Arachidonic Acid Release and Catecholamine Secretion from Digitonin-Treated Chromaffin Cells: Effects of Micromolar Calcium, Phorbol Ester, and Protein Alkylating Agents

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Abstract: The relationship between catecholamine secretion and arachidonic acid release from digitonin-treated chromaffin cells was investigated. Digitonin renders permeable the plasma membranes of bovine adrenal chromaffin cells to Ca^{2+}, ATP, and proteins. Digitonin-treated cells undergo exocytosis of catecholamine in response to micromolar Ca^{2+} in the medium. The addition of micromolar Ca^{2+} to digitonin-treated chromaffin cells that had been prelabeled with [³H]arachidonic acid caused a marked increase in the release of [³H]arachidonic acid. The time course of [³H]arachidonic acid release paralleled catecholamine secretion. Although [³H]arachidonic acid release and exocytosis were both activated by free Ca^{2+} in the micromolar range, the activation of [³H]arachidonic acid release occurred at Ca^{2+} concentrations slightly lower than those required to activate exocytosis. Pretreatment of the chromaffin cells with N-ethylmaleimide (NEM) or p-bromophenacyl bromide (BPB) resulted in dose-dependent inhibition of 10 μM Ca^{2+}-stimulated [³H]arachidonic acid release and exocytosis. The IC_{50} of NEM for both [³H]arachidonic acid release and exocytosis was 40 μM. The IC_{50} of BPB for both events was 25 μM. High concentrations (5–20 mM) of Mg^{2+} caused inhibition of catecholamine secretion without altering [³H]arachidonic acid release. A phorbol ester that activates protein kinase C, 12-O-tetradecanoylphorbol-13-acetate (TPA), caused enhancement of both [³H]arachidonic acid release and exocytosis. The findings demonstrate that [³H]arachidonic acid release is stimulated during catecholamine secretion from digitonin-treated chromaffin cells and they are consistent with a role for phospholipase A_2 in exocytosis from chromaffin cells. Furthermore the data suggest that protein kinase C can modulate both arachidonic acid release and exocytosis. Key Words: Catecholamine secretion—Arachidonic acid release—Chromaffin cells—Digitonin. Frye R. A. and Holz R. W. Arachidonic acid release and catecholamine secretion from digitonin-treated chromaffin cells: Effects of micromolar calcium, phorbol ester, and protein alkylating agents. J. Neurochem. 44, 265–273 (1985).

Exocytosis is a ubiquitous mechanism for the secretion of prepackaged hormones, neurotransmitters, and other substances. The release from cells of the contents of the secretory vesicles by exocytosis was first demonstrated biochemically in early studies of Ca^{2+}-dependent catecholamine secretion from the adrenal medulla (for review, see Viveros, 1975). However, the biochemical events that mediate exocytosis are still largely unknown. Phospholipase A_2 (EC 3.1.1.4; phosphatide 2-acylhydrolase) is a Ca^{2+}-dependent enzyme that releases unsaturated fatty acids from the sn- 2-position of phospholipid. Because lysophospholipids (Ahkong et al., 1972) and unsaturated fatty acids such as arachidonic acid (Creutz, 1981) are membrane fusogens, it is possible that this enzyme plays a role in exocytosis. In a previous study we found that stimulation of intact chromaffin cells with a nicotinic cholinergic agonist or by depolarization with elevated K^+ caused arachidonic acid release that was correlated with catecholamine secretion with regard to time course and Ca^{2+} dependency (Frye and Holz, 1984). Activation of arachidonic acid release occurs during exocytosis in several other systems

including platelets (Bills et al., 1976), mast cells (Roberts et al., 1979), and pituitary cells (Naor and Catt, 1981).

The plasma membranes of cultured bovine adrenal medullary chromaffin cells can be rendered leaky by digitonin treatment which allows micromolar Ca^{2+} and ATP in the extracellular medium to enter the chromaffin cells and directly stimulate exocytosis (Dunn and Holz, 1983; Wilson and Kirshner, 1983). The plasma membrane also becomes permeable to proteins. Thus, the preparation allows direct access to the intracellular events occurring during exocytosis. In the present study we have characterized arachidonic acid release from digitonin-treated chromaffin cells and have correlated the release with the exocytosis of catecholamine. The effects of Ca^{2+}, Mg^{2+}, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), and the protein alkylating agents N-ethylmaleimide (NEM) and p-bromophenacyl bromide (BPB) on both arachidonic acid release and exocytosis were investigated.

