

Phorbol Esters Enhance Exocytosis from Chromaffin Cells by Two Mechanisms

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Abstract: Treatment with phorbol esters such as 12-*O*-tetradecanoylphorbol acetate (TPA) rapidly enhances [³H]nor-epinephrine secretion from digitonin-permeabilized adrenal chromaffin cells. When TPA treatment was prolonged for several hours, a second distinct enhancing effect was observed. This later enhancement was most prominent at intracellular Ca²⁺ concentrations of 3–30 μM, and did not require the continued presence of membrane-bound protein kinase C for its expression. The effect could be elicited by as little as 30-min exposure to TPA, followed by several hours in TPA-free medium. This effect of TPA was blocked by actinomycin D and cycloheximide, indicating a requirement for RNA and

protein synthesis. Similar effects were seen when intact cells that had been pretreated with TPA were stimulated to secrete by depolarizing concentrations of K⁺. Thus, protein kinase C enhances secretion by two mechanisms. One is rapid and probably reflects the effects of immediate protein phosphorylation. The other occurs over several hours and requires gene transcription and protein synthesis. **Key Words:** Protein kinase C—Phorbol ester—Chromaffin cell—Digitonin-permeabilized—Exocytosis—Catecholamine. **Bittner M. A. and Holz R. W.** Phorbol esters enhance exocytosis from chromaffin cells by two mechanisms. *J. Neurochem.* **54**, 205–210 (1990).

Protein kinase C has been proposed to play a role in exocytosis from a variety of cell types, including platelets (Castagna et al., 1982), neutrophils (Kajikawa et al., 1983), and adrenal chromaffin cells (Knight and Baker, 1983; Pocotte et al., 1985; Brocklehurst et al., 1985). The phorbol ester 12-*O*-tetradecanoylphorbol acetate (TPA) causes translocation of protein kinase C to membrane and persistent activation of the enzyme in chromaffin (TerBush and Holz, 1986) and other cells (Kraft and Anderson, 1983). In permeabilized chromaffin cells, the activation of protein kinase C results in secretion becoming more sensitive to Ca²⁺ (Knight and Baker, 1983; Pocotte et al., 1985). Prolonged activation of protein kinase C by micromolar TPA is known to down-regulate the enzyme in a number of cell types (Ballester and Rosen, 1985; Melloni et al., 1986; Matthies et al., 1987). In chromaffin cells the loss of substantial amounts of protein kinase C had little or no effect on Ca²⁺-dependent catecholamine secretion (Bittner and Holz, 1986). In this study we examine the effects on secretion of prolonged exposure

to much lower concentrations of TPA. We demonstrate for the first time an effect of TPA on exocytosis which requires gene transcription, and does not depend on the continuous presence of activated protein kinase C.

MATERIALS AND METHODS

Primary dissociated cells from bovine adrenal medulla were prepared, purified by differential plating, and maintained as monolayer cultures in 6.4-mm diameter collagen-coated culture wells at a density of 500,000 cells/cm². Cells were prepared and plated in the presence of 1.3 μg/ml Fungizone (Squibb). At least 12 h prior to the start of an experiment, the culture medium was replaced by medium without Fungizone. Experiments were performed 4–8 days after preparation of cultures.

The physiological salt solution (PSS) used in some experiments contained either normal K⁺ [145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 15 mM HEPES (pH 7.4), and 0.5 mM ascorbate] or elevated K⁺ [95 mM NaCl, 56 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 15 mM HEPES (pH 7.4), and 0.5 mM ascorbate]. The potassium glutamate permeabilizing so-

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Abbreviations used: Act D, actinomycin D; BSA, bovine serum albumin; CHX, cycloheximide; KGEP, potassium glutamate, EGTA, PIPES-containing solution; LTP, long-term potentiation; PDBu, phorbol dibutyrate; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PSS, physiological salt solution; TPA, 12-*O*-tetradecanoylphorbol acetate.

