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Evidence Implicating Protein Kinase C in Exocytosis from Electropermeabilized Bovine Chromaffin Cells

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Summary. The calcium sensitivity of exocytosis from electropermeabilized chromaffin cells is increased by activators of protein kinase C, such as TPA and certain phorbol esters, diacylglycerols, and mezerein. A range of putative inhibitors of protein kinase C block both the phorbol ester-sensitive component of secretion and also the underlying insensitive component. These inhibitors are also shown to inhibit medulla protein kinase C activity in vitro. The extent of secretion is reduced when electropermeabilized cells are exposed to Ca^{2+} levels much in excess of 50 μM. The onset of inhibition is faster than the relatively slow rate of Ca-dependent exocytosis and is insensitive to inhibitors of proteolysis. Adrenal medulla protein kinase C activity is also irreversibly inhibited by high Ca^{2+} concentrations. Both the secretory response and the protein kinase C activity in vitro have similar nucleotide and cation specificities. Although these data do not definitely establish an involvement of protein kinase C in exocytosis, none argue against it.

Key Words exocytosis · secretion · calcium · protein kinase C · adrenal medulla · catecholamine

Introduction

In 1983 we reported that certain phorbol esters which can replace diacylglycerol in activating protein kinase C (Castagna et al., 1982) also increase the sensitivity to calcium of ATP-dependent exocytosis in electrically-permeabilized bovine adrenal medullary cells (Knight & Baker, 1983). Since that time, many secretory systems have been shown to be stimulated both by phorbol esters and membrane permeant analogues of diacylglycerol (Kikkawa & Nishizuka, 1986; Rink, Sanchez & Hallam, 1983; Shapira et al., 1987), and many of these effects persist in permeabilized cells consistent with an involvement of protein kinase C in exocytosis (Knight, Niggli & Scrutton, 1984; Brocklehurst & Pollard, 1985; TerBush & Holz, 1986; Hu et al., 1987).

In this paper we examine in some detail the characteristics of phorbol ester activation of exocytosis in bovine chromaffin cells. On the assumption that protein kinase C is the endogenous receptor for these molecules (Nishizuka, 1984), our experiments throw some light on the way in which protein kinase C interacts with the machinery of exocytosis in this tissue.

Materials and Methods

Preparation of Cells and Method of Electropermeabilization

Cells were prepared and rendered permeable by exposure to brief high voltage pulses as described previously (Baker & Knight, 1978; Knight & Baker, 1982; 1985). The cells were suspended in either a potassium glutamate-based medium or one containing sucrose in place of potassium glutamate (Knight & Baker, 1982). Unless otherwise stated in the legends, the cells were suspended in the glutamate-based medium. The experiments were performed at room temperature, between 20 and 24°C.

Measurement of Secretion

Catecholamine, dopamine-B-hydroxylase (DBH), and lactate dehydrogenase (LDH) were measured as described previously (Knight & Baker, 1982). The EGTA in the samples was diluted to 0.4 mM with water so as to reduce the inhibitory effect of this ligand on the DBH assay.

Phorbol Esters and Analogues of Diacylglycerol

As these compounds are hydrophobic they were first dissolved in dimethylsulphoxide (DMSO), and this stock solution was diluted to give a final DMSO concentration of 0.3%. All controls contained DMSO at the same final concentration. Phorbol esters were obtained from Sigma, and the diacylglycerol analogues, 1-oleoyl,2-acetylglycerol (OAG), 1,2- and 1,3-dioctanoylglycerol

* Deceased.

(diC_8) were kindly provided by Dr. Y. Nishizuka (Kobe University, Japan).

Other Chemicals

[^{32}P]ATP was obtained from Amersham, DE52 anion exchange resin from Whatman, diacylglycerol kinase inhibitor (R59022) from Janssen Life Science Products, and protein kinase inhibitors H7 and W7 from Seikagaku America. The synthetic peptide GSRRRRG was kindly provided by Dr. P. Parker, (Imperial Cancer Research Fund, London) and inositol phosphates by Dr. R. Irvine (ARC, Babraham, Cambridge, UK).

