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Phorbol ester stimulates catecholamine synthesis in isolated bovine adrenal medullary cells

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In isolated bovine adrenal medullary cells, the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), an activator of protein kinase C, stimulated [^{14}C]catecholamine synthesis from [^{14}C]tyrosine, but not from [^{14}C]DOPA. This stimulatory effect of TPA on [^{14}C]catecholamine synthesis was not dependent upon extracellular Ca^{2+}, and TPA did not affect the uptake of $^{45}Ca^{2+}$ or the release of catecholamine by the cells. TPA also did not affect the intracellular cyclic AMP (cAMP) level. 4α-Phorbol 12,13-didecanoate, which is not an activator of protein kinase C, did not stimulate the synthesis of [^{14}C]catecholamine from [^{14}C]tyrosine. The stimulatory effect of TPA on [^{14}C]catecholamine synthesis was additive with that of carbamylcholine, but not with that of dibutyryl cAMP (DB-cAMP). From these results, it was suggested that protein kinase C is involved in the regulation of tyrosine hydroxylase activity and that this regulatory mechanism might be similar to that involving cAMP.

Phorbol ester *Protein kinase C* *Catecholamine synthesis* *Adrenal medullary cell*

1. INTRODUCTION

The phorbol ester, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), which is an activator of protein kinase C, is useful for studying the role of this enzyme in cellular responses [1–3]. TPA is reported to stimulate catecholamine release from 'permeable' adrenal medullary cells caused by low concentrations of Ca^{2+} [4–6], but to have no effect on catecholamine release from the intact cells caused by acetylcholine [4,7].

Here, we examined the effect of TPA on the synthesis of catecholamine by isolated bovine adrenal medullary cells, to determine the role of protein kinase C in catecholamine biosynthesis.

2. MATERIALS AND METHODS

Bovine adrenal medullary cells were isolated by sequential digestion of adrenal medullary slices with collagenase as in [8]. For most experiments, the cells ($\sim 2 \times 10^6$ cells/ml) were incubated at 37°C with or without test compounds in 2 ml of medium consisting of 154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.4), 10 mM glucose and 5 mg/ml bovine serum albumin. For some experiments, calcium-free medium was prepared by adding 1 mM EGTA to this medium instead of 2.2 mM $CaCl_2$.

For determination of catecholamine synthesis, isolated cells were incubated with [^{14}C]tyrosine (final concentration, 2×10^{-5} M, 175×10^4 cpm). In some experiments, L-[^{14}C]DOPA (2×10^{-5} M, 340×10^4 cpm) was used as substrate instead of [^{14}C]tyrosine. After incubation, the tubes were rapidly chilled in ice, and the cells were separated from the medium and homogenized in 5 ml of 0.4 N perchloric acid (PCA). The ^{14}C-labelled catecholamine in the supernatant was measured by ion-exchange chromatography on a Duolite C-25 column (H^+ form, 0.4 × 7.0 cm) as in [9].

For determination of cAMP, cells were in-

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cubated in the medium in the presence of 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM). The reaction was stopped by addition of 0.5 ml of ice-cold 25% trichloroacetic acid, and the cAMP was separated on an ion-exchange column (AG 500 WX8, H-type 200-400 mesh) and measured by the protein binding method [10].

For determination of $^{45}Ca^{2+}$ uptake, a suspension of the isolated cells was added to incubation medium containing 3 μCi $^{45}Ca^{2+}$ in the presence or absence of reagents. After incubation, the tubes were immediately chilled on ice and then centrifuged and the precipitated cells were washed 3 times with Ca^{2+}-free medium. The $^{45}Ca^{2+}$ taken up into the cells was extracted with 0.4 N PCA and counted in a liquid scintillation counter.

For determination of catecholamine release, the catecholamine content of the cells and medium was determined by the fluorometrical method [9,11]. The results were expressed as percentages of the total amount of cellular catecholamine.

TPA and 4α-phorbol 12,13-didecanoate were dissolved in dimethyl sulfoxide (DMSO). All solutions contained 0.5% (v/v) DMSO. The sources of the materials used were as follows: L-[U-^{14}C]-tyrosine, L-[^{14}C]DOPA, $^{45}CaCl_2$ and [^{3}H]cAMP (Radiochemical Centre, Amersham, England); TPA, 4α-phorbol 12,13-didecanoate, DB-cAMP, IBMX(Sigma); carbamylcholine (Nakarai Chemical Co.) and forskolin (Calbiochem-Behring).