lution (KGEP) used for secretion experiments contained 139 mM potassium glutamate, 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.6), 5 mM EGTA with 20 μ M digitonin, 1 mM MgCl₂, 2 mM MgATP, 0.5% bovine serum albumin (BSA), and CaCl₂ to yield the desired concentrations of free Ca²⁺. At the end of an experiment, the incubation solutions were removed, the cells were lysed in 1% Triton X-100, and the [³H]norepinephrine in the incubation and cell solutions was determined by liquid scintillation spectrometry. The total [³H]norepinephrine was the sum of the [³H]norepinephrine released into the medium and the [³H]norepinephrine remaining in the cells. Secretion is expressed as (³H]norepinephrine in the medium/total [³H]norepinephrine) \times 100. Ca²⁺-dependent release was calculated by subtracting the percentage of [³H]norepinephrine released in the absence of Ca²⁺ from the percentage of [³H]norepinephrine released in the presence of Ca²⁺. Similarly, K⁺-stimulated release was calculated by subtracting the percentage of [³H]norepinephrine released in PSS with normal (5.6 mM) K⁺ from the percentage of [³H]norepinephrine released in PSS with elevated (56 mM) K⁺. Unless otherwise indicated, experiments were performed at 25°C.

The homogenizing buffer for the protein kinase C assay contained 139 mM potassium glutamate, 5 mM EGTA, 20 mM PIPES (pH 6.6), 50 μ g/ml leupeptin, and 2 mM dithiothreitol. Samples (0.02 ml) were diluted 10-fold into an assay solution with the following final concentrations of components: 14 mM potassium glutamate, 20 mM PIPES (pH 6.6), 10 mM MgCl₂, 10 mM dithiothreitol, 0.8 mg/ml BSA, 5 μ g/ml leupeptin, 25 μ M 2-mercaptoethanol, 0.01% Triton X-100, 30 μ M ATP (~300,000 cpm of [γ -³²P]ATP), 50 μ g/ml histone Type III-S and 0.5 mM EGTA \pm 0.8 mM CaCl₂, 167 μ g/ml phosphatidylserine, and 26.7 μ g/ml 1,2-diolein. After 10 min at 30°C, the incubation was stopped by the addition of 5% trichloroacetic acid containing 0.25% sodium tungstate, 15 mM NaH₂PO₄, and 2 mM ATP. BSA was added, and the precipitate was centrifuged, and redissolved in 0.5 M NaOH. The precipitation/resuspension procedure was repeated twice, and the radioactivity in the resuspended pellets was determined. Protein kinase C activity was defined as the difference between the activity in the presence of Ca²⁺ and phosphatidylserine/diglyceride and the activity in the presence of phosphatidylserine/diglyceride without Ca²⁺. The kinase activity measured was >90% Ca²⁺- and phospholipid/diglyceride-dependent. The amounts of phorbol ester carried over from cell incubations did not affect the assay.

Data are expressed as means \pm standard errors of the mean. Significance between groups was determined by Student's *t* test. Error bars smaller than symbols were omitted from figures.

Leupeptin and TPA were obtained from Calbiochem, digitonin from Fluka Chemical, phosphatidylserine from Avanti, [³H]norepinephrine from New England Nuclear, and [γ -³²P]ATP from Amersham. Other reagents were purchased from Sigma Chemical.

RESULTS

Pretreatment of cultured chromaffin cells with 30 nM TPA for 30 min enhanced Ca²⁺-dependent [³H]norepinephrine secretion (Fig. 1A). The relative enhancement was particularly marked at low Ca²⁺

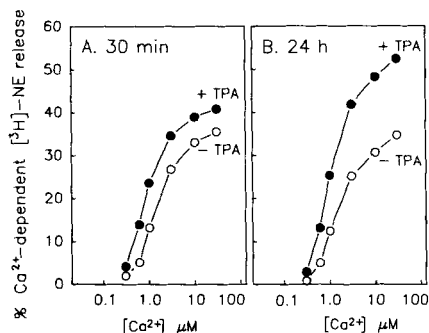


FIG. 1. Comparison of the TPA-induced enhancement of secretion at 30 min and 24 h. Cultured chromaffin cells were labeled with [³H]norepinephrine for 2 h, rinsed for 30 min in culture medium, and then incubated in culture medium with or without 30 nM TPA and 0.01% dimethyl sulfoxide for 30 min (A) or 24 h (B). Secretion was stimulated by permeabilizing the cells in KGEP with 20 μ M digitonin for 15 min in the presence of various Ca²⁺ concentrations. The incubation solutions were removed, the cells were lysed in 1% Triton X-100, and the [³H]norepinephrine in the incubation and cell solutions was determined by liquid scintillation spectrometry. The values for [³H]norepinephrine release in the absence of Ca²⁺ at 30 min were: no TPA, 5.0 \pm 0.3; 30 nM TPA, 5.6 \pm 0.3; and at 24 h the values were: no TPA, 6.4 \pm 0.4; 30 nM TPA, 4.6 \pm 0.2. *n* = 3 wells/group.

