The phorbol ester 12-0-tetradecanoylphorbol 13-acetate (TPA) caused phosphorylation of phosphoproteins of 56-kDa which co-migrated with and had identical pI values to subunits of tyrosine hydroxylase. The phosphorylation was closely correlated with an increase of $[\text{H}]3,4\text{-dihydroxyphenylalanine (DOPA)}$ production which is a reflection of increased tyrosine hydroxylase activity. Only those phorbol esters which activate protein kinase C induced phosphorylation of the 56-kDa proteins and increased $[\text{H}]$DOPA production. Neither TPA-induced phosphorylation of the 56-kDa proteins nor TPA-induced enhancement of $[\text{H}]$ DOPA production required extracellular Ca++. TPA caused increases in phosphorylation of the 56-kDa proteins and increases in $[\text{H}]$DOPA production over similar concentration ranges (10–1000 nm). TPA did not increase cellular cAMP. The data suggest that phorbol ester-induced phosphorylation of intracellular tyrosine hydroxylase, possibly by protein kinase C, results in increased tyrosine hydroxylase activity.

Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine biosynthesis (1). In situ secretagogue-induced phosphorylation and activation of tyrosine hydroxylase has been observed in a variety of tissues including rat pheochromocytoma (2, 3), rat superior cervical ganglion (4), suspended bovine chromaffin cells (5, 6), and primary cultures of bovine chromaffin cells (7). In pheochromocytoma cells (2, 3, 8), rat superior cervical ganglion (9), and bovine chromaffin cells (7), there is evidence for both Ca++-dependent and cAMP-dependent phosphorylation and activation of tyrosine hydroxylase. Studies with purified bovine striatal tyrosine hydroxylase (10), purified rat pheochromocytoma tyrosine hydroxylase (12), partially purified rat caudate nucleus tyrosine hydroxylase (13), and purified bovine adrenal tyrosine hydroxylase (14) have demonstrated that cAMP-dependent protein kinase phosphorylates and activates tyrosine hydroxylase. A Ca++/calmodulin-dependent protein kinase phosphorylates purified bovine adrenal tyrosine hydroxylase and is in the presence of a specific protein increases tyrosine hydroxylase activity (15). A Ca++/calmodulin-dependent protein kinase also phosphorylates purified rat pheochromocytoma tyrosine hydroxylase (8).

Recently it has been found that the Ca++/phospholipid-dependent protein kinase (protein kinase C) phosphorylates and activates partially purified tyrosine hydroxylase from pheochromocytoma cells (16). Protein kinase C is Ca++-dependent and requires acidic phospholipids for activity (17, 18). Diglyceride in the presence of phosphatidylinserine increases the Ca++ sensitivity of the enzyme to micromolar or perhaps submicromolar concentrations (19). 12-O-Tetradecanoylphorbol 13-acetate (TPA) can substitute for diglyceride in vitro (20) and similarly increases the Ca++ sensitivity of the enzyme. In intact platelets TPA activates protein kinase C and protein phosphorylation and enhances serotonin secretion (20, 21). TPA also increases protein phosphorylation and secretion in intact bovine chromaffin cells (22) and secretion in bovine chromaffin cells with plasma membrane permeabilized by either intense electric fields (23) or digitonin (22).

In the present study, phorbol esters were used to investigate whether protein kinase C can regulate catecholamine biosynthesis within adrenal chromaffin cells by phosphorylation and activation of tyrosine hydroxylase.

**MATERIALS AND METHODS**

**Cell Preparation—**Primary dissociated cells from bovine adrenal medulla were prepared and maintained as monolayer cultures in Eagle's minimal essential medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal calf serum as previously described (24). The culture medium contained 100 units/ml of penicillin, 100 µg/ml of streptomycin, 50 µg/ml of gentamycin, and 1.3 µg/ml of Fungizone (Squibb, Princeton, NJ) to prevent bacterial and fungal contamination. The culture medium also contained 10 µM cytokine arabinoside to inhibit fibroblast growth. Cells were usually cultured as monolayers in 16-mm diameter plastic culture wells (Costar, Cambridge, MA) at a density of 250,000 cells/cm². In some experiments cells were plated in 6.4-mm diameter wells (Costar) at a density of 500,000 cells/cm². In experiments in which cellular cAMP was measured, cells were purified by differential plating and cultured in collagen-coated culture cells (16-mm diameter). 200 µl of sterile calfskin collagen (Calbiochem-Behring) solution (50 µg/ml of 0.1% acetic acid) was applied to each 16-mm diameter well. Cells were plated after evaporation of the acetic acid. Experiments were performed 4–14 days after preparation. There were approximately 50 nmol of catecholamine/million cells. Immediately before an experiment, cells were incubated for 1 h with physiological salt solution (PSS) containing 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 sodium ascorbate except where otherwise indicated. An experiment was initiated by replacing the medium with new solution. All experiments were performed at 25°C.