Temperature-Jump Experiments

Aliquots of cell suspension (200 μl) were incubated with CaEGTA buffers and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) at 0°C in 1.5 ml microanalysis tubes and then transferred to a warm water bath. The temperature of the cell suspension, measured with a small thermocouple, rose to 23°C within 20 sec. The tubes were removed at this temperature, which was also the temperature of the room.

Isolation and Assay of Protein Kinase C

Fresh adrenal tissue was homogenized in cold buffer A, i.e., 20 mM Tris HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 10 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 20 μg/ml leupeptin. Cyclic AMP (40 μM final concentration) and NP 40 (1% final concentration) were added to the homogenate, which was allowed to stand (4°C) for 30 min with occasional gentle mixing. The detergent solubilized preparation obtained after centrifugation (12,000 × *g*, 4°C, 15 min) was applied to a DE-52 cellulose column (0.5 × 1 cm) equilibrated with buffer A plus cyclic AMP. The column was washed with 5 volumes of buffer A plus cyclic AMP, 5 volumes of Buffer A, and the enzyme eluted with 0.1 M NaCl in Buffer A. Cyclic AMP treatment reduces contamination of cyclic AMP-dependent protein kinase by dissociating the holoenzyme, so preventing it binding to the anion exchange column (Lubben & Traugh, 1983). When adrenal cells were used, they were washed after treatment, resuspended in Buffer A and sonicated briefly (3 × 10 sec) on ice. Cytosol and membrane fractions were separated by centrifugation (120,000 × *g*, 4°C, 15 min). Membranes were solubilized with NP 40 then both cytosol and membrane protein kinase C isolated as described above.

Protein kinase C activity was determined as described elsewhere (Kraft & Anderson, 1983*a*), i.e., by subtracting the amount of [^{32}P] incorporated into histone H1 (Type III S, 100 μg) in the absence of added phospholipids from the amount incorporated in the presence of phospholipids. Protein kinase C activity is expressed as the amount of [^{32}P] transferred to histone in pmol/min/mg protein. Unless indicated otherwise, CaEGTA buffers (10 mM) giving a free Ca^{2+} concentration of 20 μM were used in all experiments. The Mg^{2+} concentration in the assay was 10 mM. The concentration of [^{32}P]ATP used was 100 μM (approximately 10^6 cpm). Phosphatidylserine (60 μg) and diolein (10 μg) were added as appropriate.

Measurements of Ca^{2+} and phospholipid-dependent phosphate incorporation into histone were unreliable using crude medulla homogenate. On the rare occasions that we saw some sensitivity to Ca^{2+} and phospholipid, the activity was very low and not linear with time. Column chromatography was necessary to extract the Ca^{2+} and phospholipid-sensitive kinase from the crude homogenate and from other kinases and endogenous activators. The activity of the extracted kinase was linear with respect to time and amount of enzyme used. Furthermore, chromatography was essential in order to completely remove the phorbol ester (TPA) in those experiments in which cells were pretreated with this agent. The bulk of the assays were done at pH 6.6 (40 mM PIPES), the same pH as all secretion experiments, rather than pH 7.4 as is more usual. Protein kinase C activity was similar at both pH's (20 mM Tris, pH 7.4, 814 SEM, 42 pmol/min/mg protein; 40 mM PIPES, pH 6.6, 1007 SEM, 11 pmol/min/mg protein, $n = 3$). OAG, 1,2-dioctanoyl glycerol (30 μM), TPA (60 nM) and mezerein (1 μM) were able to substitute for diolein in the assay.

Results

Effects of TPA on the Ca Sensitivity of Catecholamine Release

The main effect of the phorbol ester TPA on exocytosis in electrically permeabilized bovine adrenal medullary cells is to bring about a modest leftward shift in the Ca-activation curve with rather little effect at very low and very high concentrations of Ca^{2+} (Fig. 1; Knight & Baker, 1983). The effect is half-maximal at approximately 2 nM, which is in the concentration range reported to stimulate protein kinase C (Castagna et al., 1982). It is not necessary to pretreat with TPA to see this effect because addition of TPA to cells pre-exposed to intermediate concentrations of Ca^{2+} also brings about additional catecholamine release (Knight & Baker, 1983). This increased catecholamine release seems to occur by exocytosis because it is associated with the vesicular enzyme dopamine-B-hydroxylase, but not the cytosolic marker lactate dehydrogenase (Fig. 1).