concentrations (0.3–1.0 μ M), with a several-fold increase in Ca²⁺-dependent secretion. When the preincubation with phorbol ester was continued for 24 h, the enhancement of secretion at 10 and 30 μ M Ca²⁺ was greatly increased without a significant change in the enhancement at \leq 1 μ M Ca²⁺ (Fig. 1B). The increased enhancement of secretion at 10–30 μ M Ca²⁺ was also observed after an 8-h preincubation with TPA (data not shown). Similar effects were seen with phorbol dibutyrate (PDBu), but not with the inactive phorbol ester, 4 β -phorbol 13-monoacetate (data not shown).

After short (1–30 min) preincubations with TPA, the immediate enhancement of secretion is correlated with an increase in the amount of protein kinase C that becomes bound to the membrane (TerBush and Holz, 1986; TerBush et al., 1988). We therefore examined the subcellular distribution of protein kinase C after 18–24 h of 30 nM TPA. Whereas prolonged incubation with higher concentrations of TPA (e.g., \geq 1 μ M) decreases protein kinase C activity by 85–90% (Bittner and Holz, 1986), 30 nM TPA had little or no effect on the total amount of protein kinase C measurable after 18–24 h of TPA treatment.¹ In the experiment shown in Fig. 2, chromaffin cells were preincubated with 30 nM TPA for 18 h, at which time some of the cultures had the TPA-containing medium re-

¹ In the experiments described in this article, 18–24 h treatment with 30 nM TPA had no effect on the total protein kinase C activity. In a few other studies, this concentration of TPA reduced the total measurable protein kinase C activity by 25–30%.

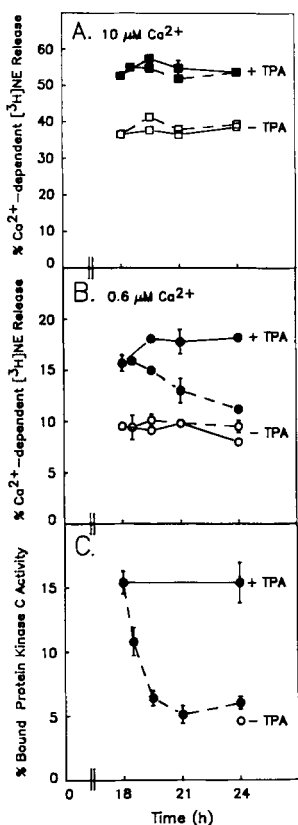


FIG. 2. Effect of removal of TPA on long-term enhancement of secretion. Chromaffin cells were labeled with $[^3\text{H}]\text{norepinephrine}$ (A and B). Some groups were treated continuously for 18–24 h with (filled symbols, solid lines) or without (unfilled symbols, solid lines) 30 nM TPA and 0.01% dimethyl sulfoxide in culture medium. In other groups, after an 18-h incubation with 30 nM TPA, medium was removed and replaced with drug-free medium (filled symbols, dashed lines). Some of the cultures without TPA for 18 h also received fresh, drug-free medium at this time (unfilled symbols, dashed lines). At the indicated times, the Ca^{2+} -dependent $[^3\text{H}]\text{norepinephrine}$ release (A and B) was determined and expressed as the percentage of the total catecholamine. Ca^{2+} concentrations were 10 μM (A) or 0.6 μM (B). $n = 3$ wells/group. **C:** Parallel cultures were treated exactly as described above, except that cells were not labeled with $[^3\text{H}]\text{norepinephrine}$. All cultures, except the group indicated by the open circle, were treated with 30 nM TPA for 18 h. At 18 h, TPA was either maintained in the culture medium (solid line), or the culture medium was removed and replaced with TPA-free medium (dashed line). At the indicated times, intact cells were rinsed briefly with PSS containing 5.6 mM K^+ , scraped into homogenizing buffer, sonicated to lyse the cells, and immediately fractionated into membrane and supernatant fractions by centrifugation. Both fractions were suspended in buffer containing 0.1% Triton X-100, and assayed for protein kinase C activity. Data in C were combined from two separate experiments. Total protein kinase C activity was unaffected by TPA treatment. Total activities (nmol $[^{32}\text{P}]\text{ATP}$ transferred/10 min/150,000 cells) for each point were: 24 h, -TPA: 1.29 ± 0.12 ; 24 h, +TPA: 1.30 ± 0.12 ; 18 h, +TPA: 1.20 ± 0.12 ; 18 h, +TPA, 30 min, -TPA: 1.20 ± 0.14 ; 18 h, +TPA, 90 min, -TPA: 1.25 ± 0.03 ; 18 h, +TPA, 3 h, -TPA: 1.18 ± 0.14 ; 18 h, +TPA, 6 h, -TPA: 1.35 ± 0.12 . $n = 4$ wells/group.

