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ROLE OF Ca^{2+}/PHOSPHOLIPID-DEPENDENT PROTEIN KINASE IN CATECHOLAMINE SECRETION FROM BOVINE ADRENAL MEDULLARY CHROMAFFIN CELLS

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Abstract—The role of Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) in catecholamine secretion from bovine adrenal medullary chromaffin cells was examined using four protein kinase C inhibitors: polymyxin B, sphingosine, staurosporine, and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7). For this purpose, digitonin-permeabilized chromaffin cells were used. Secretion of catecholamines from these cells was stimulated by the addition of micromolar amounts of exogenous free Ca^{2+}. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and arachidonic acid, activators of protein kinase C, enhanced the catecholamine secretion evoked by Ca^{2+}. But phorbol-12,13-diacetate, a phorbol ester analog that does not activate protein kinase C, had no effect on Ca^{2+}-evoked secretion. Polymyxin B at a low concentration (1 μM) abolished the enhancement of secretion by TPA or arachidonic acid without affecting the secretion evoked by Ca^{2+}. However, polymyxin B at higher concentrations (10–100 μM) greatly reduced Ca^{2+}-evoked catecholamine secretion. Sphingosine (10 μM–1 mM), staurosporine (100 nM–1 μM), and H-7 (100–500 μM) inhibited TPA- or arachidonic acid-enhanced secretion but not Ca^{2+}-evoked secretion. In cells in which protein kinase C was down-regulated by TPA, specific binding of [^{3}H]phorbol-12,13-dibutyrate to the cells almost disappeared and the enhancement of secretion by TPA was no longer observed, whereas Ca^{2+}-evoked secretion was maintained. These results strongly suggest that protein kinase C is not essential for the Ca^{2+}-dependent catecholamine secretion from bovine adrenal chromaffin cells, but acts instead as a modulator.

Ca^{2+} plays a very important role in hormone secretion and neurotransmitter release. In bovine adrenal medulla, stimulation of the nicotinic acetylcholine receptor in the cell membrane by a physiological secretagogue, acetylcholine, causes an influx of extracellular Ca^{2+} into the cells, increases the intracellular free Ca^{2+} concentration, and consequently induces catecholamine secretion by exocytosis [1–4]. However, the intracellular mechanism by which Ca^{2+} triggers such exocytotic secretion is still largely unknown.

Several studies have suggested that protein phosphorylation in cells is involved in the secretory process in a number of systems, including adrenal medullary cells [5, 6], neural tissues [7, 8], platelets [9], and mast cells [10]. It has been reported that Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) participates in Ca^{2+}-dependent cell functions in many systems [11]. Protein kinase C is activated when it becomes membrane bound in the presence of Ca^{2+} and diacylglycerol or phorbol ester. In bovine adrenal chromaffin cells, it has been shown that Ca^{2+} influx into the cells causes diacylglycerol accumulation from phosphatidylinositol hydrolysis, and an extremely rapid translocation of protein kinase C to the cell membranes [12]. Several reports have demonstrated that phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA)†, a protein kinase C activator, stimulates the secretion of catecholamines from cultured bovine chromaffin cells and potentiates the secretion induced by Ca^{2+} ionophore or high K^+ medium in the presence of Ca^{2+} [13–16]. TPA also enhances catecholamine secretion from digitonin-permeabilized or electrically permeabilized chromaffin cells evoked by micromolar amounts of free Ca^{2+} [17, 18]. These findings indicate that the activation of protein kinase C may participate in Ca^{2+}-dependent catecholamine secretion from chromaffin cells. In previous experiments, we have reported that polymyxin B, a selective inhibitor of protein kinase C, completely inhibits catecholamine secretion from cultured cells induced by acetylcholine, high K^+ medium, TPA, or Ca^{2+}-ionophore ionomycin [16]. These results further indicate that the activation of protein kinase C may be involved in exocytotic secretion. However, the inhibitory effect of polymyxin B on acetylcholine- or high K^+-induced secretion of catecholamines has been considered to result from blockade of the influx of extracellular Ca^{2+} into the cells stimulated by these secretagogues, rather than an effect on the intracellular secretion mechanism. Furthermore, polymyxin B at high concentrations inhibits not only protein kinase C but also Ca^{2+}/calmodulin-dependent protein kinase [19]. Therefore, it is still unclear whether or not activation of protein kinase C is responsible for catecholamine secretion from bovine adrenal chromaffin cells stimulated by acetylcholine.

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† Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; KRH, Krebs–Ringer–HEPES; and EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Recently, many potent or selective inhibitors of protein kinase C have been found. Therefore, to analyze the role of protein kinase C in catecholamine secretion from adrenal chromaffin cells, we chose four protein kinase C inhibitors which include a polypeptide antibiotic, polymyxin B [19], a sphingoid long-chain base, sphingosine [20], a fungal alkaloid, staurosporine [21], and a synthetic isoquinoline sulfonamide derivative, H-7 [22]. These agents have been shown to inhibit protein kinase C activity *in vitro* and to suppress cellular responses to protein kinase C activators or agonists in several tissues [20, 23–25].