MATERIALS AND METHODS

Cell preparation

Cells disaggregated from bovine adrenal medullae (Fenwick et al., 1978; Kilpatrick et al., 1980) were added to 6.4-mm diameter uncoated culture wells (Costar, Cambridge, MA) at a density of 150,000 cells/well in 0.3 ml of Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Sterile Systems, Logan, UT), 10 μM cytosine arabinoside (to inhibit fibroblast proliferation), gentamicin (50 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (2.5 μg/ml) (Squibb, Princeton, NJ). In some experiments chromaffin cells were purified by centrifugation through a Percoll density gradient (Kilpatrick et al., 1980) or by differential plating (Waymire et al., 1983). Cortisol released into the culture medium was measured by radioimmune assay. After 4 days at 34°C in 5% CO_2/95% air, chromaffin cells formed monolayers containing approximately 40 nmol catecholamine/10^6 cells. The culture medium was replaced after 4 days and experiments were performed on days 5–10.

Catecholamine secretion

Cells were incubated for 5 h with 40 μl/well of culture medium described above without cytosine arabinoside containing 0.5% (wt/vol) fatty acid-free bovine serum albumin (BSA), 0.5 mM ascorbate, and 1.0 μCi/ml l-[7-^{3}H]norepinephrine (Amersham, Chicago, IL). Cells were then incubated three times for 10 min with 0.25 ml/well physiological salt solution containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 15 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4), and 0.5% (wt/vol) fatty acid-free BSA.

Experiments were performed at 25°C. Unless otherwise indicated the cells were incubated for 5 min with 40 μl of potassium glutamate solution containing 139 mM potassium glutamate, 20 mM 1,4-piperazine diethanesulfonic acid (PIPES) (pH 6.6), 1 mM $MgCl_2$, 1 mM MgATP, 20 μM digitonin, 0.5% (wt/vol) fatty acid-free BSA, 5 mM

EGTA, and various amounts of $CaCl_2$ to achieve free Ca^{2+} concentrations calculated according to Portzehl et al. (1964). After removal of the potassium glutamate incubation solution containing the released [^{3}H]-norepinephrine, the [^{3}H]norepinephrine remaining in the cells was liberated by the addition of 40 μl of 1% Triton X-100. The radioactivity in the solutions was measured in a scintillation counter. Catecholamine secretion was expressed as the percentage of the total radioactivity released into the medium.

Arachidonic acid release

Cells were incubated for 5 h with 40 μl/well of culture medium described above without cytosine arabinoside containing 0.5% wt/vol fatty acid-free BSA and 10 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (100 μCi/mmol) (Amersham, Chicago, IL). [^{3}H]Arachidonic acid-labeled cells were rinsed three times with physiological salt solution and treated in an identical fashion as [^{3}H]norepinephrine-labeled cells as described above. The 40-μl aqueous incubation solution containing the released [^{3}H]arachidonic acid was removed from the cells and applied directly to the preadsorbent zone of the TLC plate (Analtech, Newark, DE) which contained 10 μg of previously applied nonradioactive arachidonic acid to allow subsequent iodine vapor visualization. Following separation with ethylacetate/acetic acid (100:1), the arachidonic acid band was scraped and counted to determine released [^{3}H]arachidonic acid. Experiments in which ethylacetate extracts of the test solutions were applied to the TLC plates gave identical results. The radioactivity remaining in the cells was solubilized in 1% Triton X-100 and counted. Results of the [^{3}H]arachidonic acid release experiments are expressed as percent of the total radioactivity per well released into the medium as free [^{3}H]arachidonic acid.

In experiments in which the distribution of radioactivity in cell lipids was determined, the lipids of the cell monolayer were dissolved in 75 μl of ice cold methanol which was added to the well immediately following the removal of the incubation solution. The labeled lipids in the methanol extracts were fractionated by one-dimensional, sequential, ascending TLC. The plates were first run for 15 min in chloroform/methanol/water (95:35:6). The plates were then run for 25 min in hexane/diethyl ether/ethanol/NH_3 (75:20:5:0.5). The lipids were separated with the following approximate R_f values: phosphatidylinositol/phosphatidylserine (0.05), phosphatidylcholine (0.10), phosphatidylethanolamine (0.25), arachidonic acid (0.45), diacylglycerol (0.60), and triacylglycerol/cholesterol ester (0.80).

Data are expressed as mean ± SEM. Error bars smaller than point symbols were omitted from figures. BSA, ATP, TPA, and NEM were purchased from Sigma (St. Louis, MO), BPB from Aldrich (Milwaukee, WI), and digitonin from Fluka (Hauppauge, NY).