**Protein Phosphorylation—**Intracellular phosphorylation in cells was determined by incubation of cells in 6.4- or 16-mm diameter wells containing 12-0-tetradecanoylphorbol 13-acetate (TPA); DOPA, 3,4-dihydroxyphenylalanine; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′,N′-tetraacetic acid; PSS, physiological salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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1The abbreviations used are: TPA, 12-O-tetradecanoylphorbol 13-acetate; DOPA, 3,4-dihydroxyphenylalanine; EGTA, ethylene glycol bisβ-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PSS, physiological salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
for 30 min in PSS containing [32P]phosphate (0.25–0.5 mCi/ml, carrier-free). Cells were subsequently incubated in [32P]phosphate-free PSS containing various drugs unless otherwise indicated. In some experiments to improve resolution on the autoradiograms there was a 10-min incubation in [32P]phosphate-free PSS before addition of solutions with or without drugs. Experiments were usually terminated by replacing the medium with 0.10–0.25 ml of stop solution (5% sodium dodecyl sulfate, 2% mercaptoethanol, 5% glycerol, 62 mM Tris-Cl (pH 6.7), and 0.5 mg/ml of bromphenol blue). Cellular proteins were further denatured by incubation at 90 °C for 3 min. The Tris-C1 (pH 6.7), and 0.5 mg/ml of bromphenol blue). Cellular proteins were further denatured by incubation at 90 °C for 3 min. The solution, 0.08 ml, was analyzed for phosphoprotein by sodium dodecyl sulfate-polyacrylamide (6.9% or 5-20% gradient) slab gel electrophoresis according to O’Farrell (27).

 Autoradiograms of one-dimensional SDS-PAGE were analyzed by densitometry (EC910 Den- sitometer, EC-Apparatus Corp., St. Petersburg, FL). Peak heights (in arbitrary units) on densitometric tracings were measured after subtracting background densities in neighboring regions of the tracing, as described previously (26).

 In Situ [3H]DOPA Production—In situ tyrosine hydroxylase activity was analyzed by measuring the conversion of [3H]tyrosine to [3H] DOPA according to previously published methods (28, 29). Cells cultured in 16-mm diameter dishes were incubated with 0.4 ml of 150 μm bromocriptine (an inhibitor of DOPA decarboxylase) in PSS for 30 min at 25 °C and then replaced with 0.2 ml of PSS containing 150 μm bromocriptine, 10 or 50 μm [3H]tyrosine (7 μCi/ml, side chain, 23-[3H]tyrosine), and various drugs for indicated times. The reaction was terminated with 0.1 ml of 1.2 N perchloric acid. In some experiments 35-mm diameter dishes were used with 0.8 ml of bromocriptine-containing PSS, 0.6 ml of [3H]tyrosine containing PSS, and 0.2 ml of perchloric acid. After perchloric acid addition the solution was added to 6 ml of a solution containing 1 M Tris base, 0.04 M KH2P04, 1% EDTA, 15 mg of Na2S2O5 (pH 8.6), and 25 μl of a solution containing 0.01 M HCl, 0.25 mg/ml tyrosine, and 0.25 mg/ml dopamine. The resulting solution was added to 0.8-cm diameter columns containing 200 mg of acid alumina. Columns were washed with 20 ml of distilled water prior to elution of [3H]DOPA with 3 ml of 0.3 N HCl. [3H]DOPA in aliquots were measured by scintillation counting and data expressed as picomoles of [3H]DOPA/mg of protein.

 Cyclic AMP was measured by a radioimmune assay according to Vaitukatris et al. (30).