3. RESULTS AND DISCUSSION

Fig.1 shows the effect of the phorbol ester TPA at concentrations of 10^{-9} to 10^{-6} M on the synthesis of [^{14}C]catecholamine from [^{14}C]tyrosine in isolated bovine adrenal medullary cells on incubation for 15 min. Stimulation of [^{14}C]catecholamine synthesis was detectable at a TPA concentration as low as 10^{-9} M, and maximal at 10^{-7} M. The time courses of [^{14}C]catecholamine synthesis with or without TPA (10^{-7} M) are shown in fig.2. Basal and TPA-stimulated [^{14}C]catecholamine synthesis were linear during incubation for at least 45 min. TPA did not stimulate [^{14}C]catecholamine synthesis when [^{14}C]DOPA was used as substrate instead of [^{14}C]tyrosine (not shown), indicating that TPA stimulated catecholamine synthesis through an effect on the hydroxylation of tyrosine to

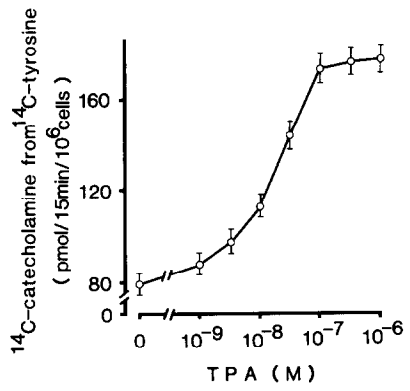

Fig.1. Dose-response curve for TPA-induced formation of [^{14}C]catecholamine from [^{14}C]tyrosine. Cells were incubated for 15 min with TPA(10^{-9} - 10^{-6} M) in the normal medium. Values are means ± SD for 4-6 experiments.

DOPA, the rate-limiting step in catecholamine synthesis.

Next we determined whether the stimulatory effect of TPA on catecholamine synthesis was dependent on extracellular Ca^{2+}. As shown in table 1, the increase in [^{14}C]catecholamine synthesis caused by TPA was not affected by omission of Ca^{2+} from the medium. The increase in the synthesis of [^{14}C]catecholamine by carbamylcholine was dependent on extracellular Ca^{2+}, but that by DB-cAMP was not.

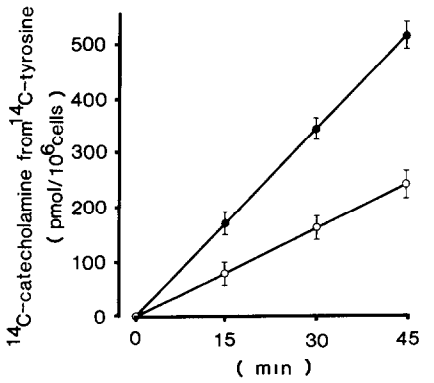

Fig.2. Time course for TPA-induced formation of [^{14}C] catecholamine from [^{14}C]tyrosine. Cells were incubated for various periods with (●—●) or without (○—○) TPA(10^{-7} M) in the normal medium. Values are means ± SD for 4-6 experiments.

Increase in Ca^{2+} uptake into adrenal medullary cells is known to stimulate the synthesis and release of catecholamine. Therefore, we examined whether TPA stimulates the uptake of Ca^{2+} by the cells. The effects of TPA and carbamylcholine on $^{45}Ca^{2+}$ uptake and catecholamine release are shown in table 2. TPA did not stimulate $^{45}Ca^{2+}$ uptake or catecholamine release by the cells. Moreover, it did not affect the increases in $^{45}Ca^{2+}$ uptake and catecholamine release caused by carbamylcholine. Thus the increase in catecholamine synthesis caused by TPA did not seem to be due to increased uptake of Ca^{2+} by the cells.

The synthesis of [^{14}C]catecholamine from [^{14}C]tyrosine is also known to be stimulated by cAMP [12]. Therefore, the effect of TPA on the cAMP level in the cells was examined. As shown in table 3, TPA did not increase the intracellular cAMP level. On the other hand, forskolin, an activator of adenylate cyclase, increased the cAMP level, and stimulated [^{14}C]catecholamine synthesis from [^{14}C]tyrosine in the isolated cells [13]. Therefore, the stimulation of catecholamine synthesis by TPA was apparently not mediated by formation of intracellular cAMP.

It is reported that phorbol esters, such as TPA, activate protein kinase C, but that 4α-phorbol 12,13-didecanoate does not [1]. The synthesis of [^{14}C]catecholamine from [^{14}C]tyrosine was not increased by 4α-phorbol 12,13-didecanoate (10^{-5}–10^{-7} M) (not shown). Therefore, provided that TPA is specific for protein kinase C, the synthesis of catecholamine from tyrosine is likely to be regulated by protein kinase C activity.