RESULTS

Lipid distribution of radioactivity in [^{3}H]arachidonic acid-labeled chromaffin cell cultures

The monolayer cultures of bovine chromaffin cells incorporated [^{3}H]arachidonic acid primarily

TABLE 1. *[³H]Norepinephrine secretion and lipid distribution of radioactivity in chromaffin cells labeled with [³H]arachidonic acid*

	NE secretion	AA medium	AA cell	PC cell	PE cell	PI/PS cell	TG/CE cell	DG cell
PSS	2.2 ± 0.2	0.146 ± 0.012	0.226 ± 0.014	39.7 ± 0.6	33.7 ± 0.5	6.4 ± 0.2	19.1 ± 0.2	0.65 ± 0.01
Digitonin (0 Ca²⁺)	2.7 ± 0.2	0.188 ± 0.003	0.212 ± 0.007	38.4 ± 0.4	33.7 ± 0.2	6.6 ± 0.1	20.0 ± 0.7	0.84[b] ± 0.03
Digitonin (10 µM Ca²⁺)	11.8 ± 0.3	0.553[a] ± 0.018	0.383[a] ± 0.017	38.1 ± 0.4	33.4 ± 0.4	6.5 ± 0.1	20.2 ± 0.6	0.98[c] ± 0.04

Cultured chromaffin cells, which were prelabeled with [³H]norepinephrine or [³H]arachidonic acid for 5 h, were incubated with physiological salt solution (PSS) or a 20 µM digitonin-containing potassium glutamate solution with or without 10 µM free Ca²⁺. Catecholamine secretion, arachidonic acid release into the medium, and distribution of radioactivity in the indicated cell lipids were determined after 5 min. Lipid results are expressed as percentage of the radioactivity in the indicated lipids. There were four wells/group.

PC, Phosphatidylcholine; PE, phosphatidyl ethanolamine; PI/PS, phosphatidylinositol and phosphatidylserine; TG/CE, triacylglycerol and cholesterol ester; DG, diacylglycerol.

[a] $p < 0.001$ versus digitonin and 0 Ca²⁺. [b] $p < 0.001$ versus PSS. [c] $p < 0.05$ versus digitonin, 0 Ca²⁺.

into the phospholipid pools; however, some of the incorporated radioactivity was associated with a neutral lipid fraction (Table 1). Less than 1% of the radioactivity associated with the cells was in the form of free [³H]arachidonic acid. Treatment of the cells with 20 µM digitonin in the absence of Ca²⁺ caused little change in the distribution of [³H]arachidonic acid in the cells and a small increase in the amount of [³H]arachidonic acid released into the medium. Digitonin treatment in the presence of 10 µM free Ca²⁺ caused an almost twofold increase in the level of cell-associated free [³H]arachidonic acid and a nearly three-fold increase in the amount of free [³H]arachidonic acid released into the medium (Table 1). In the presence of digitonin, Ca²⁺ caused only a small increase (17 ± 5%) in the level of labeled diacylglycerol when the rates of [³H]arachidonic acid release and catecholamine secretion were maximal (Fig. 1). Because most (about 70%) of the Ca²⁺-dependent [³H]arachidonic acid production was released into the medium, in the subsequent experiments only [³H]arachidonic acid released into the medium was measured. Therefore the data may somewhat underestimate the total Ca²⁺-dependent [³H]-arachidonic acid produced. In parallel experiments performed under identical conditions with [³H]norepinephrine-labeled chromaffin cells, the presence of 10 µM Ca²⁺ caused a large increase in catecholamine secretion (Table 1).

In one experiment it was determined that more polar derivatives of [³H]arachidonic acid were released from the cells. The radioactivity represented 16% that of released free [³H]arachidonic acid and these compounds were not further investigated.

It is possible that cells such as fibroblasts and adrenal cortical cells that contaminate adrenal medullary chromaffin cell cultures could contribute to the [³H]arachidonic acid release that we observed.

Indeed, digitonin-treated monolayer cultures of bovine adrenal cortical cells labeled with [³H]-arachidonic acid released [³H]arachidonic acid in response to 10 µM Ca²⁺ in a similar manner to that observed with the chromaffin cell cultures (data not shown). However it is unlikely that contaminating cells in the adrenal chromaffin cell cultures were the major source of the released arachidonic acid. Differential plating increases the percentage of chromaffin cells in the preparation from approximately 65% to at least 95% as determined by histochemistry and electron microscopy (Waymire et
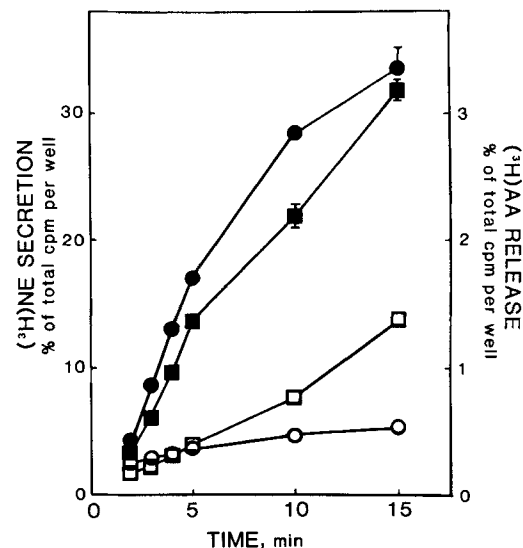