 Phorbol esters were stored in aliquots of 1 mg/ml dimethylsulfoxide at −20 °C. Phorbol esters in dimethyl sulfoxide were diluted 1:100

![Fig. 1. Two-dimensional PAGE autoradiography of the effects of 1,1-dimethyl-4-phenylpiperazinium (DMPP) and TPA on protein phosphorylation. Chromaffin cells which had been labeled with [32P] phosphate for 30 min were incubated in: PSS for 5 min (A); in PSS containing DMPP (10 μM) for 5 min (B); in PSS for 30 min (C); or in PSS containing TPA (100 nM) for 30 min (D). Phosphorylated proteins were separated by two-dimensional PAGE (5–20%) (9 μg of protein/gel). The pH values attained are indicated along the horizontal axis and the molecular weight markers are indicated on left and right vertical axis. The pl values for the multiple p56 phosphoproteins (identified previously as tyrosine hydroxylase (TH)) 1, 2, and 3 correspond to pl 6.37, 6.27, and 6.15, respectively.](image-url)
into H₂O before dilution (1:100) into incubation medium. Data are expressed as mean ± S.E. Significance was tested by Student's t test.

RESULTS

Identification of Tyrosine Hydroxylase as a Substrate for Phorbol Ester-stimulated Protein Phosphorylation—Two-dimensional electrophoresis reproducibly resolved greater than 15 spots that were phosphorylated upon incubation with TPA (100 nM) for 30 min (Fig. 1). These proteins have been described previously (22). Of particular interest were the effects of TPA on phosphorylation of multiple spots at 56,000 daltons. In unstimulated cells there were relatively small amounts of phosphorylated protein with a molecular mass of 56,000 daltons and pI values of 6.37 and 6.27 (Fig. 2). TPA (100 nM) caused [³²P]phosphate incorporation into three proteins of 56,000 daltons with pI values of 6.37, 6.27, and 6.15 (Fig. 2). Nicotinic receptor stimulation of chromaffin cells resulted in phosphorylation of three identical proteins (Fig. 2). Previous studies have demonstrated that specific tyrosine hydroxylase antibody binds to these three phosphoproteins at 56,000 daltons on Western blots of two-dimensional SDS-PAGE (7). Thus, the data strongly suggest that these phosphoproteins at 56 kDa are phosphorylated upon incubation with TPA are subunits of tyrosine hydroxylase. They will be referred to as tyrosine hydroxylase in the remainder of this paper.

Time Course of Tyrosine Hydroxylase Phosphorylation—The time course of phosphorylation of tyrosine hydroxylase was investigated by one-dimensional SDS-PAGE of [³²P]-labeled proteins, and subsequent autoradiography and densitometry of the band at 56 kDa. TPA (100 nM) induced phosphorylation of tyrosine hydroxylase that was significant by 5 min and was maximal by 15 min (Fig. 2).

Effects of TPA on in Situ [¹H]DOPA Production—In vitro phosphorylation of tyrosine hydroxylase by protein kinase C results in the activation of the enzyme (16). If incubation of chromaffin cells with TPA results in phosphorylation of tyrosine hydroxylase in situ, then TPA should also cause in situ activation of [¹H]DOPA production, which is a reflection of tyrosine hydroxylase activity. Indeed, TPA (300 nM) after a 5-min delay caused an increase in the rate of [¹H]DOPA production in the presence of the aromatic acid decarboxylase inhibitor brocresine (Fig. 3). TPA (30 nM to 1 µM) caused a dose-dependent increase in [¹H]DOPA production (Fig. 4).

Effects of Various Phorbol Esters on Tyrosine Hydroxylase Phosphorylation and [¹H]DOPA Production—Both TPA and 4-β-phorbol 12,13-dibutyrate which activate protein kinase C in vitro (20) stimulated phosphorylation of tyrosine hydroxylase and increased [¹H]DOPA production (Table I). 4-α-Phorbol 12,13-didecanoate which does not activate protein kinase C in vitro stimulated neither tyrosine hydroxylase

![Fig. 2. Time course of TPA-stimulated tyrosine hydroxylase phosphorylation.](image)

![Fig. 3. TPA-stimulated [¹H]DOPA production.](image)
TPA-induced phosphorylation of a 56-kDa protein was unaltered by removal of extracellular Ca$^{2+}$ (22). As indicated above, this protein is likely to be tyrosine hydroxylase. Similarly, removal of extracellular Ca$^{2+}$ with or without EGTA had little or no effect on the TPA-induced increase in $[^{3}H]$DOPA production (Table II).

The cAMP content of monolayers of purified chromaffin cells was 0.53 ± 0.03 nmol/0.5 million cells and was not significantly altered by TPA (100 nM) between 5 and 30 min. Thus, it is unlikely that TPA acts indirectly by activating a cAMP-dependent protein kinase.