placed with TPA-free medium (dashed line, filled symbols). During the 6 h following the removal of TPA, the percentage of membrane-bound protein kinase C returned to the level seen in untreated cells (unfilled circle) (Fig. 2C). The enhancement of secretion at 0.6 μM Ca^{2+} was also reversed during the same period (Fig. 2B).

In contrast to the reversibility of the effects of TPA on secretion stimulated by 0.6 μM Ca^{2+} , TPA's enhancement of secretion at 10 μM Ca^{2+} persisted long after the removal of TPA (Fig. 2A). Even a 30-min exposure to 30 nM TPA followed by a 15.5-h incubation without TPA was sufficient to elevate secretion in response to 10 μM Ca^{2+} (Table 1). Thus, the enhancement of secretion at 10 μM Ca^{2+} , which requires time to develop, does not require the continued presence of membrane-bound protein kinase C. In contrast, the enhancement seen at 0.6–1 μM Ca^{2+} is related to membrane-bound enzyme.

Experiments similar to those shown in Fig. 2 were conducted with PDBu (100 nM). As might be expected with this more water-soluble phorbol ester, on removal of PDBu both the loss of membrane-bound protein kinase C and the loss of the enhancement of secretion at 0.6 μM Ca^{2+} were more rapid than with TPA. Because the effects on secretion were more consistent with TPA than with PDBu, most experiments were performed using TPA.

The immediate enhancement of secretion from permeabilized cells induced by a 30-min incubation with TPA did not require protein synthesis. For example, in a typical experiment cells were preincubated with or without the protein synthesis inhibitor cycloheximide (CHX) (1 $\mu\text{g}/\text{ml}$) for 30 min, followed by incubation with or without TPA (30 nM) for an additional 30 min (in the continuing presence or absence of 5 $\mu\text{g}/\text{ml}$ CHX). TPA increased the secretion stimulated by 1 μM Ca^{2+} from $14.1 \pm 0.7\%$ to $22.5 \pm 0.5\%$ in the absence of CHX and from $14.6 \pm 0.3\%$ to $23.8 \pm 0.6\%$ in the presence of CHX. At 10 μM Ca^{2+} , TPA increased Ca^{2+} -dependent secretion from $34.9 \pm 0.6\%$ to $39.4 \pm 0.7\%$ in the absence of CHX and $34.6 \pm 0.5\%$ to

TABLE 1. Effect of a brief preincubation with TPA on the long-term enhancement of secretion

| Treatment | Percent Ca^{2+} -dependent secretion |
|-----------------------------------|---|
| - TPA, 16 h | 28.0 ± 0.3 |
| + TPA, 30 min, then - TPA, 15.5 h | 35.5 ± 0.4^a |
| + TPA, 16 h | 39.4 ± 0.3^a |

Cultured chromaffin cells were labeled with $[^3\text{H}]\text{norepinephrine}$ for 2 h, rinsed for 30 min in culture. Some cells were incubated for 16 h with or without 30 nM TPA. Other cells were incubated with medium containing 30 nM TPA for 30 min followed by 15.5 h in TPA-free medium. Secretion was stimulated by permeabilizing the cells in KGEP with or without 10 μM Ca^{2+} for 15 min. $n = 3$ wells/group.

^a $p < 0.001$ versus - TPA, 16 h.

39.8 ± 0.7% in the presence of CHX. However, the enhancement of secretion induced after longer incubations in TPA was inhibited by CHX (Fig. 3A). TPA alone caused a continuing increase in Ca²⁺-dependent secretion over 10 h. CHX caused a small inhibition of secretion in the absence of TPA, and significantly inhibited the TPA-induced increment in secretion by 10 h.