FIG. 1. Time course of arachidonic acid release and exocytosis. Chromaffin cells labeled with [³H]norepinephrine or [³H]arachidonic acid were incubated for the indicated times with a potassium glutamate solution containing 20 µM digitonin in the presence (filled symbols) or absence (open symbols) of 10 µM Ca²⁺. Catecholamine secretion (●,○) and arachidonic acid release (■,□) were determined. There were four wells/group.

al., 1983). Monolayer cultures of these cells had virtually no visually detectable fibroblast-like cells (data not shown). Cortisol secretion into the culture medium, a measure of cortical cell contamination, was reduced by 90% in monolayers from cells purified by differential plating. The release of [^{3}H]arachidonic acid caused by micromolar Ca^{2+} and the potentiation of release caused by the phorbol esters TPA (see below) were virtually identical in cultures of unpurified chromaffin cells and of these highly purified chromaffin cells (data not shown). Furthermore unpurified cells and cells purified by centrifugation through a Percoll gradient (Fig. 4 for example) also released [^{3}H]arachidonic acid in response to micromolar Ca^{2+} in a similar fashion. Thus arachidonic acid release in cultures of unpurified chromaffin cells is largely accounted for by release from chromaffin cells.

Time course of arachidonic acid release and exocytosis

Chromaffin cell cultures labeled with either [^{3}H]norepinephrine or [^{3}H]arachidonic acid were incubated with digitonin in the presence or absence of 10 μM free Ca^{2+}. A slow release of arachidonic acid occurred in the absence of Ca^{2+}. Ca^{2+} (10 μM) stimulated arachidonic acid release with a similar time course as catecholamine secretion (Fig. 1). Ca^{2+}-stimulated arachidonic acid release and exocytosis occurred most rapidly during the first 5 min.

Ca^{2+} dose response of arachidonic acid release and exocytosis

Low concentrations of free Ca^{2+} stimulated both arachidonic acid release and norepinephrine secretion in digitonin-treated cells. The activation of arachidonic acid release occurred at free Ca^{2+} levels lower than those required to activate exocytosis (Fig. 2). At 1.0 μM Ca^{2+} arachidonic acid release was almost fully activated whereas exocytosis was only partially activated. Raising the free Ca^{2+} concentration from 1.0 μM to 10 μM caused a large increase in exocytosis and a much smaller increment in arachidonic acid release (Fig. 2). In a similar experiment (data not shown) 1.0 mM free Ca^{2+} caused 60% greater Ca^{2+}-dependent catecholamine secretion than 10 μM Ca^{2+}; under the same conditions 1.0 mM Ca^{2+} caused only 28% greater Ca^{2+}-dependent arachidonic acid release than 10 μM Ca^{2+}.

Effect of NEM and BPB on arachidonic acid release and exocytosis

NEM is a nonspecific sulfhydryl alkylating agent that inhibits arachidonate release from platelet membranes (Silk et al., 1981) and catecholamine secretion from digitonin-treated cells (Wilson and Kirshner, 1983). BPB, another protein alkylating agent, inhibits phospholipase A_2 (Volverk et al.,
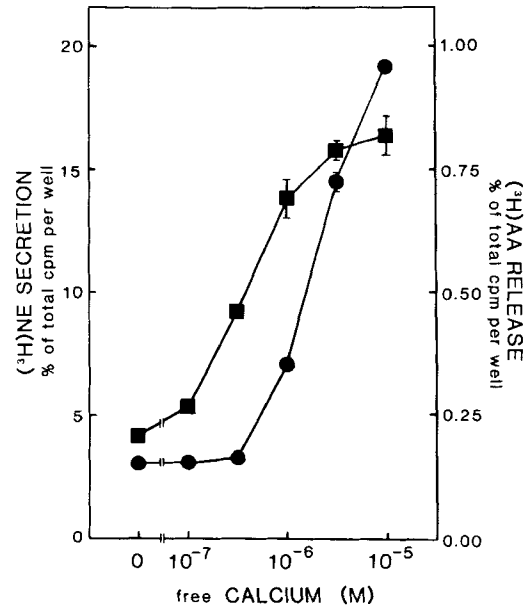

FIG. 2. Ca^{2+} dose response of arachidonic acid release and exocytosis. Chromaffin cells labeled with [^{3}H]norepinephrine or [^{3}H]arachidonic acid were incubated with a potassium glutamate solution containing 20 μM digitonin plus the indicated free Ca^{2+} concentrations for 5 min. Zero Ca^{2+} contained 5 mM EGTA and no added Ca^{2+}. Catcholamine secretion (●) and arachidonic acid release (■) were determined after 5 min. There were four wells/group.