Effects of Mezerein and Other Phorbol Esters on Catecholamine Release

A variety of phorbol esters and related compounds have been tested for their effects on exocytosis, and only those analogues that activate protein kinase C, e.g., mezerein, TPA and 4β phorbol-12,13 dibenzoate, are effective at increasing the Ca sensitivity of exocytosis (Fig. 2*a*). Phorbols such as 4β phorbol-13, monoacetate and 4β phorbol are without effect. The order of effectiveness of the compounds tested is also the same as that found for activation of protein kinase C (Catagna et al., 1982; Miyaka et al., 1984; Ebeling et al., 1985).

Sensitivity to Analogues of Diacylglycerol

Under physiological conditions, protein kinase C is activated by diacylglycerol generated by breakdown of membrane phospholipids (Hokin & Hokin, 1953). Because of its extreme hydrophobicity, diacylglycerol is difficult to deliver to permeabilized cells; but a number of analogues exist that seem to enter cells much more readily. Of these, 1-oleoyl,2-acetylglycerol (OAG) is without effect on the Ca sensitivity of exocytosis, whereas, 1,2-dioctanoylglycerol brings about a leftward shift closely resembling that seen with TPA (Fig. 2*b*). The shift is half maximal at a 1,2-dioctanoylglycerol concentration close to 90 μM. 1,3-dioctanoylglycerol is much less effective on a mole-for-mole basis than the 1,2 analogue.

Do TPA, Mezerein, and 1,2-Dioctanoylglycerol Act at the Same Site?

If TPA, mezerein and 1,2-dioctanoylglycerol act on protein kinase C to increase the sensitivity of exocytosis to Ca^{2+}, they should act at the same site on the enzyme; as a result their actions should be additive at low concentrations, but not so once the site is fully occupied by one or other activator. This is examined quantitatively for TPA and mezerein in Fig. 2*a* and for TPA and 1,2-dioctanoylglycerol in Fig. 2*b* and *c*. These data provide very strong evidence that all these molecules act at the same site.

Ca Sensitivity Is Not Affected by Inositol, Inositol 1,4,5-Trisphosphate, or Inositol 1,3,4,5-Tetrakisphosphate

One physiological source of diacylglycerol is the phosphatidylinositol family of phospholipids, which on hydrolysis liberate various phosphorylated inositols in addition to diacylglycerol (*see* Berridge & Irvine, 1984; Batty, Nahorski & Irvine, 1985). We have examined inositol (1 mM), inositol 1,4,5-triphosphate (25 μM) and inositol 1,3,4,5-tetrakisphosphate (25 μM), all of which are without effect on the Ca sensitivity of exocytosis (*data not shown*).

TPA and Dioctanoylglycerol Are Still Effective in the Presence of Aspirin and Indomethacin

There is evidence in some tissues that activation of protein kinase C can in turn activate phospholipase