Treatment of chromaffin cells with digitonin enables Ca^{2+} and relatively small molecules in the extracellular medium to enter the cell without affecting the function of the intracellular chromaffin granules [26, 27]. Exocytotic secretion from digitonin-permeabilized chromaffin cells is stimulated by the addition of micromolar amounts of free Ca^{2+} to the medium in the presence of Mg^{2+}-ATP. Therefore, using the permeabilized cells, we have access to the intracellular action sites of Ca^{2+} in stimulus–secretion coupling and can observe the direct action of the inhibitors in the cell.

In this experiment, we examined the effects of four protein kinase C inhibitors on catecholamine secretion from digitonin-permeabilized adrenal chromaffin cells evoked by micromolar amounts of Ca^{2+}.

MATERIALS AND METHODS

Tissue culture instruments were obtained from the Falcon Plastics Co. (Cockeysville, MD, U.S.A.). Eagle's Minimum Essential Medium was from Nissui Seiyaku (Tokyo, Japan). Calf serum, digitonin, and arachidonic acid were obtained from Nakarai Chemicals Ltd. (Kyoto, Japan). Polymyxin B was purchased from the Taito Pfizer Co. Ltd. (Tokyo, Japan). TPA, phorbol-12,13-diacetate, phorbol-12,13-dibutyrate and sphingosine were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Staurosporine was from the Calbiochem Co. (San Diego, CA, U.S.A.). H-7 was from Seikagaku Inc. (Tokyo, Japan). [20 (n)-^{3}H]Phorbol-12,13-dibutyrate (10–20 Ci/mmol) was from Amersham International Ltd. (Arlington Heights, IL, U.S.A.). All other chemicals were of the highest grade available from commercial sources.

Primary cell culture and preparation of digitonin-permeabilized chromaffin cells. Bovine adrenal glands were provided by the Iwate Slaughterhouse. Adrenal chromaffin cells were isolated by digestion of collagenase using a method described previously [28]. The isolated cells were suspended immediately in Eagle's Minimum Essential Medium containing 10% calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (0.3 μg/mL), and were plated on a 35-mm dish at a density of 2×10^6 cells per dish. The cells were maintained at 37° in a CO_2 incubator (95% air/5% CO_2) and were used for experiments at 4 days of culturing. A total of 2×10^6 cells contained 36.3 ± 2.4 μg of catecholamines, as epinephrine and norepinephrine.

Permeabilized cells were prepared by digitonin treatment of the cultured cells as described by Dunn and Holz [26] and Wilson and Kirshner [27]. Briefly, after 4 days in culture the cells were washed twice with pre-warmed Krebs–Ringer–HEPES (KRH) buffer (pH 7.4) containing 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, and 5.6 mM glucose. The cells were then preincubated for 10 min at 37°, and incubated for 5 min with 20 μg/mL digitonin in potassium glutamate–HEPES buffer (pH 7.4), consisting of 150 mM potassium glutamate, 15 mM HEPES-KOH, 5 mM EGTA, and 5.6 mM glucose. The digitonin-treated cells were washed with potassium glutamate–HEPES buffer and incubated for 5 min, with or without the test agents in potassium glutamate–HEPES buffer containing 2 mM ATP, 2 mM $MgSO_4$, and either 5 mM EGTA or 5 mM EGTA and various amounts of $CaCl_2$. The concentrations of free Ca^{2+} were adjusted by use of an EGTA–Ca^{2+} buffer and were calculated according to Portzehl *et al.* [29].

Secretion of catecholamines from digitonin-permeabilized cells. After incubation, the reaction was terminated by transferring the incubation medium to tubes in an ice-cold bath. Catecholamines secreted into the medium were extracted with 0.4 M perchloric acid and adsorbed to aluminum hydroxide. Their amounts were estimated by the ethylenediamine condensation method [30], using a fluorescence spectrophotometer (Hitachi 650-10S) at an excitation wavelength of 420 nm and an emission wavelength of 540 nm. At those wavelengths, epinephrine and norepinephrine showed the same fluorescence intensity. The amount of catecholamines secreted from the cells was expressed as percent of total cellular catecholamines.

Down-regulation of protein kinase C and binding of [^{3}H]phorbol-12,13-dibutyrate in the cells. For down-regulation of protein kinase C [31], the cultured cells were incubated with 1 μM TPA in the culture medium for 24 hr at 37° in a CO_2 incubator, and were subjected to digitonin treatment.

