PKC inhibition, and the second pathway is activated by phorbol ester, blocked by PKC inhibition, and virtually unaffected by intracellular Ca^{2+} chelation (10). Thus, activation of any receptor coupled to phospholipase C has the potential to activate this Ca^{2+}-independent, PKC-mediated secretory pathway. PKC potentiation of exocytosis is better studied in non-excitable cells that lack voltage-gated channels, but to date little is known about the possible importance of this pathway in excitable cells beyond the studies of gonadotrophs noted above.

In the present study, we have examined the effects of PKC activation on catecholamine secretion from the clonal pheochromocytoma cell line PC12. This excitable cell line is derived from adrenal chromaffin tissue and has proved valuable as a model system for studying stimulus-evoked exocytosis. PKC activation has long been known to enhance evoked catecholamine release from these cells (12, 13), but the underlying mechanisms remain unknown, as do the effects of PKC under non-stimulating conditions. Our results indicate that PKC activation evokes secretion under resting conditions (*i.e.* in the absence of recognized stimuli) and that this arises because of a specific, hyperpolarizing shift in the activation of L-type Ca^{2+} channels.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2, 95% air in RPMI 1640 culture medium containing L-glutamine. Medium was supplemented with 20% fetal calf serum and 1% penicillin/streptomycin (Life Technologies, Inc.). Cells were passaged every 7 days by resuspension (1:2) in fresh medium and used for up to 20 passages. The prolonged period without medium change enhanced evoked catecholamine release (14). When used for experiments, cells were transferred to smaller flasks in 10 ml of medium, and 1 μM dexamethasone (Sigma, from a stock solution of 1 mM in ultrapure water) was applied for 72–96 h to further enhance catecholamine secretion (15).

Each experimental day, aliquots of PC12 cells were plated onto poly-D-lysine-coated 22 x 22-mm coverslips at a density of 0.5–1.0 × 10^5 cells per coverslip and allowed to adhere for ~1 h. Fragments of coverslip were then transferred to a recording chamber (volume of ~80 μl), which was continually perfused under gravity (flow rate of 1–2 ml/min) with a control solution of (in mM): NaCl 135, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2.5, Hepes 5, and glucose 10 (pH 7.4, osmolarity adjusted to ~300 mosM with sucrose, 21–24 °C). Ca^{2+}-free solutions contained 1 mM EGTA and no added Ca^{2+}. All drugs were applied in the perfusate. The effects of PKC activation were investigated using the phorbol esters 12-*O*-tetra-

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[1] The abbreviations used are: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 4α-PDD, 4α-phorbol-12,13-didecanoate; BIM, bisindolylmaleimide; ω-CgTx, ω-conotoxin GVIA; ω-AgaTx, ω-agatoxin IVA.