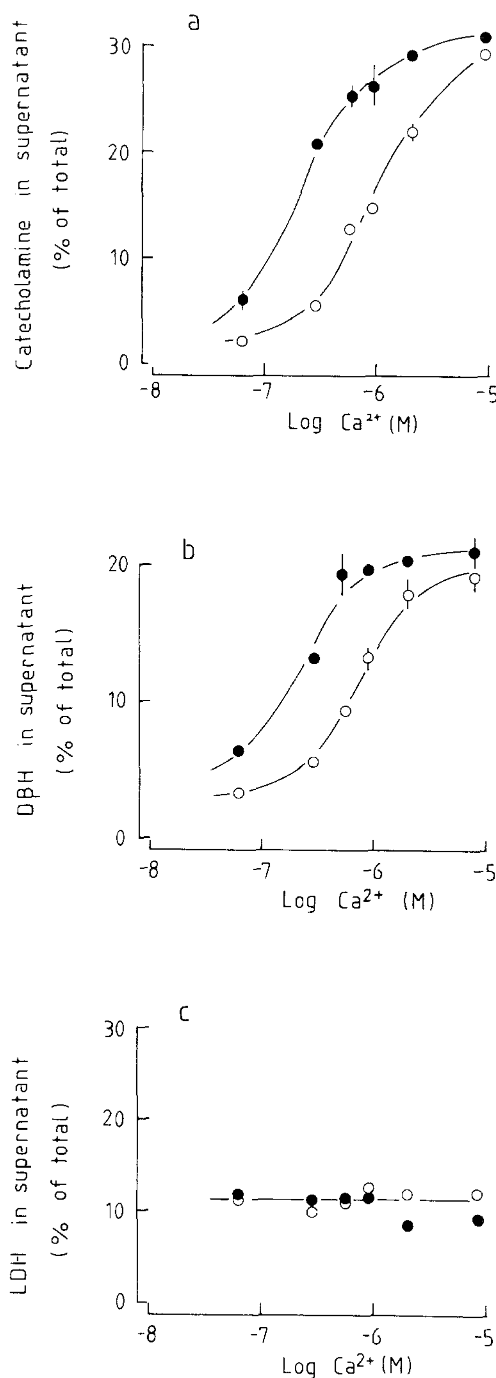

Fig. 1. TPA alters the Ca sensitivity to exocytosis. Cells suspended in a sucrose-based medium containing 5 mM MgATP and 0.2 mM EGTA were rendered permeable and immediately diluted into a similar medium (○), or one containing TPA (●), the final concentration being 160 nM. After 10 min incubation, the cells were challenged with the Ca^{2+} shown by the addition of 1.2 mM CaEGTA buffers, and 20 min later centrifuged. The catecholamine (*a*), dopamine-B-hydroxylase (*b*), and lactate dehydrogenase (*c*) content of the supernatant was measured and is expressed as a percentage of the total in the cell suspension. Data are means of 3 determinations, the error bars the SEM

A_2 (Touqui et al., 1986; Maraganore, 1987), to produce prostaglandins and other membrane-active agents via a route that is sensitive to both aspirin and indomethacin. Neither of these agents (1 mM), however, have any effect on the increase in Ca sensitivity brought about by TPA and dioctanoylglycerol, suggesting that their actions do not require activation of the cyclo-oxygenase pathway. In addition, exogenous arachidonate (0.1–10 μM) has no effect on the shape of the Ca-activation curve nor does it modify the TPA-induced shift. Higher concentrations of arachidonate precipitate cells lysis. The mechanism by which arachidonate acts is unlikely to involve part of a normal metabolic process, as similar concentrations of eicosi fatty acids that are not readily metabolized have similar lytic effects.

Comparison of the Rates of Response to Ca^{2+} and TPA

The rather slow rate of secretion (minutes) seen when leaky cells are exposed to Ca^{2+} in the presence or absence of TPA (Knight & Baker, 1982, 1983) can be explained in terms of the rate at which the Ca^{2+} buffer diffuses through the pores of the plasma membrane and reaches its target, rather than the rate of the secretory process itself. However, a similar rather slow response is also seen when the leaky cells are triggered to secrete by a temperature jump after they have been fully equilibrated with Ca^{2+} at 0°C (Fig. 3*a*). The presence of TPA does not markedly alter this rate induced by high Ca^{2+} concentrations (Fig. 3*b*). The TPA-sensitive component of the secretory process can be observed at submicromolar Ca^{2+} levels. Here again though the rate of secretion triggered by TPA at constant Ca^{2+} is quite slow (Fig. 3*c*), it could be argued that the slow rate of secretion was not limited by the rate of fusion of the secretory granules and the plasma membrane, but rather the slow rate at which the contents of the secretory granule are emptied into the extracellular medium through the fusion pores (Breckenridge & Almers, 1987; Whitaker & Zimmerberg, 1987). If this were the case then one might expect catecholamine to continue to leave the cell once fusion had been triggered even after the conditions promoting membrane fusion