The experiment shown in Fig. 3B was performed to determine whether the small TPA-induced enhancement of secretion in the presence of CHX in Fig. 3A required continuous incubation with TPA. In addition, it was designed to determine whether untranslated messenger RNA in TPA- and CHX-treated cells can be expressed to increase secretion after TPA and CHX are removed. The experiment was done in parallel with that in Fig. 3A and was performed identically, except that, after 4 h (first arrow), the TPA-containing medium was replaced with fresh medium with or without CHX (5 µg/ml). Thus, after the initial 4-h incubation, none of the incubation media contained TPA. At 10 h (second arrow), the medium on the cells was replaced with drug-free medium (no TPA, no CHX). As discussed above, in the absence of CHX, removal of TPA after 4 h had no effect on the subsequent enhancement of secretion (filled circles), which remained elevated throughout the 22-h period. When TPA was removed from the cells after 4 h in the continued presence of CHX, secretion returned to control levels by 10 h (filled square). This drop indicates that the small enhance-

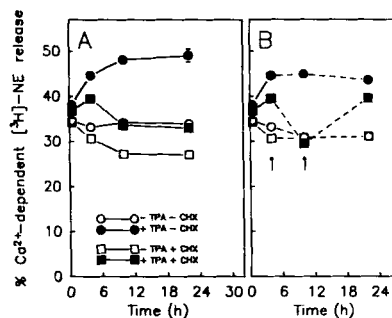


FIG. 3. Effect of inhibiting protein synthesis on the development of long-term enhancement of secretion. **A:** Chromaffin cells were labeled with [³H]norepinephrine, rinsed in culture medium with or without 1 µg/ml CHX for 30 min, and then incubated in culture medium with or without 30 nM TPA and with or without 5 µg/ml CHX for the indicated times. **B:** The same experiment as in A until 4 h. At 4 h (first arrow), the medium in all of the wells was replaced by fresh medium without TPA, either with (squares) or without (circles) 5 µg/ml CHX. At 10 h (second arrow), the medium in all wells was replaced with drug-free medium (without TPA or CHX). For both A and B, Ca²⁺-dependent secretion was determined during a 15-min incubation in KGEP containing 20 µM digitonin with and without 10 µM free Ca²⁺ as described. Secretion was expressed as the percentage of the total catecholamine. Secretion was assayed at 30 min, 4 h, 10 h, and 22 h after the initiation of TPA treatment. n = 3 wells/group.

ment observed at 4 h in the presence of CHX and TPA was probably due to an effect of membrane-bound protein kinase C. Finally, when CHX was removed from cells after 10 h (second arrow) to allow translation to occur (TPA having been removed at 4 h), secretion was enhanced at 22 h (filled square at 22 h compared to filled square at 10 h). Thus, mRNA for a stimulatory protein was probably being transcribed during the incubation with phorbol ester and CHX, but was not translated until CHX was removed.

The RNA synthesis inhibitor actinomycin D (Act D) was used to examine the effect of inhibiting RNA synthesis on the long-term enhancement by TPA. Like CHX, Act D did not prevent an initial increase in secretion due directly to membrane-bound protein kinase C, but it did abolish the large enhancement of secretion seen at later times (data not shown). However, unlike the results with CHX in Fig. 3B, secretion did not increase in TPA-treated cells after Act D was removed. Presumably, mRNA induced by TPA in the CHX-treated cells was stable and resulted in synthesis of a protein important for secretion after CHX is removed. Act D prevented the induction of the message.

Treatment with CHX or Act D, either alone or in combination with 30 nM TPA, had no effect on the total protein kinase C activity in the cells. After a 21-h incubation with various agents, values for protein kinase C activity (nmol ³²P from [^γ-³²P]ATP incorporated/10 min/150,000 cells) were: no TPA or CHX, 2.30 ± 0.05; 30 nM TPA, 2.24 ± 0.04; 5 µg/ml CHX, 2.24 ± 0.03; TPA + CHX, 2.19 ± 0.05.