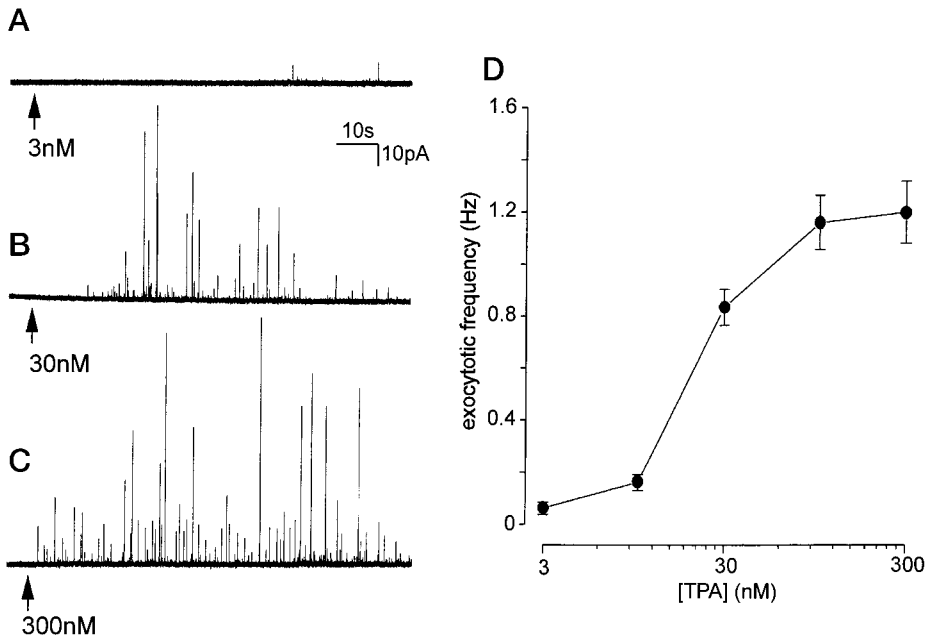

FIG. 1. **The phorbol ester TPA evokes catecholamine release from PC12 cells.** *A–C*, amperometric recordings from separate, individual PC12 cells. At the point indicated by the *arrows*, the perfusate was exchanged for one containing TPA at the concentrations indicated. *Scale bars* apply to all traces. *D*, concentration-response relationship for the effects of TPA to evoke catecholamine release. Each plotted point is the mean ± S.E. exocytotic frequency determined from between 8 and 12 cells in each case.

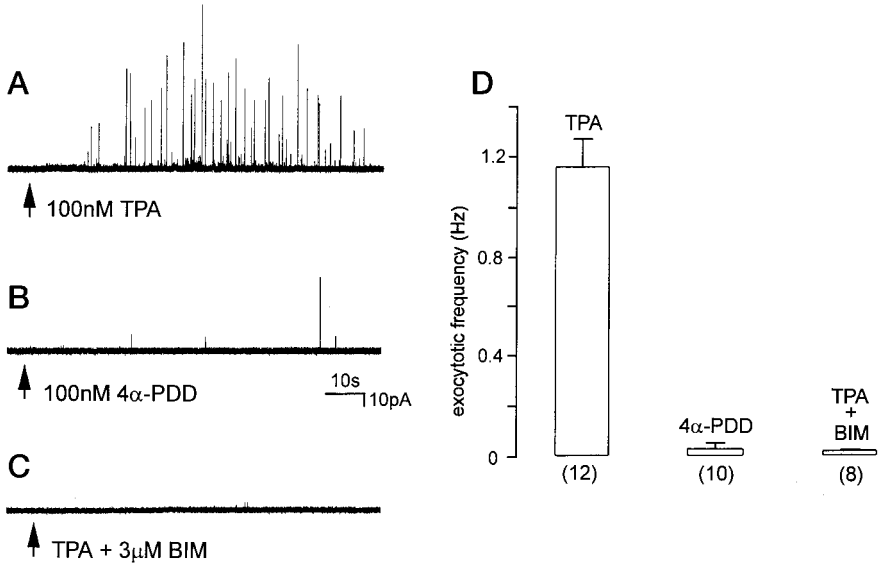

FIG. 2. **The effects of TPA are mediated by activation of PKC.** *A–C*, example amperometric recordings from individual PC12 cells during phorbol ester application. At the points indicated by the *arrows*, cells were exposed to either 100 nM TPA (*A*), 100 nM 4α-PDD (*B*), or 100 nM TPA in the presence of 3 μM BIM (*C*). *D*, bar graph showing the mean exocytotic frequency (with *vertical S.E. bar*, taken from the number of cells indicated in *parentheses* in each case) evoked by phorbol esters under the same conditions as in *A–C*, as indicated.

decanoylphorbol-13-acetate (TPA) and 4α-phorbol-12,13-didecanoate (4α-PDD). These were pre-dissolved in ethanol at 0.1 mM and added to the perfusate at the final concentrations indicated. In some studies, the PKC inhibitor bisindolylmaleimide (BIM, 3 μM) was added to the cells in the same manner.

Amperometry—Carbon fiber microelectrodes (proCFE, Dagan Instruments, Minneapolis, MN) with a diameter of 5 μm were positioned adjacent to individual PC12 cells using a Narishige MX-2 micromanipulator and were polarized to +800 mV to allow oxidation of released catecholamine. Resulting currents were recorded using an Axopatch 200A amplifier (with extended voltage range), filtered at 1 kHz, and digitized at 2 kHz before storage on computer. All acquisition was performed using a Digidata 1200 interface and Fetchex software from the pClamp 6.0.3 suite (Axon Instruments). Exocytosis is expressed as the frequency of quantal events; frequency was determined by counting the number of events over an 85-s period, 5 s after switching to test solutions, using Mini Analysis Program (Synaptosoft Inc., Leonia, NJ).