The binding of [^{3}H]phorbol-12,13-dibutyrate on the cells down-regulated by TPA was determined by a method described elsewhere [31]. After preincubation of the cells with 1 μM TPA for 24 hr at 37°, the cells were washed with KRH buffer and incubated for 30 min at 30° with 1 mL of KRH buffer containing 22 nM [^{3}H]phorbol-12,13-dibutyrate (0.63 μCi). The cells were washed in ice-cold KRH buffer and solubilized in 1 mL of 10% Triton X-100; then the solution was transferred to a counting vial. Radioactivity was determined by a liquid scintillation spectrometer (Aloka LSC-900). Nonspecific binding was defined in the presence of 2 μM TPA. Specific binding was expressed as cpm bound/2×10^6 cells.

Statistics. Statistical calculations were made according to the methods of Snedecor and Cochran [32]. Differences were considered significant when P, calculated by Student's t test, was < 0.05.

RESULTS

Effects of free Ca^{2+} and TPA on catecholamine secretion from digitonin-permeabilized bovine adrenal chromaffin cells. As shown in our previous report [16], polymyxin B (100 μM), an inhibitor of

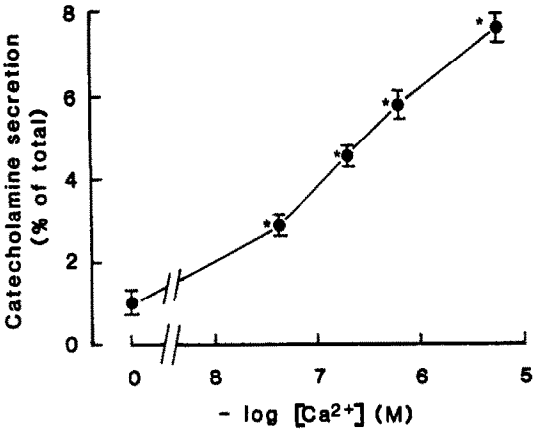

Fig. 1. Effects of different concentrations of free Ca^{2+} on catecholamine secretion from digitonin-permeabilized cells. After preincubation of the cultured cells for 10 min at 37°, the cells were incubated for 5 min with 20 μ/mL digitonin in potassium glutamate–HEPES buffer. Digitonin-permeabilized cells were incubated for 5 min at 37° with different concentrations of free Ca^{2+} in potassium glutamate–HEPES buffer. Catecholamines secreted from the cells were determined as described in Materials and Methods. Catecholamine secretion is shown as a percentage of the total cellular catecholamine content (36.3 ± 2.4 μg). Values are means ± SD from four experiments. Key: (*) significantly different from control ($P < 0.001$).

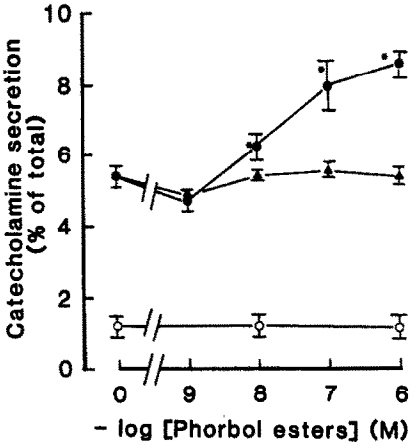

Fig. 2. Effects of different concentrations of TPA and phorbol-12,13-diacetate on catecholamine secretion from digitonin-permeabilized cells. The digitonin-permeabilized cells were incubated for 5 min at 37° with different concentrations of TPA in Ca^{2+}-free (with 5 mM EGTA, ○) or 600 nM free Ca^{2+}-containing medium (●), or of phorbol-12,13-diacetate in 600 nM free Ca^{2+}-containing medium (▲). Catecholamines secreted from the cells were determined as described in Materials and Methods. Catecholamine secretion is shown as a percentage of the total cellular catecholamine content (36.3 ± 2.4 μg). Values are means ± SD from four experiments. Key: (*) significantly different from control ($P < 0.02$).

protein kinase C, completely inhibited catecholamine secretion from cultured bovine adrenal chromaffin cells treated with acetylcholine in the presence of Ca^{2+}. However, this inhibitory effect was due to blockade of the Ca^{2+} influx into the cells, as the rise in the cytosolic free calcium concentration induced by acetylcholine was abolished by polymyxin B (100 μM). To examine the direct effects of protein kinase C inhibitors on Ca^{2+}-dependent catecholamine secretion, we used cultured bovine adrenal chromaffin cells treated with a low concentration of digitonin (20 μg/mL). Digitonin-permeabilized cells are stimulated to secrete catecholamines by exocytosis by the addition of micromolar amounts of Ca^{2+} to the medium [26, 27]. Under our experimental conditions, the basal (spontaneous) secretion of catecholamines in the presence of 5 mM EGTA was 1.10 ± 0.30% of total catecholamines (Fig. 1). A significant increase in catecholamine secretion (2.80% of total catecholamines) was observed with 43 nM free Ca^{2+}, and the secretion at 5.6 μM free Ca^{2+}, the highest concentration tested, was 7.60% of total catecholamines. In this experiment, we used a concentration of 600 nM free Ca^{2+} (5.85% secretion of total catecholamines).