1974) and phospholipase C (Hoffman et al., 1982). A comparison was made of the effects of these agents on [^{3}H]arachidonic acid release and catecholamine secretion from digitonin-treated cells. Chromaffin cells were incubated for 15 min in physiological salt solution (without BSA) in the presence or absence of the protein alkylating agents and then were incubated in potassium glutamate solution with 20 μM digitonin in the presence or absence of 10 μM Ca^{2+}. Pretreatment of chromaffin cells with either agent caused simultaneous inhibition of Ca^{2+}-dependent arachidonic acid release and exocytosis (Fig. 3). Inhibition by NEM of arachidonic acid release and exocytosis occurred between 25 and 100 μM (IC$_{50}$ = 40 μM), and inhibition by BPB occurred between 10 and 50 μM (IC$_{50}$ = 25 μM).

Effects of Mg^{2+} on arachidonic acid release and exocytosis

Mg^{2+} at high concentrations (5–20 mM) caused a strong inhibition of Ca^{2+}-stimulated catecholamine secretion (Fig. 4). The presence of 20 mM Mg^{2+} caused a 75% inhibition of 10 μM Ca^{2+}-stimulated exocytosis. In contrast Mg^{2+} had no significant effect on arachidonic acid release. A similar effect of Mg^{2+} on catecholamine secretion from digitonin-treated chromaffin cells had been previously observed (Wilson and Kirshner, 1983).
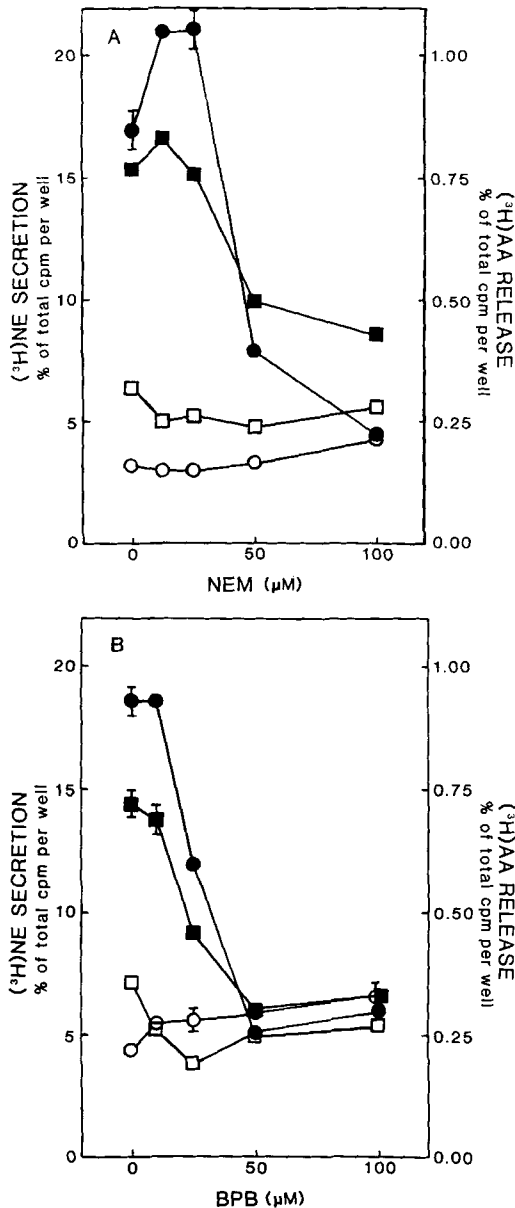
FIG. 4. Effects of Mg^{2+} on arachidonic acid release and exocytosis. Percoll-purified chromaffin cells labeled with [³H]norepinephrine or [³H]arachidonic acid were incubated with a potassium glutamate solution containing 20 μM digitonin in the presence (filled symbols) or absence (open symbols) of 10 μM Ca^{2+}, plus added Mg^{2+} as indicated. After 5 min, catecholamine secretion (●,○) and arachidonic acid release (■,□) were determined. There were four wells/group.