Electrophysiology—Ca^{2+} channel currents were recorded using the whole-cell patch clamp technique. The perfusate was of composition (in mM): NaCl 110, CsCl 5, $MgCl_2$ 0.6, $BaCl_2$ 20, Hepes 5, glucose 10, and tetraethylammonium chloride 20 (pH 7.4). Osmolarity of the perfusate was adjusted to 300 mosM by addition of sucrose. Patch pipettes (5–7-megohms resistance) were filled with a solution of (in mM): CsCl 130, EGTA 1.1, $MgCl_2$ 2, $CaCl_2$ 0.1, NaCl 10, Hepes 10, and Na_2ATP 2 (pH 7.2). Membrane potential was monitored using perforated patch recordings, for which pipettes were filled with 120 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM NaCl, 11 mM EGTA, 11 mM Hepes, and 240 μg/ml amphotericin B (from a stock solution of 60 mg/ml in dimethyl sulfoxide) (pH 7.2). Cells were perfused with the control solution used for amperometric recordings (containing 5 mM K^+; see "Cell Culture").

$[Ca^{2+}]_i$ Measurements—Cells were preincubated for 1 h at 21–24 °C in control solution containing 4 μM Fura-2/AM. Samples were then placed in the perfusion chamber, and changes in $[Ca^{2+}]_i$ were indicated from the fluorescence emitted at 510 nm due to alternate excitation at 340 and 380 nm using Joyce Loebl PhoCal apparatus (Applied Imaging). Calibration of fluorescence signals into absolute $[Ca^{2+}]_i$ values was performed as described previously (16).

All data are expressed as means ± S.E., and statistical comparisons were made using unpaired *t* tests, with $p < 0.05$ being considered significant.

RESULTS

To investigate the effects of PKC activation on catecholamine release, the PKC-activating phorbol ester TPA was bath-applied during amperometric recordings. Fig. 1*A* indicates that TPA caused the appearance of spike-like amperometric events, each of which corresponds to the oxidization of the released contents of a single vesicle of catecholamine (17, 18). The effects of TPA were clearly concentration-dependent (Fig. 1*B*),

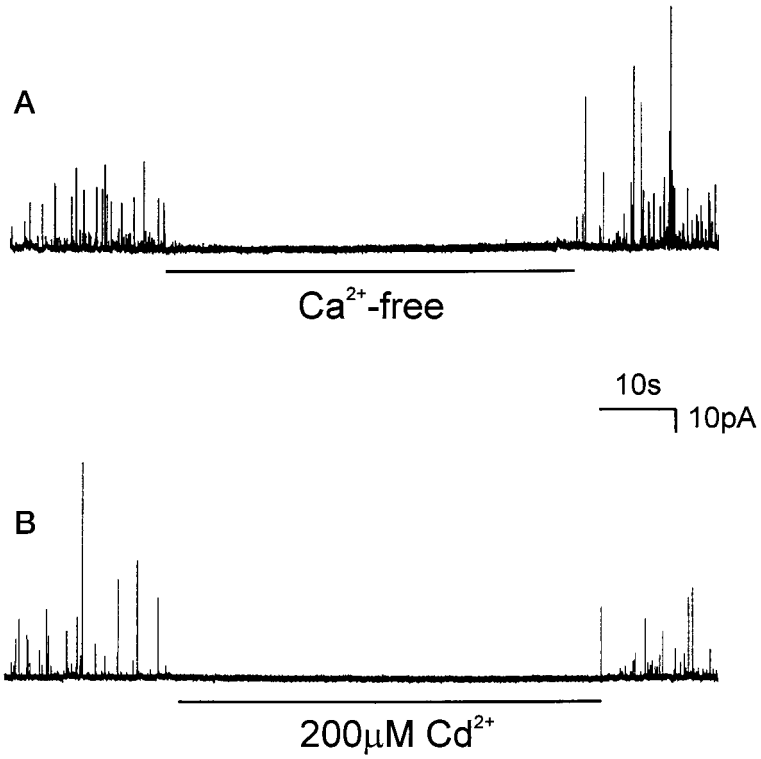

FIG. 3. **Ca^{2+} dependence of PKC-mediated exocytosis.** Both traces (*A* and *B*) show ongoing secretion evoked from 2 different PC12 cells by exposure to 100 nM TPA. For the periods indicated by the *horizontal bars*, Ca^{2+} was removed from the perfusate and replaced with 1 mM EGTA (*A*), or Cd^{2+} (200 μM) was applied in the continued presence of Ca^{2+} (*B*). *Scale bars* apply to both traces.