**TABLE II**

Effect of extracellular calcium on TPA-stimulated $[^{3}H]$DOPA production

<table>
<thead>
<tr>
<th>Concentration</th>
<th>EGTA</th>
<th>TPA</th>
<th>pmol $[^{3}H]$DOPA/30 min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 mM Ca$^{2+}$</td>
<td>0</td>
<td>0</td>
<td>145 ± 11</td>
</tr>
<tr>
<td>2.2 mM Ca$^{2+}$</td>
<td>0</td>
<td>300 nM</td>
<td>339 ± 23$^*$</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>121 ± 5</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>300 nM</td>
<td>315 ± 11$^*$</td>
</tr>
<tr>
<td>0</td>
<td>1 mM</td>
<td>0</td>
<td>124 ± 6</td>
</tr>
<tr>
<td>0</td>
<td>1 mM</td>
<td>300 nM</td>
<td>296 ± 13$^*$</td>
</tr>
</tbody>
</table>

* $p < 0.001$ versus 2.2 mM Ca$^{2+}$.
$^*$ $p < 0.001$ versus 0 Ca$^{2+}$.
$^1p < 0.001$ versus 0 Ca$^{2+}/1$ mM EGTA.

**FIG. 4. Dose-response of TPA-stimulated $[^{3}H]$DOPA production.** Cells in 35-mm dishes were incubated for 30 min in PSS containing 150 μM brocresine and various concentrations of TPA. The incubation medium was changed to the same buffer with or without 300 nM TPA for 15 min. Cells were then incubated in the preceding media with or without 300 nM TPA and 10 μM $[^{3}H]$tyrosine (2.5 × 10$^6$ cpm/ml). $[^{3}H]$DOPA was assayed after 30 min. All solutions contained brocresine (150 μM). There were five wells (50 μg of protein/well) in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>$[^{3}P]$Phosphate incorporation (cpm)</th>
<th>pmol $[^{3}H]$DOPA/30 min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>6.5 ± 1.6</td>
<td>127 ± 4</td>
</tr>
<tr>
<td>TPA</td>
<td>32.5 ± 5.7$^*$</td>
<td>178 ± 12$^*$</td>
</tr>
<tr>
<td>4-β-Phorbol 12,13-dibutyrate</td>
<td>260.0 ± 4.3$^*$</td>
<td>193 ± 12$^*$</td>
</tr>
<tr>
<td>4-β-Phorbol 12,13-didecanoate</td>
<td>10.1 ± 1.7</td>
<td>122 ± 9$^*$</td>
</tr>
<tr>
<td>4-α-Phorbol 12,13-didecanoate</td>
<td>7.4 ± 2.5</td>
<td>112 ± 8$^*$</td>
</tr>
</tbody>
</table>

* $p < 0.01$ versus no drug.

phosphorylation nor $[^{3}H]$DOPA production. 4-β-Phorbol 12,13-didecanoate which is less potent in vitro activator of protein kinase than TPA or 4-β-phorbol 12,13-dibutyrate (20) caused neither phosphorylation of tyrosine hydroxylase nor increased $[^{3}H]$DOPA production. 4-β-Phorbol 12,13-didecanoate which is less potent than TPA in activating protein kinase C in vitro enhanced phosphorylation of tyrosine hydroxylase in situ. 4-β-Phorbol 12,13-didecanoate which is less potent than TPA in activating protein kinase C in vitro (20) failed to cause phosphorylation of tyrosine hydroxylase in situ. The lack of effect on tyrosine hydroxylase phosphorylation is correlated with its inability to stimulate secretion from chromaffin cells (data not shown) and its lesser ability than TPA to shift the subcellular distribution of protein kinase C from cytosolic to membrane-bound enzyme which is required for activation of the enzyme. Thus, it is likely that 4-β-phorbol 12,13-didecanoate did not induce the phosphorylation of tyrosine hydroxylase because it did not activate intracellular protein kinase C.

**DISCUSSION**

**Phorbol Ester-induced Phosphorylation of Tyrosine Hydroxylase**—The phorbol ester TPA caused phosphorylation of a variety of chromaffin cell proteins. The most predominant and consistent TPA-induced phosphorylation was of proteins of 56 kDa which co-migrated with and had identical PI values (6.37, 6.27, and 6.15) to the nicotinic agonist-stimulated 56-kDa phosphoprotein that had been identified as tyrosine hydroxylase by immunochemical methods (7). These data strongly suggest that the 56-kDa proteins phosphorylated by TPA in chromaffin cells were subunits of tyrosine hydroxylase.