Next, we examined the effects of TPA on the stimulation by carbamylcholine and DB-cAMP of [^{14}C]catecholamine synthesis from [^{14}C]tyrosine. The stimulation of catecholamine synthesis by acetylcholine (carbamylcholine) is known to depend on the presence of Ca^{2+} in the medium (table 1) and is thought to be mediated by Ca^{2+}-calmodulin-dependent protein kinase [14–16], while cAMP-induced stimulation of catecholamine synthesis is thought to be mediated by cAMP-dependent protein kinase [15,17,18]. As shown in table 4, the increase in synthesis of [^{14}C]catecholamine by TPA was additive with that by carbamylcholine, but not with that by

Table 2

Effects of TPA and carbamylcholine on $^{45}Ca^{2+}$ uptake and catecholamine release by isolated cells

	$^{45}Ca^{2+}$ uptake (nmol/10^6 cells)	Catecholamine release (%)
Control	1.2 ± 0.1	0.5 ± 0.1
TPA	1.3 ± 0.1	0.7 ± 0.1
Carbamylcholine	15.2 ± 1.7	11.4 ± 1.1
Carbamylcholine + TPA	15.5 ± 1.9	11.2 ± 1.0

Cells were incubated for 15 min with or without TPA (10^{-7} M) and carbamylcholine (10^{-4} M) in the normal medium. Values are means ± SD for 4–6 experiments

Table 1

Effect of extracellular Ca^{2+} on TPA-, carbamylcholine- and DB-cAMP-induced catecholamine formation

	Control	TPA	Carbamylcholine	DB-cAMP
Ca^{2+} (2.2 mM)	80 ± 5	170 ± 10	176 ± 10	181 ± 10
Ca^{2+} (0 mM)	76 ± 4	168 ± 9	78 ± 5	184 ± 11

Cells were incubated for 15 min with or without TPA(10^{-7} M), carbamylcholine (10^{-4} M) and DB-cAMP (7 mM) in normal and Ca^{2+}-free media. The formation of [^{14}C]catecholamine from [^{14}C]-tyrosine is expressed in pmol/15 min per 10^6 cells. Values are means ± SD for 4–6 experiments

Table 3

Effects of TPA and forskolin on the intracellular cAMP level

	cAMP (pmol/10^6 cells)
Control	3.6 ± 0.4
TPA	3.8 ± 0.4
Forskolin	32.4 ± 3.1

Cells were incubated for 15 min with or without TPA (10^{-7} M) and forskolin (10^{-5} M) in the normal medium containing 0.5 mM IBMX. Values are means ± SD for 4–6 experiments. The increase in the cAMP level caused by forskolin (10^{-5} M) reached a maximum after 5–10 incubation, and then decreased slowly. The level of cAMP was still high after 15 min

Table 4

Effect of TPA on carbamylcholine- and DB-cAMP-induced catecholamine formation

	Formation of [^{14}C]catecholamine from [^{14}C]tyrosine (pmol/15 min per 10^6 cells)
Control	80 ± 5
TPA	170 ± 10
Carbamylcholine	176 ± 10
DB-cAMP	181 ± 10
TPA + carbamylcholine	254 ± 12
TPA + DB-cAMP	184 ± 11
Carbamylcholine + DB-cAMP	281 ± 14
TPA + carbamylcholine + DB-cAMP	283 ± 14

Cells were incubated for 15 min with or without TPA (10^{-7} M), carbamylcholine (10^{-4} M) and DB-cAMP (7 mM) in the normal medium. The concentrations of these agents were the lowest that produced maximal increase in [^{14}C]catecholamine synthesis from [^{14}C]tyrosine. Values are means ± SD for 4–6 experiments

DB-cAMP. The increase in synthesis of [^{14}C]catecholamine by carbamylcholine was also additive with that by DB-cAMP. Moreover, the increase in the synthesis of [^{14}C]catecholamine produced by carbamylcholine plus DB-cAMP was not affected by the addition of TPA. These results suggested that the stimulation of catecholamine synthesis by TPA might be mediated by a similar mechanism to that involving cAMP. In this respect, a recent report showing that tyrosine hydroxylase purified from rat pheochromocytoma was phosphorylated by protein kinase C and that the phosphorylated site was identical to that by cAMP-dependent protein kinase [19] is of great interest.

To summarize, the present results suggest that protein kinase C plays a role in the regulation of tyrosine hydroxylase activity, probably by a similar mechanism to that cAMP is involved. The physiological significance of this regulatory mechanism requires further investigation.

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