TPA, a phorbol ester, is known to enhance catecholamine secretion evoked by free Ca^{2+} in digitonin-permeabilized and electrically permeabilized chromaffin cells [17, 18]. As shown in Fig. 2, we also obtained similar results. The increase in catecholamine secretion evoked by 600 nM free Ca^{2+} was potentiated by TPA in a concentration-dependent manner. TPA at 100 nM, which is a submaximal concentration (7.93% secretion of total catecholamines), was used in the experiment below. TPA did not enhance catecholamine secretion in the absence of external Ca^{2+} (Fig. 2). Ca^{2+}-evoked secretion was also potentiated by phorbol-12,13-dibutyrate, a phorbol ester analog that activates protein kinase C (data not shown), but not by its "inactive" analog phorbol-12,13-diacetate (Fig. 2) [33]. The stimulatory concentrations of phorbol-12,13-dibutyrate were very similar to those of TPA.

Effects of protein kinase C inhibitors on Ca^{2+}- *and* Ca^{2+} *plus TPA-evoked catecholamine secretion.* Figure 3 shows the effects of different concentrations of the four inhibitors, polymyxin B, sphingosine, staurosporine, and H-7, on catecholamine secretion from digitonin-permeabilized chromaffin cells stimulated by addition of 600 nM free Ca^{2+} or 600 nM Ca^{2+} plus 100 nM TPA to the medium. As shown in Fig. 3A, polymyxin B decreased both Ca^{2+}- and Ca^{2+} plus TPA-evoked catecholamine secretion in a concentration-dependent manner. Polymyxin B at low concentrations (100 nM and 1 μM) inhibited only the secretion stimulated by Ca^{2+} plus TPA; polymyxin B at 100 nM greatly reduced TPA-enhanced secretion (79% inhibition), and at 1 μM completely abolished it without affecting Ca^{2+}-evoked secretion. On the other hand, polymyxin B at higher concentrations (10 and 100 μM) decreased Ca^{2+}-evoked secretion; polymyxin B at 10 μM reduced it (36% inhibition), and at 100 μM completely suppressed it. Polymyxin B itself had no effect on the basal secretion of catecholamines in non-stimulated cells. The other three protein kinase C inhibitors, sphingosine (10 μM–1 mM), staurosporine (100 nM–1 μM), and H-7 (100–500 μM), depressed only the secretion of catecholamines enhanced by 100 nM