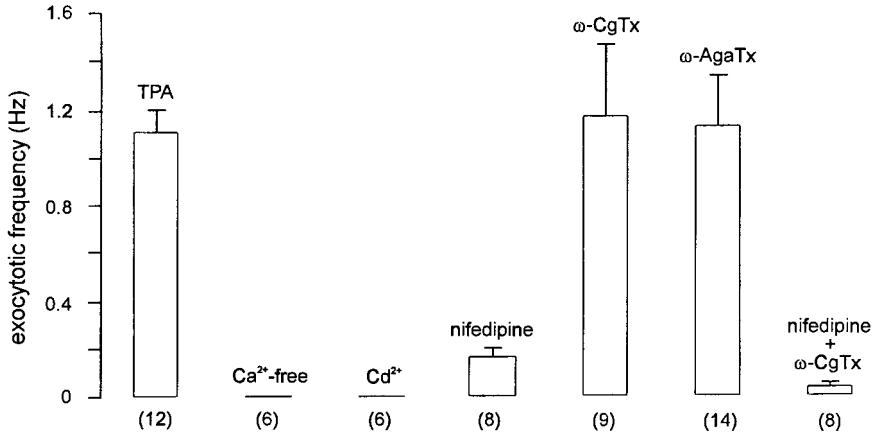

FIG. 4. **Bar graph showing the mean number of secretory events (with *vertical S.E. bars*) recorded over an 85-s period.** Shown are the mean number of secretory events evoked by TPA in the absence of Ca^{2+} channel blockers (*left-hand bar*), in Ca^{2+}-free solution (containing 1 mM EGTA), in the presence of 200 μM Cd^{2+} or 2 μM nifedipine, and following a 10-min pretreatment period with 1 μM ω-CgTx, 200 nM ω-Aga-IVA, or nifedipine together with ω-CgTx. The number of cells tested in each case is shown in *parentheses*.

with detectable exocytosis evident at a TPA concentration as low as 3 nM and saturation of the response at 100 nM TPA. As shown in Fig. 2, two pieces of evidence indicate that the effects of TPA to evoke catecholamine secretion were largely mediated by activation of PKC. Firstly, the effects of TPA were not mimicked by 4α-PDD, a phorbol ester which is not biologically active with respect to PKC activation (Fig. 2, *B* and *D*). Secondly, the secretagogue action of TPA could be almost completely inhibited by co-application of 3 μM BIM (Fig. 2, *C* and *D*). Although very small amounts of secretion were observed in response to 4α-PDD or to TPA in the presence of BIM, the results of Fig. 2 together indicate that the dominant effect of TPA to evoke catecholamine secretion was via PKC activation.

To examine whether Ca^{2+} influx was required for PKC-mediated exocytosis from PC12 cells, we first initiated secretion by exposure of cells to 100 nM TPA and then, in its continued presence, exchanged the perfusate for one which had no added Ca^{2+} but contained 1 mM EGTA. Under these conditions (exemplified in Fig. 3*A*, representative of six such recordings), exocytosis was abruptly, completely, and reversibly inhibited. Similarly, when, instead of removing extracellular Ca^{2+}, we added 200 μM Cd^{2+} to the perfusate, complete and reversible inhibition of secretion was observed (*e.g.* Fig. 3*B*, representative of six recordings). These findings indicated that PKC-mediated secretion from PC12 cells was fully dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels.