et al., 1983). A similar Ca^{2+}-potentiating effect of TPA on secretion from suspended chromaffin cells rendered leaky by high electric fields has recently been reported (Knight and Baker, 1983). We also observed a stimulation by TPA of exocytosis. We found that pretreatment of the cells with TPA in physiological salt solution was required to give large and reproducible effects (data not shown). Consequently, in the following experiments the cells were pretreated for 15 min with physiological saline containing the indicated TPA concentrations prior to treatment with the digitonin-containing solution. Treatment of the chromaffin cells with TPA over a range of 10–300 nM caused a dose-dependent increase in arachidonic acid release and catecholamine secretion (Fig. 5). Treatment with 300 nM TPA increased the 10 μM Ca^{2+}-stimulated component of arachidonic acid release and exocytosis by 65 and 72%, respectively. A significant stimulation of arachidonic acid release and catecholamine secretion in the absence of Ca^{2+} was also observed. The effects of various Ca^{2+} concentrations on catecholamine secretion and arachidonic acid release from digitonin-treated chromaffin cells in the presence and absence of 100 nM TPA were examined (Fig. 6A and B). Catecholamine secretion and arachidonic acid release were significantly increased by TPA between 10^{-7}–10^{-5} M Ca^{2+}. Treatment with TPA lowered the threshold concentration of free Ca^{2+} that was required to activate exocytosis.

FIG. 3. Effect of NEM and BPB on arachidonic acid release and exocytosis. Chromaffin cells labeled with [³H]-norepinephrine or [³H]arachidonic acid were incubated for 15 min with physiological salt solution (without BSA) with the indicated concentration of NEM (A) or BPB (B) or solvent control (0.5% ethanol). The physiological salt solution was removed and replaced with potassium glutamate solution containing 20 μM digitonin in the presence (filled symbols) or absence (open symbols) of 10 μM Ca^{2+}. Catecholamine secretion (●,○) and arachidonic acid release (■,□) were determined after 5 min. There were four wells/group.

Effect of TPA on arachidonic acid release and exocytosis

The phorbol ester TPA is a direct activator of protein kinase C (Castagna et al., 1982). In platelets activation of protein kinase C with TPA potentiates the effect of Ca^{2+} to induce exocytosis (Yamanishi
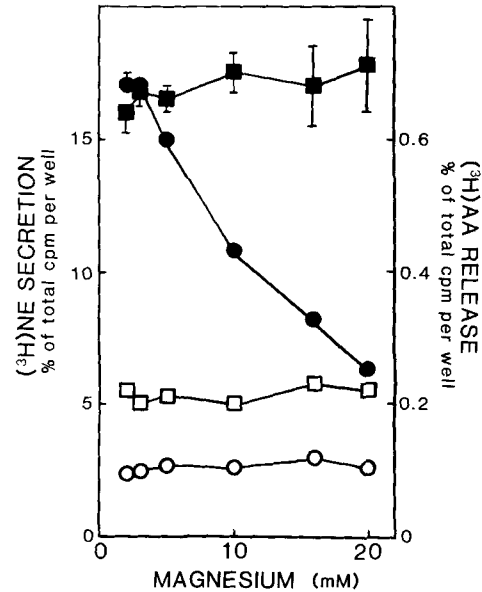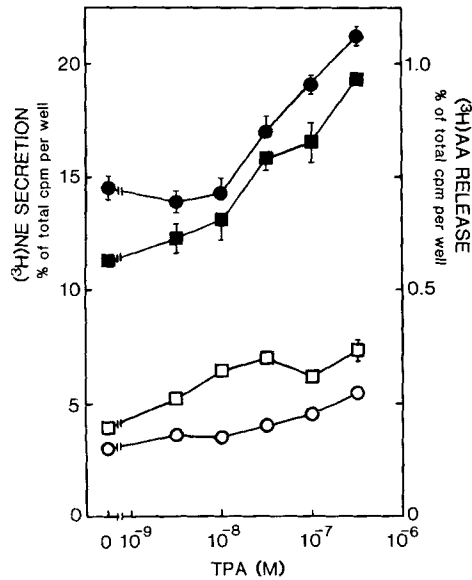
FIG. 5. Stimulation of arachidonic acid release and exocytosis by TPA. Chromaffin cells labeled with [^{3}H]-norepinephrine or [^{3}H]arachidonic acid were incubated for 15 min with physiological salt solution containing the indicated concentrations of TPA. The solution was then removed and replaced with a potassium glutamate solution containing 20 μM digitonin in the presence (filled symbols) or absence (open symbols) of 10 μM Ca^{2+} and the continued presence of the indicated concentration of TPA. After 5 min catecholamine secretion (●,○) and arachidonic acid release (■,□) were determined. There were four wells/group.