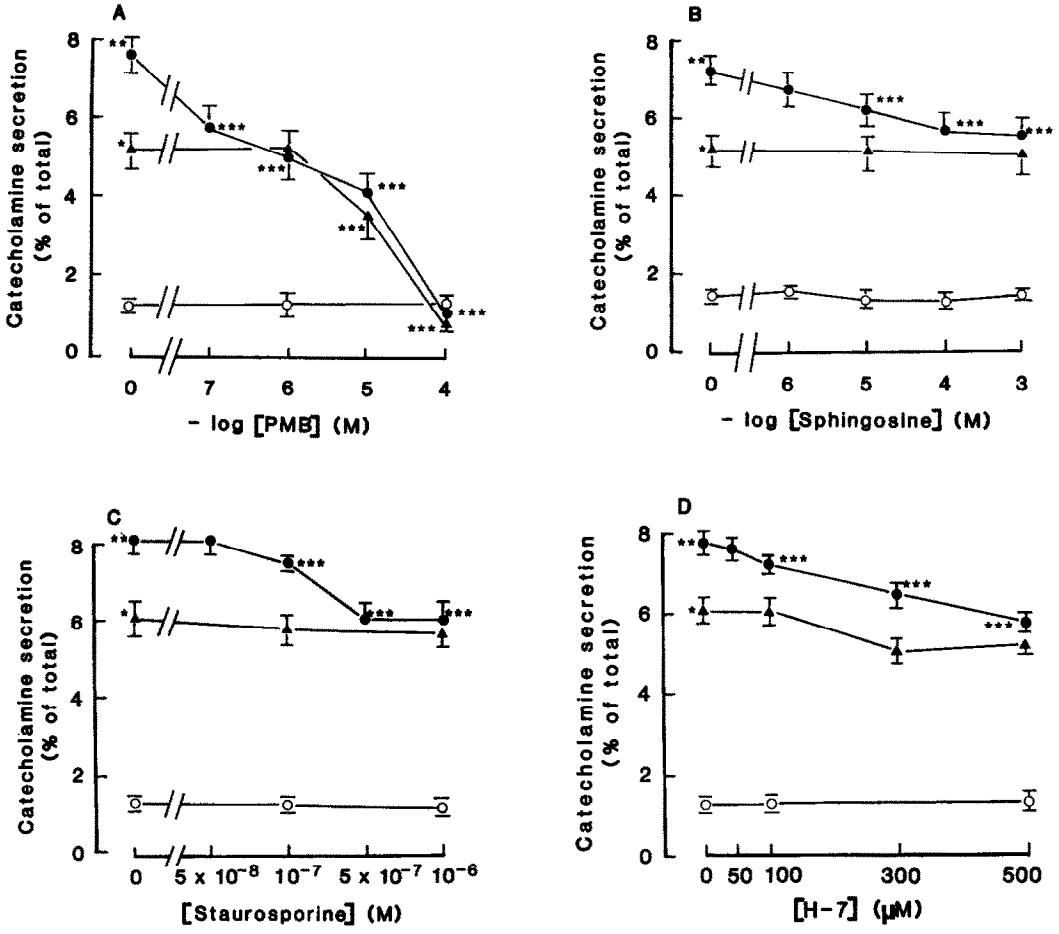

Fig. 3. Effects of different concentrations of polymyxin B (PMB), sphingosine, staurosporine, and H-7 on catecholamine secretion evoked by 600 nM Ca^{2+} or 600 nM Ca^{2+} + 100 nM TPA. After pre-incubation of the cultured cells for 10 min at 37°, the cells were incubated for 5 min with 20 μg/mL digitonin in potassium glutamate–HEPES buffer (first incubation). Digitonin-permeabilized cells were incubated for 5 min at 37° with different concentrations of PMB (A), sphingosine (B), staurosporine (C), or H-7 (D) in Ca^{2+}-free (with 5 mM EGTA, ○), 600 nM free Ca^{2+} (▲)-, or 600 nM free Ca^{2+} + 100 nM TPA-containing medium (●, second incubation). Catecholamines secreted from the cells were determined as described in Materials and Methods. Catecholamine secretion is shown as a percentage of the total cellular catecholamine content (36.3 ± 2.4 μg). Values are means ± SD from four experiments. Key: (*) significantly different from control ($P < 0.001$); (**) significantly different from Ca^{2+}-evoked secretion ($P < 0.01$); and (***) significantly different from Ca^{2+} + TPA-evoked secretion ($P < 0.01$).

TPA without affecting the secretion evoked by 600 nM free Ca^{2+} (Fig. 3, B–D). These inhibitors had no effect on the basal secretion. The inhibition of TPA-induced secretion by sphingosine was 48% at 10 μM and 76 and 81% at 100 μM and 1 mM respectively. The inhibition by staurosporine was 15% at 100 nM, and at 500 nM the inhibition was almost complete. The inhibition by H-7 was 35% at 100 μM and 300 and 500 μM concentrations produced 82 and 100% inhibition respectively. On the other hand, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004) at 100 and 300 μM had no effect on TPA-enhanced and Ca^{2+}-evoked secretion, and at 500 μM slightly depressed only TPA-enhanced secretion (data not shown). The inhibition of protein kinase C by HA1004 ($K_i = 40$) is less potent than that by H-7 ($K_i = 6.0$) [22]. These results suggest that the secretion of catecholamines from digitonin-permeabilized chromaffin cells induced by TPA in the presence of free Ca^{2+} is mediated via activation of protein kinase C.

Effects of protein kinase C inhibitors on Ca^{2+} *plus arachidonic acid-evoked catecholamine secretion.* To further confirm a relationship between catecholamine secretion and protein kinase C activation, we examined the influence of the protein kinase C inhibitors on the secretion from digitonin-treated chromaffin cells evoked by another activator of protein kinase C, arachidonic acid. Unsaturated fatty acids are known to activate protein kinase C *in vitro* [34]. In digitonin-permeabilized chromaffin cells, arachidonic acid enhanced the secretion from the

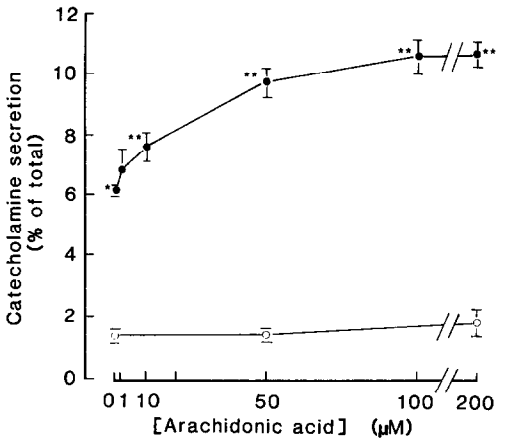

Fig. 4. Effects of different concentrations of arachidonic acid on catecholamine secretion. The permeabilized cells were incubated for 5 min at 37° with different concentrations of arachidonic acid in Ca^{2+}-free (with 5 mM EGTA, ○) or 600 nM free Ca^{2+}-containing medium (●). Catecholamines secreted from the cells were determined as described in Materials and Methods. Catecholamine secretion is shown as a percentage of the total cellular catecholamine content (36.3 ± 2.4 μg). Values are means ± SD from four experiments. Key: (*) significantly different from control ($P < 0.001$); and (**) significantly different from Ca^{2+}-evoked secretion ($P < 0.01$).

cells evoked by 600 nM Ca^{2+} in a concentration-dependent manner, but it had little effect on the basal secretion in the absence of external free Ca^{2+} (Fig. 4). A significant enhancement of secretion was observed with 10 μM arachidonic acid. The enhanced catecholamine secretion was maximal at 100 μM arachidonic acid (Fig. 4). A submaximal concentration, 50 μM arachidonic acid, which resulted in about 1.5-fold enhanced increase in secretion, was used in the experiment below.