We and others have previously shown that PC12 cells possess functional L-, N-, and P-type Ca^{2+} channels (19, 20). In response to various stimuli, including depolarization via exposure to high K^+-containing solutions, Ca^{2+} influx through ω-CgTx-sensitive N-type channels is of primary importance, whereas Ca^{2+} influx through other channel types contributes far less to exocytosis (with the notable exception of acidic stimuli, which evoke secretion mediated primarily via P-type Ca^{2+} channels (21)). To investigate whether PKC-mediated secretion was mediated by any specific Ca^{2+} channel type, we investigated the ability of 100 nM TPA to evoke secretion in the presence of various selective Ca^{2+} channel blockers. Fig. 4 summarizes our findings. In contrast to the effects of other secretagogues (20, 21), PKC-mediated secretion was unaffected by pretreatment of cells with either ω-CgTx or ω-AgaTx. Instead, secretion was reduced by approximately 85% in the presence of nifedipine (Fig. 4). A minor contribution to secretion by N-type channels was seen by pretreatment of cells with

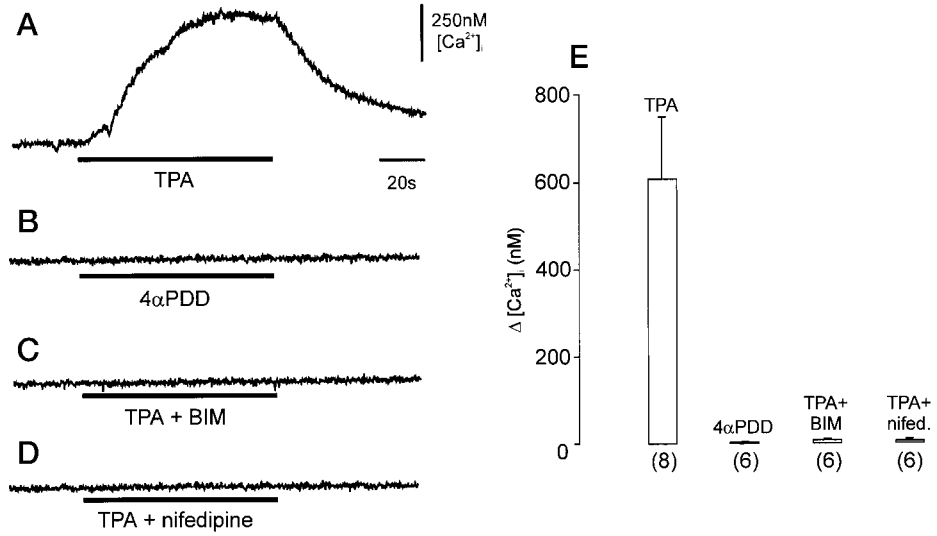

FIG. 5. **PKC activation raises $[Ca^{2+}]_i$ in PC12 cells.** *A–D*, $[Ca^{2+}]_i$ measurements determined in four separate PC 12 cells. During recordings, cells were exposed (for the periods indicated by the *horizontal bars*) to 100 nM TPA (*A*), 100 nM 4α-PDD (*B*), 100 nM TPA in the presence of 3 μM BIM (*C*), or 100 nM TPA in the presence of 2 μM nifedipine (*D*). *Scale bars* apply to all traces. *E*, bar graph showing the mean ± S.E. rises of $[Ca^{2+}]_i$ under the four conditions illustrated in *A–D*. Values were calculated in each case from the number of cells indicated in *parentheses*.