The effects of TPA suggest that protein kinase C-mediated protein phosphorylation can modulate arachidonic acid release, and exocytosis and may constitute a potential ATP-requiring step in the process of exocytosis. Chromaffin cells made leaky with digitonin become depleted of cytosolic ATP and become largely dependent upon addition of exogenous ATP for Ca^{2+}-dependent catecholamine secretion (Dunn and Holz, 1983; Wilson and Kirshner, 1983). In some experiments under such ATP-depleted conditions, we have observed a partial ATP dependency for Ca^{2+}-dependent arachidonic acid release (data not shown); however, the effect of ATP on arachidonic acid release has been variable and in other experiments no ATP dependency for arachidonic acid release was observed when catecholamine secretion was stimulated over two-fold by exogenous ATP.

DISCUSSION

Ca^{2+}-dependent arachidonic acid release from digitonin-treated chromaffin cells

We have previously demonstrated that treatment of chromaffin cells with low concentrations of digitonin renders the plasma membrane permeable to Ca^{2+}, ATP, and proteins and yet preserves the exocytotic release of catecholamine that can be in-
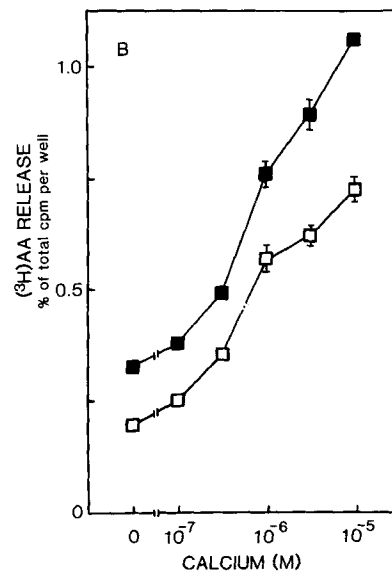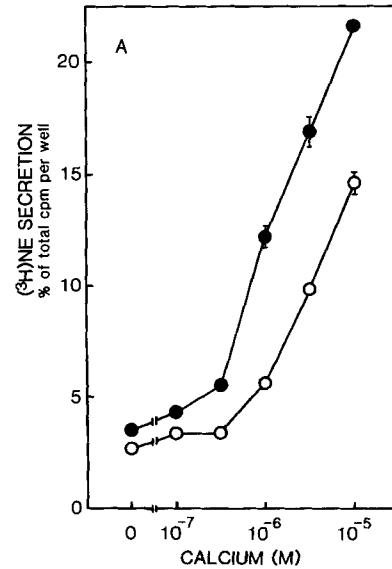

FIG. 6. TPA effect on Ca^{2+} dose response of exocytosis and arachidonic acid release. Chromaffin cells labeled with [^{3}H]norepinephrine or [^{3}H]arachidonic acid were incubated for 15 min with physiological salt solution in the presence (filled symbols) or absence (open symbols) of 100 nM TPA. The solution was then removed and replaced with a potassium glutamate solution containing 20 μM digitonin plus the indicated Ca^{2+} concentration with the same concentration of TPA as in the initial incubation. Catecholamine secretion (**A**) and arachidonic acid release (**B**) were determined after 5 min. There were four wells/group. TPA caused statistically significant enhancement of both [^{3}H]norepinephrine secretion and arachidonic acid release at all Ca^{2+} concentrations (p < 0.01).

duced by micromolar Ca^{2+} in the medium (Dunn and Holz, 1983). In this study we have taken advantage of the direct access to the cell interior in digitonin-treated chromaffin cells to study more directly the biochemical events associated with exocytosis. We found that micromolar Ca^{2+} stimulated

a three- to five-fold increase in the release of [³H]arachidonic acid from prelabelled cellular lipids coincident with catecholamine secretion. The amount of [³H]arachidonic acid release was greater than the amount of free [³H]arachidonic acid in the cells and, therefore, resulted from the degradation of arachidonic acid-containing lipids, probably either by phospholipase A_2 or by activation of phospholipase C and subsequent activity of diacylglycerol and monoacylglycerol lipase. In platelets there is considerable uncertainty concerning the relative importance of these pathways (Bell et al., 1979; Walenga et al., 1981). Because the latter pathway, if it is important, is associated with a several-fold increase in diacylglycerol (Rittenhouse-Simmons, 1979) that was not observed in the present experiments, it is likely that phospholipase A_2 rather than the phospholipase C-diacylglycerol lipase pathway was responsible for [³H]arachidonic acid release.