Figure 5 shows the effects of polymyxin B, sphingosine, staurosporine, and H-7 on catecholamine secretion from the permeabilized cells evoked by 600 nM Ca^{2+} plus 50 μM arachidonic acid. Polymyxin B at low concentrations (100 nM and 1 μM) depressed only the secretion enhanced by arachidonic acid (Fig. 5A). The other inhibitors, sphingosine (10 μM–1 mM), staurosporine (100 nM–1 μM), and H-7 (100–500 μM), also inhibited only the secretion enhanced by arachidonic acid (Fig. 5, B–D). The inhibitory concentrations of these substances in arachidonic acid-enhanced secretion of catecholamines were very similar to those in TPA-enhanced secretion.

Effect of down-regulated of protein kinase C on catecholamine secretion. When the cells were treated with 1 μM TPA for 24 hr at 37°, specific binding of [^{3}H]phorbol-12,13-dibutyrate to the cells was greatly reduced to 19% of total specific binding (Fig. 6A). This suggests that protein kinase C in the cells is almost entirely down-regulated by long-term exposure of the cells to phorbol ester.

As shown in Fig. 6B, treatment of the cells with 1 μM TPA for 24 hr abolished the enhancement of catecholamine secretion from digitonin-permeabilized cells evoked by TPA, whereas this treatment had no effect on the secretion evoked by 600 nM free Ca^{2+}.

DISCUSSION

Although many protein kinase C inhibitors have been found, they are not highly specific for protein kinase C. Therefore, inhibition of cell function by one kinase C inhibitor cannot be attributed directly to the inhibition of protein kinase C. To solve this problem, the effects of two or more inhibitors on the same cell function should be compared. We chose four protein kinase inhibitors, polymyxin B, sphingosine, staurosporine, and H-7. Protein kinase C possesses the catalytic site and the regulatory site to which the negative phospholipid, phosphatidylserine, binds. Therefore, the inhibitors can be grouped roughly into two types. It has been reported that polymyxin B and sphingosine act on the regulatory site, but at a low concentration of phosphatidylserine sphingosine also interacts at the catalytic site of the enzyme [19, 35, 36]. On the other hand, staurosporine and H-7 act on the catalytic site [21, 22, 36].

Our results have demonstrated that all four protein kinase C inhibitors, polymyxin B at low concentrations (100 nM and 1 μM), sphingosine (10 μM–1 mM), staurosporine (100 nM–1 μM), and H-7 (100–500 μM), almost completely inhibited only the enhancement of Ca^{2+}-evoked secretion by TPA (Fig. 3, A–D). These results indicate that the stimulatory effect of TPA on the secretion is attributable to activation of protein kinase C. Therefore, the phosphorylation of some proteins by activated protein kinase C probably induces catecholamine secretion in adrenal chromaffin cells. This is confirmed by the finding that the secretion enhanced by another protein kinase C activator, arachidonic acid, also was almost completely abolished by the four kinase inhibitors (Fig. 5, A–D). On the other hand, neither polymyxin B at low concentrations nor sphingosine, staurosporine, and H-7 inhibited catecholamine secretion from digitonin-treated cells evoked by micromolar amounts of free Ca^{2+} (Fig. 3, A–D). Furthermore, the effect of Ca^{2+} on the secretion persisted in cells in which protein kinase C had been greatly reduced by down-regulation following long-term treatment with TPA, although that of TPA was abolished (Fig. 6). These results strongly suggest that the activation of protein kinase C is not involved in the process of Ca^{2+}-dependent catecholamine secretion in bovine adrenal chromaffin cells. Therefore, protein kinase C probably modulates but is not essential for Ca^{2+}-dependent secretion. This view is also supported by the finding of Holz and Senter [37] that trypsin at low concentrations (3–10 μg/mL) specifically inhibits catecholamine secretion enhanced by TPA but not evoked by Ca^{2+}, and that trypsin at higher concentrations (30–50 μg/mL) is required for inhibition of Ca^{2+}-evoked secretion in digitonin-permeabilized cells. However, protein kinase C has at least seven isoenzymes, which show different affinities for Ca^{2+}, phospholipids, or unsaturated fatty acids and are present in tissues in different distributions [38–40]. It is possible that each