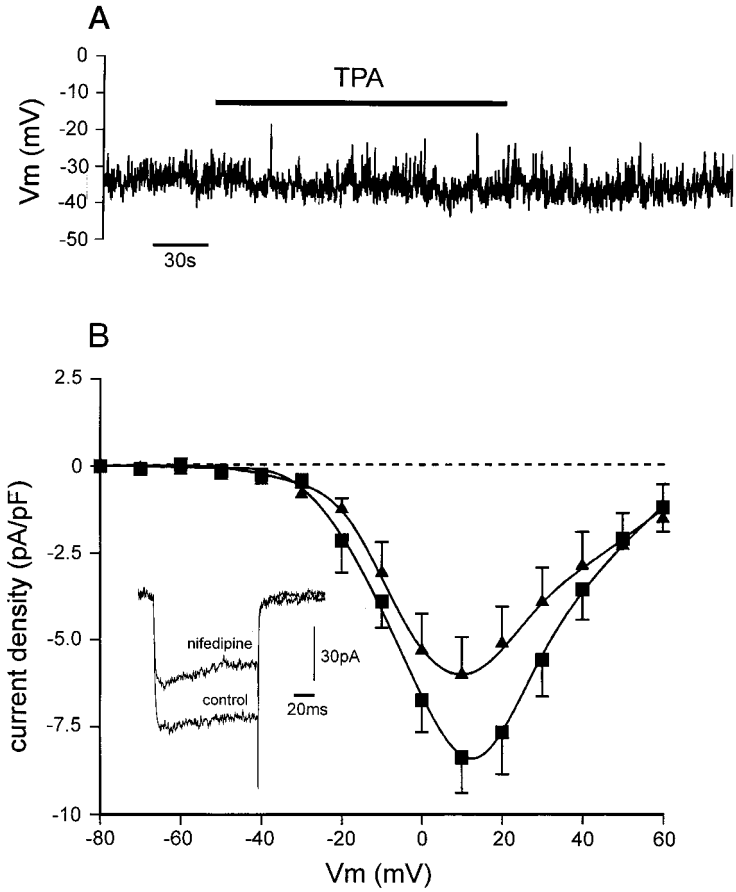

FIG. 6. *A*, example recording of membrane potential from a PC12 cell, recorded using the perforated patch technique. TPA (100 nM) was applied for the period indicated by the *horizontal bar*. *B*, Ca^{2+} channel current-voltage relationships obtained from PC12 cells before (*squares*) and during (*triangles*) bath application of 2 μM nifedipine. Each plotted point is the mean (with *vertical S.E. bar*) taken from 10 cells in each case. The *inset* shows example currents evoked by step depolarizations to +10 mV before and during nifedipine application, as indicated. *Vm*, membrane voltage; *pF*, picofarads.

ω-CgTx in the presence of nifedipine, but the most important finding of this pharmacological study was that Ca^{2+} influx through L-type channels was the major contributory route of Ca^{2+} influx in PKC-mediated catecholamine secretion.

To examine the involvement of L-type Ca^{2+} channels in PKC-mediated secretion in further detail, we investigated the effects of TPA on $[Ca^{2+}]_i$. As illustrated in Fig. 5*A*, bath application of 100 nM TPA caused large, reversible rises of $[Ca^{2+}]_i$ of around 600 nM (basal $[Ca^{2+}]_i$ was 136 ± 12 nM; n = 26). By contrast, such effects were not observed in cells exposed to 100 nM 4α-PDD (Fig. 5*B*) or to TPA in the presence of 3 μM BIM (Fig. 5*C*), indicating that the ability of TPA to raise $[Ca^{2+}]_i$ was due to activation of PKC. Furthermore, the effects of TPA on $[Ca^{2+}]_i$ were blocked by co-application of 2 μM nifedipine. These findings, summarized in Fig. 5*E*, confirm the conclusion that PKC activation stimulates Ca^{2+} influx specifically through L-type Ca^{2+} channels.

The simplest interpretation of the findings described above is that PKC activation leads to membrane depolarization that is sufficient to activate L-type but not other types of voltage-gated Ca^{2+} channels. To investigate this, we firstly used perforated patch recordings to monitor the membrane potential of PC12 cells while bathed in solutions containing 5 mM K^+ (as used for amperometric and fluorometric recordings). Fig. 6*A*, representative of six such recordings, illustrates the finding that TPA (100 nM) was completely without effect on membrane potential.