Relationship between [³H]arachidonic acid release and catecholamine release from digitonin-treated cells

Ca^{2+}-induced catecholamine secretion was always accompanied by increased [³H]arachidonic acid release. [³H]Arachidonic acid release was well correlated with catecholamine secretion with regard to time course, inhibition by the two protein-alkylating agents NEM and BPB, and stimulation by the phorbol ester TPA (see below). However [³H]arachidonic acid release was activated at a somewhat lower free Ca^{2+} concentration (0.3 μM) than catecholamine secretion (approximately 1 μM).[1] In addition, 5–20 mM Mg^{2+} strongly inhibited Ca^{2+}-dependent catecholamine secretion but had no effect on Ca^{2+}-dependent [³H] arachidonic acid release. Thus although arachidonic acid release may be directly involved in secretion, its release was not sufficient to cause secretion.

In previous experiments with intact (untreated with digitonin) chromaffin cells (Frye and Holz, 1984) nicotinic agonist or depolarization by elevated K^+ caused stimulation of catecholamine secretion that was quantitatively similar to that induced in the present experiments by 10 μM Ca^{2+}; however [³H]arachidonic acid release in intact cells was stimulated only 30% compared with the three- to five-fold increase caused by 10 μM Ca^{2+} in the digitonin-treated chromaffin cells. It is possible that a greater activation of phospholipase A_2 occurred in

digitonin-treated chromaffin cells than in intact cells during stimulation. However it is also possible that in the intact cells a large fraction of the free arachidonic acid produced by secretagogue-induced increases in cytosolic Ca^{2+} concentration was rapidly converted to arachidonyl coenzyme A by arachidonyl coenzyme A synthetase (Wilson et al., 1982; Reddy and Bazan, 1983) and then reincorporated into lipids before it could exit from the cells. In the digitonin-treated cells a much larger fraction of the free arachidonic acid released within cells may have escaped recycling into lipids by rapidly exiting into the medium, perhaps via digitonin-induced holes in the plasma membrane.

In another study we found that BPB inhibited secretion from intact chromaffin cells stimulated by a nicotinic agonist, elevated K^+, or Ba^{2+} (Frye and Holz, 1983). Because BPB also inhibited the Ca^{2+} uptake associated with the stimulation of secretion, we could not determine if BPB was able to block exocytosis at a point subsequent to Ca^{2+} influx. However, in other systems (Smolen and Weissman, 1980; Marone et al., 1981) BPB causes an inhibition of Ca^{2+} ionophore-induced exocytosis that is consistent with our present conclusions, that BPB can inhibit arachidonic acid release and exocytosis at a step subsequent to the increase in cytosolic Ca^{2+} perhaps by inhibition of phospholipase A_2 (Volverk et al., 1974).

Effects of the phorbol ester TPA

TPA is a potent, direct activator of protein kinase C (Castagna et al., 1982; Niedel et al., 1983; Donnelly and Jensen, 1983; Kikkawa et al., 1983). We found that TPA enhanced both arachidonic acid release and exocytosis, especially when present with micromolar Ca^{2+}. Experiments that are currently underway in our laboratory suggest that the effects of phorbol ester on digitonin-treated chromaffin cells are associated with increased protein phosphorylation. The data are consistent with the finding that protein kinase can activate phospholipase in sonicates of macrophages (Wightman et al., 1982). Stimulation of arachidonic acid release by TPA has been observed in other systems (Ohuchi and Levine, 1978; Mufson et al., 1979; Naor and Catt, 1981). One possible mechanism by which TPA could activate arachidonic acid release is by inducing protein kinase C-mediated phosphorylation of lipomodulin (macrocortin), a 40,000-dalton protein. Unphosphorylated, but not phosphorylated, lipomodulin inhibits phospholipase A_2 (Hirata et al., 1980; Blackwell et al., 1982; Hirata, 1981).

In summary our experiments with digitonin-treated chromaffin cells describe a low micromolar Ca^{2+} dependency for the activation of cellular arachidonic acid release and demonstrate that arachidonic acid release occurs coincident with cate-

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[1] In another system an analogous result has been reported. A basophil-derived cell line undergoes arachidonic acid release and exocytosis when treated with the Ca^{2+} ionophore A23187; the level of added Ca^{2+} needed to activate arachidonic acid release was lower than required to activate exocytosis (McGivney et al., 1981).

cholamine secretion. The data support the possibility that phospholipase A_2 is involved in exocytosis but do not establish a causal relationship. More work is required to characterize the biochemical mechanisms that modulate cellular arachidonic acid release and to determine the relationships of these processes to exocytosis.

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