Protein kinase C phosphorylates and activates tyrosine hydroxylase in vitro (16). The phosphorylation of tyrosine hydroxylase induced by phorbol esters is likely to be caused directly or indirectly by activation of protein kinase C since only those phorbol esters which activate protein kinase C in vitro enhanced phosphorylation of tyrosine hydroxylase in situ. 4-β-Phorbol 12,13-didecanoate which is less potent than TPA in activating protein kinase C in vitro (20) failed to cause phosphorylation of tyrosine hydroxylase in situ. The lack of effect on tyrosine hydroxylase phosphorylation is correlated with its inability to stimulate secretion from chromaffin cells (data not shown) and its lesser ability than TPA to shift the subcellular distribution of protein kinase C from cytosolic to membrane-bound enzyme which is required for activation of the enzyme. Thus, it is likely that 4-β-phorbol 12,13-didecanoate did not induce the phosphorylation of tyrosine hydroxylase because it did not activate intracellular protein kinase C.

**TABLE I**

The effect of various phorbol esters on tyrosine hydroxylase phosphorylation and $[^{3}H]$DOPA production

<table>
<thead>
<tr>
<th>Group</th>
<th>$[^{3}P]$Phosphate incorporation (cpm)</th>
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<tr>
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</table>

* $p < 0.01$ versus no drug.
Phorbol Ester-induced Phosphorylation of Tyrosine Hydroxylase Is Associated with Activation of Tyrosine Hydroxylase Activity—In situ enhancement of [3H]DOPA production in cells incubated in [3H]tyrosine probably reflects enhanced intracellular tyrosine hydroxylase activity (9–10, 19). Phorbol ester-induced [3H]DOPA production was correlated with phorbol ester-induced phosphorylation of tyrosine hydroxylase as follows. 1) Only those phorbol esters which induced intracellular tyrosine hydroxylase increased the rate of [3H]DOPA production; 2) neither TPA-induced tyrosine hydroxylase phosphorylation nor TPA-induced enhancement of [3H]DOPA production required extracellular Ca2+; 3) TPA caused increases in tyrosine hydroxylase phosphorylation (22) and [3H]DOPA production over similar concentration range (10–100 μM). However, the correlations were not perfect. For example, TPA-induced phosphorylation of tyrosine hydroxylase was substantially by 30 min. Although [3H]tyrosine was added after a 30-min incubation in TPA, reproducible increases in [3H]DOPA production required 5 additional min. The delay in [3H]DOPA production may have resulted from the time necessary for [3H]tyrosine uptake into the cells or other limitations of the assay. Nevertheless, the data suggest that phorbol ester-induced phosphorylation of intracellular tyrosine hydroxylase results in increased tyrosine hydroxylase activity.

Relationship of Protein Kinase C Activation of Tyrosine Hydroxylase to Cholinergic Activation Chromaffin Cells—This study raises the possibility that protein kinase C may have a physiological role in modulating tyrosine hydroxylase activity within chromaffin cells. Nictinic receptor stimulation mediates tyrosine hydroxylase phosphorylation and activity in a calcium-dependent manner (5, 7). Proteolytic fragment analysis of bovine adrenal tyrosine hydroxylase phosphorylated upon nicotinic agonist stimulation of chromaffin cells and by protein kinase C have not been directly compared. Analyses of proteolytic fragments of bovine adrenal tyrosine hydroxylase from nicotinic agonist-stimulated chromaffin cells (31) and of partially purified rat pheochromocytoma (PC-12) (16) tyrosine hydroxylase phosphorylated by protein kinase C in vitro have been performed but not under identical conditions. At this time the identity of the protein kinases responsible for nicotinic agonist-induced phosphorylation of tyrosine hydroxylase is unclear.

A small degree of tyrosine hydroxylase phosphorylation was observed when chromaffin cells were incubated with muscarinic agonists (7). The phosphorylation may have resulted from activation of phospholipase C (32) and the production of diacylglycerol which can activate protein kinase C (19).

Thus, the naturally occurring secretagogue acetylcholine which is a mixed muscarinic-nicotinic agonist may stimulate tyrosine hydroxylase phosphorylation and activation by both protein kinase C via stimulation of muscarinic receptors and by protein kinase C or another Ca2+-dependent protein kinase via stimulation of nicotinic receptors. cAMP which rises in chromaffin cells upon nicotinic stimulation of chromaffin cells (7) (but not upon incubation by TPA) also induces phosphorylation and activation of tyrosine hydroxylase (7, 31). Hence, a number of different protein kinases may play a role in the receptor-mediated, short-term regulation of in situ tyrosine hydroxylase to replenish catecholamine stores depleted by receptor-triggered exocytosis.

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