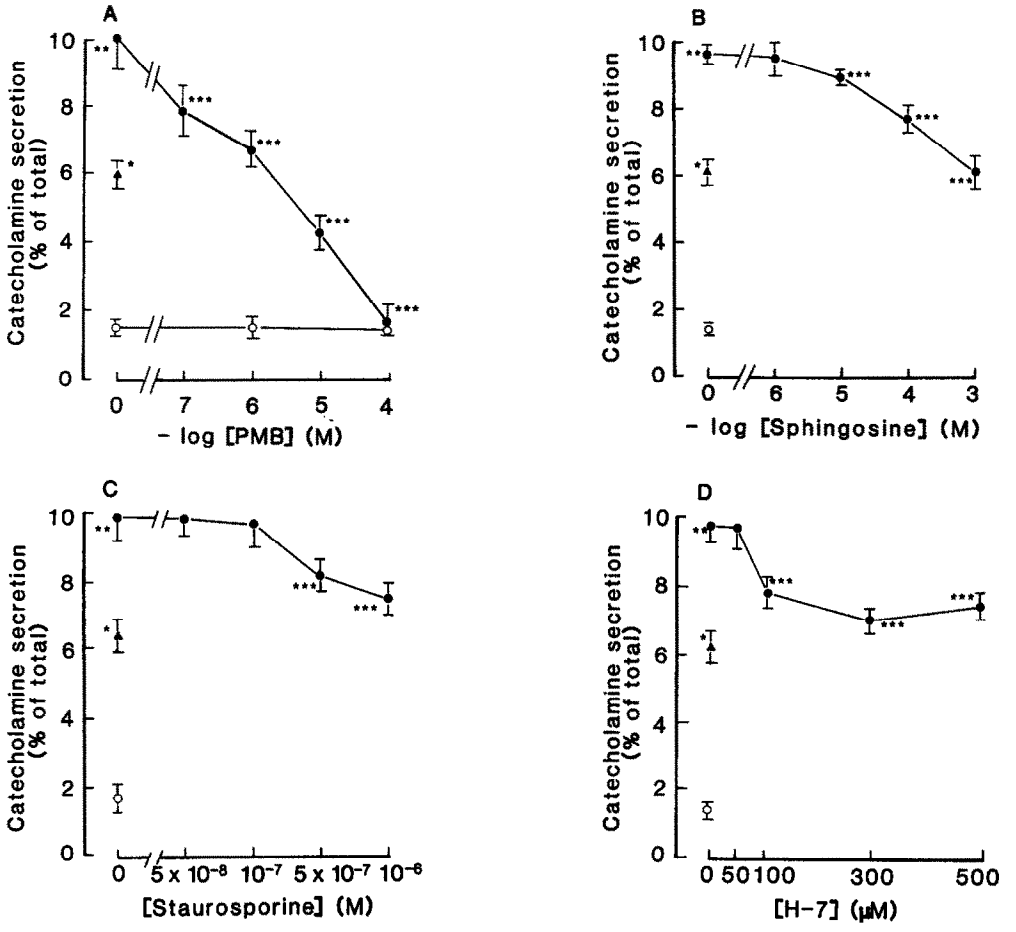

Fig. 5. Effects of different concentrations of polymyxin B (PMB), sphingosine, staurosporine, and H-7 on catecholamine secretion evoked by 600 nM Ca^{2+} + 50 μM arachidonic acid. The permeabilized cells were incubated for 5 min at 37° with different concentrations of PMB (A), sphingosine (B), staurosporine (C), or H-7 (D) in Ca^{2+}-free (with 5 mM EGTA, ○), 600 nM free Ca^{2+} (▲)-, or 600 nM free Ca^{2+} + 50 μM arachidonic acid-containing medium (●). Catecholamines secreted from the cells were determined as described in Materials and Methods. Catecholamine secretion is shown as a percentage of the total cellular catecholamine content (36.3 ± 2.4 μg). Values are means ± SD from four experiments. Key: (*) significantly different from control ($P < 0.001$); (**) significantly different from Ca^{2+}-evoked secretion ($P < 0.01$); and (***) significantly different from Ca^{2+} + arachidonic acid-evoked secretion ($P < 0.01$).

isoenzyme possesses a different affinity for the protein kinase C inhibitors. Therefore, some of these enzyme subtypes may not be inhibited by the four kinase inhibitors, and the subtypes may participate in the Ca^{2+}-dependent secretion. We need to investigate which subtypes are present in bovine adrenal chromaffin cells and their affinities for the four inhibitors.

In cells in which protein kinase C had been greatly down-regulated, the stimulatory effect of TPA on the secretion was abolished completely but that of Ca^{2+} was not affected (Fig. 6, A and B). On the basis of the results of [^{3}H]phorbol ester binding, however, 19% of total cellular protein kinase C remains in the cells treated with TPA. Therefore the possibility cannot be denied completely that the remaining enzyme is not depressed by the inhibitors and induces Ca^{2+}-evoked secretion.