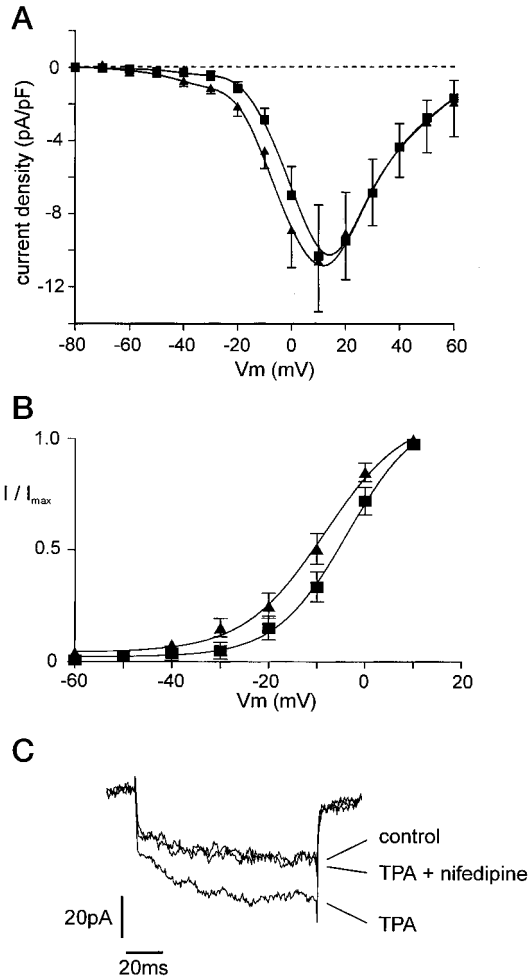

FIG. 7. *A*, Ca^{2+} channel current-voltage relationships obtained from PC12 cells before (*squares*) and during (*triangles*) bath application of 100 nM TPA. Each plotted point is the mean (with *vertical S.E. bar*) taken from 14 cells in each case. *B*, activation curves plotted from data shown in *A*. For each cell, the current was normalized to the peak amplitude (taken at +10 mV) before (*squares*) and during (*triangles*) bath application of 100 nM TPA. *C*, example currents recorded at a test potential of −20 mV before and during TPA application (100 nM) and then during application of 2 μM nifedipine in the continued presence of TPA, as indicated. *Vm*, membrane voltage.

Conventional whole-cell recordings were employed next to investigate the contribution to total Ca^{2+} current that could be attributed to L-type channels. Current-voltage relationships were constructed before and during bath application of 2 μM nifedipine. In 10 cells (Fig. 6*B*), nifedipine reduced Ca^{2+} currents by ~31% (as determined at +10 mV), and currents were similarly reduced over most of the activating range of test potentials.

Bath application of 100 nM TPA had little or no effect on Ca^{2+} current amplitudes at the peak of the current-voltage relationship, as determined in another 14 cells (Fig. 7*A*). However, a clear enhancement of amplitude was noted at more negative activating test potentials. This is seen more clearly when current amplitudes are normalized to their peak value (*i.e.* amplitude = 1 at +10 mV for each cell, and currents are then plotted as a fraction of this value at other test potentials). Clearly, TPA caused a negative shift of some 6–8 mV in current activation. To investigate this further, we examined the ability of TPA to enhance currents evoked by small step depolarizations (to −20 mV). As illustrated in Fig. 7*C* (see also Fig. 7*A*), TPA caused a marked enhancement of currents, and this enhancing effect was abolished when nifedipine was applied in the continued presence of TPA. This indicated that the negative shift in activation caused by TPA arose via a predominant, if not exclusive, effect on L-type channels.

DISCUSSION

PKC is well established as a powerful modulator of exocytosis in a variety of cell types. It is known to phosphorylate numerous proteins associated with the process of regulated secretion, including the F-actin-based cytoskeleton and sites within the SNAP/SNARE complex associated with vesicle docking and fusion (reviewed in Ref. 22); in addition, the effects of PKC on ion channel activity are widely documented (23). Most commonly, PKC activation potentiates evoked exocytosis, and this is true for PC12 cells when exocytosis is stimulated by depolarizing levels of extracellular K^+ (*e.g.* Ref. 13). However, the effects of PKC activation on secretion under non-stimulating conditions have not previously been studied in this model secretory system. We were prompted to investigate this by the recent report that PKC activation can, in some cell types, evoke exocytosis in the absence of a change in $[Ca^{2+}]_i$ (10), possibly by enhancing the Ca^{2+} sensitivity of the secretory apparatus.