The short- and long-term effects of TPA preincubation could be observed when *intact* cells were stimulated to secrete by depolarization with 56 mM K⁺. A 10-min preincubation of cells with 30 nM TPA enhanced K⁺-stimulated secretion (Fig. 4). This enhancement appeared to be due to membrane-bound protein kinase C, because it was reversed when cells were rinsed in drug-free medium for 5 h (Fig. 4). In addition, the rapid enhancement of K⁺-stimulated secretion by TPA was not blocked by inhibiting protein synthesis (Fig. 5A). Figure 5B demonstrates that the slowly developing effect of TPA results in an increase in secretion that does not require continuous incubation with TPA but does require protein synthesis. When cells were incubated ±CHX (1 µg/ml) for 30 min, then incubated ±TPA (30 nM) ±CHX (5 µg/ml) for 10 h, and finally incubated ±CHX (2 µg/ml) in the absence of TPA for 12 h, K⁺-stimulated secretion was enhanced by TPA only in those cells in which protein synthesis was not inhibited (Fig. 5B). Note that in Fig. 5B secretion was stimulated 12 h after TPA had been removed. Thus, the effects of prolonged treatment with low concentrations of TPA on secretion elicited by depolarization in intact cells were analogous to the effects described for digitonin-permeabilized cells, which required protein synthesis but not the continuous presence of membrane-bound protein kinase C.

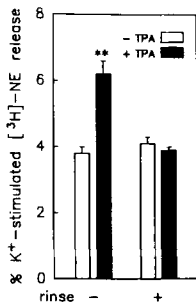


FIG. 4. Short-term effects of TPA in intact cells. Cultured chromaffin cells were labeled with [^3H]norepinephrine, and rinsed in culture medium. Cells were incubated in medium with or without 30 nM TPA for 10 min. In some of the wells, secretion was assayed immediately after this 10 min incubation ($-$ rinse), whereas in others, the treatment medium was removed, and the cells cultured in drug-free medium for 5 h before assay ($+$ rinse). Secretion induced by depolarization was determined by stimulating cells for 2 min in PSS containing either normal (5.6 mM) or elevated (56 mM) K^+ . Secretion was expressed as the percentage of the total catecholamine. K^+ -stimulated release was calculated by subtracting the percentage of [^3H]norepinephrine released in PSS with normal (5.6 mM) K^+ from the percentage of [^3H]norepinephrine released in PSS with elevated (56 mM) K^+ . $n = 3$ wells/group. $**p < 0.02$ versus $-$ TPA.

DISCUSSION

These experiments indicate that activation of protein kinase C can lead to a prolonged enhancement of the ability of cells to undergo exocytosis which does not require the continued presence of membrane-bound protein kinase C. The enhancement is detectable in intact chromaffin cells stimulated to secrete by elevated K^+ , as well as in digitonin-permeabilized cells. The effects described are not due to an alteration in the total amount of the enzyme remaining in the cells, because, as mentioned above, there was little or no change in total protein kinase C activity in these experiments. In fact, when protein kinase C was down-regulated by higher concentrations of TPA, the long-term enhancement was still evident, even when total protein kinase C activity was reduced by $>50\%$.

Many cell types contain more than one subtype of protein kinase C (Nishizuka, 1988). We have recently determined that chromaffin cells contain mainly the α subtype (B. Strulovici, M. A. Bittner, and R. W. Holz, manuscript in preparation). There are also small amounts of β and ϵ subtypes. All subtypes partially translocated to the membrane in the presence of 30 nM TPA and none were specifically down-regulated during long incubations with 30 nM TPA. Thus, the long-term effects of low concentrations of TPA cannot be explained by changes in the proportion of the various protein kinase C subtypes in the cells.

TPA can either increase (Stumpo et al., 1988; Kley, 1988) or decrease (Salehi et al., 1988) the transcription

of a number of different genes. In chromaffin cells, incubation with TPA induced the synthesis of four to eight proteins as measured in [^{35}S]methionine-labeled cells (C. L. Cooper and R. W. Holz, unpublished observations). The present study is the first to demonstrate that an effect of TPA on secretion requires gene transcription. Thus, in the permeabilized chromaffin cell, we can distinguish between two types of effects of phorbol ester treatment. The first, prominent at $\leq 1 \mu\text{M}$ free Ca^{2+} , develops within minutes, is unaffected by inhibition of protein or RNA synthesis, and is correlated with the amount of membrane-bound protein kinase C. It is probably directly and immediately dependent on protein phosphorylation. The second develops over a period of hours, is blocked by inhibition of protein and RNA synthesis, and is independent of the continuous activation of protein kinase C.