Burgoyne *et al.* [41] have reported that protein kinase C plays a major role in Ca^{2+}-activated exocytosis in digitonin-permeabilized adrenal chromaffin cells. This conclusion is based on the concept that down-regulation of protein kinase C following long-term exposure to phorbol ester is associated with a decrease in Ca^{2+}-activated catecholamine secretion from the cells. This finding is contrary to our observation that the down-regulation of protein kinase C has no effect on the Ca^{2+}-evoked secretion. Although the reason for the discrepancy is not clear, there are several differences in experimental conditions among different laboratories; in the laboratory of those authors [41], the cells were isolated by protease instead of collagenase digestion, and Dulbecco's modified Eagle's medium was used as a culture medium. These differences in culture conditions, and particularly in the methodology used for

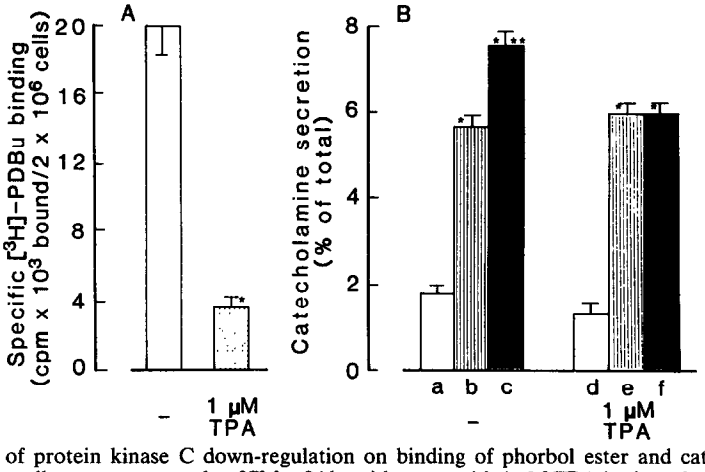

Fig. 6. Effects of protein kinase C down-regulation on binding of phorbol ester and catecholamine secretion. Intact cells were pretreated at 37° for 24 hr without or with 1 μM TPA in the culture medium. (A) Specific binding of [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]-PDBu) was determined as described in Materials and Methods. (B) After preincubation with or without 1 μM TPA, the cells were incubated with 20 μg/mL digitonin in the medium for 5 min at 37°. The permeabilized cells were then incubated for 5 min without (a and d) or with 600 nM free Ca^{2+} (b and e) or 600 nM free Ca^{2+} + 100 nM TPA (c and f) in the medium. Catecholamines secreted from the cells were determined as described in Materials and Methods. Catecholamine secretion is shown as a percentage of the total cellular catecholamine content (36.3 ± 2.4 μg). Values are means ± SD from four experiments. Key: (*) significantly different from control ($P < 0.001$); and (**) significantly different from Ca^{2+}-evoked secretion ($P < 0.001$).

digestion of the adrenal medulla, may account for these discrepancies.

Mazzei *et al.* [19] have reported that polymyxin B also inhibits Ca^{2+}/calmodulin-dependent enzyme as well as protein kinase C *in vitro*. The inhibition of Ca^{2+}/calmodulin-dependent protein kinase by polymyxin B is competitive with respect to calmodulin. However, polymyxin B is a more selective inhibitor for protein kinase C than Ca^{2+}/calmodulin-dependent protein kinase. The IC_{50} of polymyxin B for protein kinase C was 6–8 μM and for Ca^{2+}/calmodulin-dependent myosin light-chain kinase 80–100 μM under their assay conditions. Our present results showed that polymyxin B at low concentrations (100 nM–1 μM) inhibited TPA- or arachidonic acid-enhanced secretion of catecholamines and at higher concentrations (10–100 μM) inhibited Ca^{2+}-evoked secretion (Figs 3 and 5). Therefore, it is possible that a calmodulin-dependent mechanism (enzyme) may be involved in Ca^{2+}-evoked secretion of catecholamines, although the possibility that polymyxin B acts on other Ca^{2+}-dependent secretory mechanisms cannot be excluded. However, Wilson and Kirschner [27] have reported that Ca^{2+}-dependent secretion of catecholamines from digitonin-permeabilized chromaffin cells is not inhibited by calmodulin antagonists, trifluoperazine (10 μM), pimozide (10–50 μM), and *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W-13) (10–50 μM). On the other hand, it has been shown by Baker and Knight [32] that trifluoperazine (42 μM) abolishes catecholamine secretion from electrically permeabilized chromaffin cells evoked by Ca^{2+}. Recently, we also found in preliminary studies that *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (10–100 μM), a relatively specific inhibitor of calmodulin, strongly inhibits Ca^{2+}-evoked secretion from the permeabilized cells, but not *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) (10–100 μM) which has a much lower affinity for calmodulin [43]. Therefore, it is still unclear whether or not calmodulin participates in Ca^{2+}-dependent catecholamine secretion from adrenal medullary chromaffin cells. Further studies on the role of calmodulin in the Ca^{2+}-dependent secretory mechanisms of catecholamines are now in progress.

In summary, the results presented here suggest that protein kinase C is not essential for the Ca^{2+}-dependent catecholamine secretion from bovine adrenal medullary chromaffin cells, but acts instead as a modulator.

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