Our results indicate that activation of PKC by the phorbol ester TPA can indeed evoke quantal catecholamine release from PC12 cells in the absence of a recognized depolarizing stimulus (Fig. 1). However, the secretory responses were not attributable to an enhanced Ca^{2+} sensitivity of the secretory apparatus, so that secretion was stimulated at basal $[Ca^{2+}]_i$, as is the case for gonadotrophs (10). Instead, the PKC-mediated secretion we observed was entirely dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels, as indicated by the complete abolition of PKC-mediated secretion following removal of extracellular Ca^{2+} or application of Cd^{2+} (Fig. 3). Moreover, the use of selective Ca^{2+} channel blockers established that Ca^{2+} influx specifically through nifedipine-sensitive L-type Ca^{2+} channels accounted for PKC-mediated rises of $[Ca^{2+}]_i$ and exocytosis. This result was particularly surprising in light of our previous studies with these cells that have indicated that N-type channels are of primary importance in the secretory responses to raised external $[K^+]$ and acute hypoxia (20, 24, 25) and that both P/Q-type and N-type channels mediate secretion evoked by acidosis (21). These previously examined stimuli all evoke secretion via membrane depolarization, with little or no influence of L-type channels being detected. By contrast, PKC activation, as reported here, selectively stimulates Ca^{2+} influx through L-type Ca^{2+} channels without causing membrane depolarization (Fig. 6*A*). Our patch clamp studies indicate that this arises because of a leftward (hyperpolarizing) shift in the activation of Ca^{2+} channels in these cells. Furthermore, the TPA enhancement of currents evoked by small depolarizations was reversed by nifedipine, suggesting that the shift in activation was predominantly, if not exclusively, an effect on L-type channels.

Activation of protein kinase C has been shown to enhance Ca^{2+} channel activity in other preparations, including enhancement of neuronal L-type Ca^{2+} channels (6, 7). However, other studies of the effects of PKC activation on Ca^{2+} channel activity have been confounded by non-PKC-mediated actions of phorbol esters; thus, phorbol esters possibly (particularly at higher concentrations) can exert direct effects on Ca^{2+} channels (26, 27). Furthermore, detailed studies of the effects of PKC on specific neuronal Ca^{2+} channel kinetics (including effects on the voltage dependence of channel activation) are lacking. The only detailed studies have been performed using cardiac L-type channels, where PKC caused marked current enhancement (*e.g.* Ref. 28), but this current enhancement was attributed to phosphorylation of an N-terminal region lacking in neuronal L-type Ca^{2+} channels.

A recent study has shown that the neurotrophins nerve growth factor and brain-derived neurotrophic factor have an acute effect to enhance L-type Ca^{2+} channel activity in PC12 cells, and that this effect is mediated at least in part by PKC activation (29). Furthermore, the enhancement may, as in the present study, lead to activation of L-type channels without depolarization, because such treatment directly stimulates $^{45}Ca^{2+}$ uptake into PC12 cells via PKC activation (30).

The strong coupling of secretion to L-type Ca^{2+} channels described here following PKC activation might be considered surprising given that this class of Ca^{2+} channels has not been found to associate with the SNARE complex (31). By contrast, N-type Ca^{2+} channels (which more commonly mediate stimulus-secretion coupling in these cells; Refs. 20, 21) are co-localized with neurotransmitter sites (32). However, both N-type channels and SNARE proteins can be phosphorylated by PKC (33–35), and such phosphorylation of the synaptic protein interaction peptide site, located within the intracellular linking loop between domains II and III of the N-type channel α_{1B} subunit, inhibits binding of syntaxin-1 and SNAP-25 proteins (36, 37). Thus, despite co-localization of N-type channels with vesicle docking sites, PKC phosphorylation would reduce or abolish the influence of Ca^{2+} influx through these channels on exocytosis. P/Q-type channel α subunits also possess a synaptic protein interaction sequence (38), and so it is likely that their influence on secretion is also minimized. These facts, coupled with the lack of depolarization caused by TPA, would account for the lack of significant involvement of N- and P/Q-type channels in mediating secretion in the present study. Instead, the hyperpolarizing shift in activation of L-type Ca^{2+} channels appears to cause a substantial rise of $[Ca^{2+}]_i$, which is sufficient to evoke exocytosis despite the lack of an obviously close association of vesicle docking sites with L-type channels, as evidenced by our previous studies (20).

In summary, PKC activation stimulates catecholamine exocytosis in the absence of depolarizing stimuli and without causing depolarization itself. The secretory response is entirely dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels and, in contrast to the effects of other secretagogues, appears to be mediated almost entirely by L-type Ca^{2+} channels. Such a recruitment of L-type channels occurs via a hyperpolarizing shift in their voltage dependence. This modest effect on channel activity clearly has a marked functional consequence in these cells, and similar PKC-mediated effects on Ca^{2+} channels and their coupling to exocytosis are worthy of further study in other secretory systems.

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