Earlier studies have addressed the functional significance of the first effect (Knight and Baker, 1983; Poccotte et al., 1985; Brocklehurst et al., 1985; TerBush and Holz, 1986; Lee and Holz, 1986; Brocklehurst and Pollard, 1986; TerBush et al., 1988). It is possible that the persistent enhancement of secretion we observe in chromaffin cells may represent a general, delayed response to activation of protein kinase C in a variety of secretory cells. For example, long-term potentiation (LTP), the long-lived increase in synaptic strength evoked by brief high-frequency stimulation in the hippocampus, has been linked to the activation of protein kinase C (Akers et al., 1986; Malenka et al., 1986; Lovinger et al., 1986, 1987; Hu et al., 1987; Malinow et al., 1988). Lovinger and Routtenberg and their colleagues (1987) have found that protein kinase C inhib-

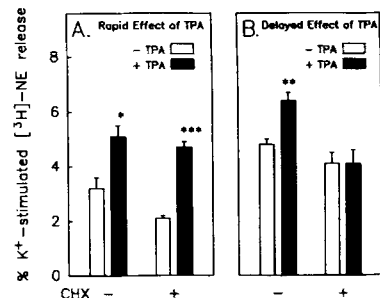


FIG. 5. Long-term enhancement of secretion in intact cells. Cultured chromaffin cells were labeled with [^3H]norepinephrine, and rinsed in culture medium. In **A**, cultures were incubated with or without CHX (1 $\mu\text{g}/\text{ml}$) for 30 min, and then incubated with or without 30 nM TPA in the continuing presence or absence of 5 μg CHX/ml for 10 min. Secretion was assayed for 2 min as described for Fig. 4. The cells in **B** were first incubated with or without CHX (1 $\mu\text{g}/\text{ml}$) for 30 min, and then incubated for 10 h with or without 30 nM TPA and in the continuing presence or absence of CHX (5 $\mu\text{g}/\text{ml}$). At 10 h, the medium was replaced with fresh medium with or without 2 μg CHX/ml, but without TPA, and cells maintained for an additional 12 h before assaying secretion as described for Fig. 4. $n = 3$ wells/group. $*p < 0.05$ versus $-$ TPA; $**p < 0.02$ versus $-$ TPA; $***p < 0.001$ versus $-$ TPA.

itors can prevent the maintenance of LTP when given prior to or shortly after LTP initiation, but are ineffective when injected 4 h after LTP. This is consistent with the long-term enhancement of secretion seen after prolonged TPA treatment in chromaffin cells, where the continued presence of activated protein kinase C is not required for the effect (Fig. 2). Several hours would provide sufficient time for newly synthesized proteins in the cell body to reach the nerve terminals via fast axonal transport. There is also evidence that protein synthesis inhibitors block the maintenance of LTP (Krug et al., 1984; Stanton and Sarvey, 1984). It is tempting to speculate that a mechanism involving protein synthesis subsequent to protein kinase C activation might play a role in the maintenance of LTP in neuronal cells.

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REFERENCES

- Akers R. F., Lovinger D. M., Colley P. A., Linden D. J., and Routtenberg A. (1986) Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science* **231**, 587-589.
- Ballester R. and Rosen O. M. (1985) Fate of immunoprecipitable protein kinase C in GH₃ cells treated with phorbol 12-myristate 13-acetate. *J. Biol. Chem.* **260**, 15194-15199.
- Bittner M. A. and Holz R. W. (1986) Evidence that protein kinase C modulates but is not necessary for Ca²⁺-dependent catecholamine secretion in digitonin-permeabilized adrenal chromaffin cells. *Soc. Neurosci. Abstr.* **12**, 998.
- Brocklehurst K. W. and Pollard H. B. (1986) Synergistic actions of Ca²⁺ and the phorbol ester TPA on K⁺-induced catecholamine release from bovine adrenal chromaffin cells. *Biochem. Biophys. Res. Commun.* **140**, 990-998.
- Brocklehurst K. W., Morita K., and Pollard H. B. (1985) Characterization of protein kinase C and its role in catecholamine secretion from bovine adrenal-medullary cells. *Biochem. J.* **228**, 35-42.
- Castagna M., Takai Y., Kaibuchi K., Sano K., Kikkawa U., and Nishizuka Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257**, 7847-7851.
- Hu G.-Y., Hvalby O., Walaas S. I., Albert K. A., Skjeflo P., Andersen P., and Greengard P. (1987) Protein kinase C injection into hippocampal pyramidal cells elicits features of long-term potentiation. *Nature* **328**, 426-429.
- Kajikawa N., Kaibuchi K., Matsubara T., Kikkawa U., Takai Y., Nishizuka Y., Itoh K., and Tomioka C. (1983) A possible role of protein kinase C in signal-induced lysosomal enzyme release. *Biochem. Biophys. Res. Commun.* **116**, 743-750.
- Kley N. (1988) Multiple regulation of proenkephalin gene expression by protein kinase C. *J. Biol. Chem.* **263**, 2003-2008.
- Knight D. E. and Baker P. B. (1983) The phorbol ester TPA increases the affinity of exocytosis for Ca in leaky adrenal medullary cells. *FEBS Lett.* **160**, 98-100.
- Kraft A. A. and Anderson W. B. (1983) Phorbol esters increase the amount of Ca²⁺, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* **301**, 621-623.
- Krug M., Lossner B., and Ott T. (1984) Anisomycin blocks the late phase of long-term potentiation in the dentate gyrus of freely moving rats. *Brain Res. Bull.* **13**, 39-42.
- Lee S. A. and Holz R. A. (1986) Protein phosphorylation and secretion in digitonin-permeabilized adrenal chromaffin cells: effects of micromolar Ca²⁺, phorbol esters and diacylglycerol. *J. Biol. Chem.* **261**, 17089-17098.
- Lovinger D. M., Colley P. A., Akers R. F., Nelson R. B., and Routtenberg A. (1986) Direct relation of long-term synaptic potentiation to phosphorylation of protein F1, a substrate for membrane protein kinase C. *Brain Res.* **399**, 205-211.
- Lovinger D. M., Wong K. L., Murakami K., and Routtenberg A. (1987) Protein kinase C inhibitors eliminate long-term potentiation. *Brain Res.* **436**, 177-183.
- Malenka R. C., Madison D. V., and Nicoll R. A. (1986) Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* **321**, 175-177.
- Malinow R., Madison D. V., and Tsien R. W. (1988) Persistent protein kinase activity underlying long-term potentiation. *Nature* **335**, 820-824.
- Matthies H. J. G., Palfrey H. C., Hirning L. D., and Miller R. J. (1987) Down regulation of protein kinase C in neuronal cells: effects on neurotransmitter release. *J. Neurosci.* **7**, 1198-1206.
- Melloni E., Pontremoli S., Michetti M., Sacco O., Sparatore B., and Horrecker B. L. (1986) The involvement of calpain in the activation of protein kinase C in neutrophils stimulated by phorbol myristic acid. *J. Biol. Chem.* **261**, 4101-4105.
- Nishizuka Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665.
- Pocotte S. L., Frye R. A., Senter R. A., TerBush D. R., Lee S. A., and Holz R. W. (1985) Effects of phorbol ester on catecholamine secretion and protein phosphorylation in adrenal medullary cell cultures. *Proc. Natl. Acad. Sci. USA* **82**, 930-934.
- Salehi Z., Taylor J. D., and Nield J. E. (1988) Dioctanoylglycerol and phorbol esters regulate transcription of *c-myc* in human promyelocytic leukemia cells. *J. Biol. Chem.* **263**, 1898-1903.
- Stanton P. K. and Sarvey J. M. (1984) Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. *J. Neurosci.* **4**, 3080-3088.
- Stumpo D. J., Stewart T. N., Gilman M. Z., and Blackshear P. J. (1988) Identification of *c-fos* sequences involved in induction by insulin and phorbol esters. *J. Biol. Chem.* **263**, 1611-1614.
- TerBush D. R. and Holz R. W. (1986) Effects of phorbol esters, diglyceride, and cholinergic agonists on the subcellular distribution of protein kinase C in intact or digitonin-permeabilized adrenal chromaffin cells. *J. Biol. Chem.* **261**, 17099-17106.
- TerBush D. R., Bittner M. A., and Holz R. W. (1988) Ca²⁺ influx causes rapid translocation of protein kinase C to membranes: studies of the effects of secretagogues in adrenal chromaffin cells. *J. Biol. Chem.* **263**, 18873-18879.