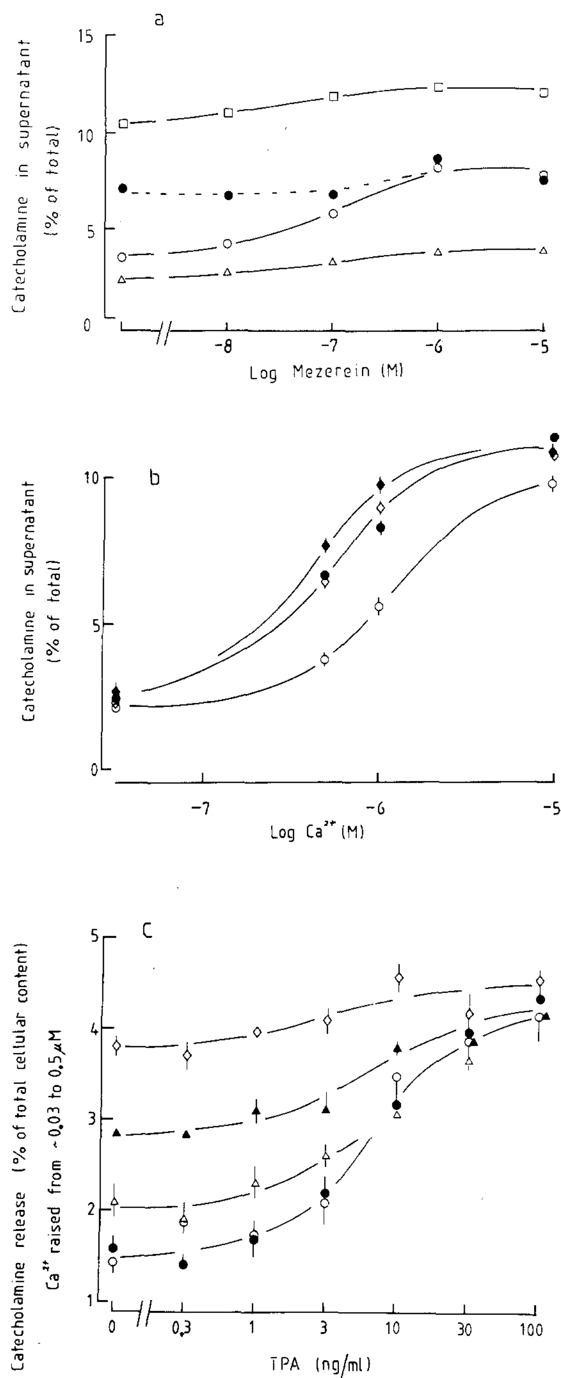

Fig. 2. Effect of mezerein and 1,2-dioctanoylglycerol (diC_8) on the Ca sensitivity of secretion in the presence and absence of TPA. (*a*) Electropermeabilized cells in buffer containing 4 mM MgATP and 0.5 mM EGTA were incubated with various concentrations of mezerein for 15 min before being challenged for a further 20 min with CaEGTA buffers corresponding to 0.05 μM Ca^{2+} (△), 0.5 μM Ca^{2+} (○), or 10 μM Ca^{2+} (□). Cells were incubated with various concentration of mezerein together with a saturating concentration (60 nM) of TPA and subsequently challenged with 0.5 μM Ca^{2+} (●). (*b*) Leaky cells as in *a* were preincubated with either 154 nM TPA (●), 190 μM diC_8 (◇), 154 nM TPA + 190 μM diC_8 (♦) for 3 min before being challenged with 15 mM CaEGTA buffers for a further 25 min. Cells with no additives (○). The data are the means of 2 determinations, the error bars being the range. (*c*) Catecholamine released over 25 min as a result of the calculated Ca^{2+} being raised from 0.03 to 0.5 μM in the presence of the various concentrations of TPA shown, together with various concentrations of diC_8 (◇, 190 μM; ▲, 95 μM; △, 24 μM; ●, 12 μM; ○, 0 μM)

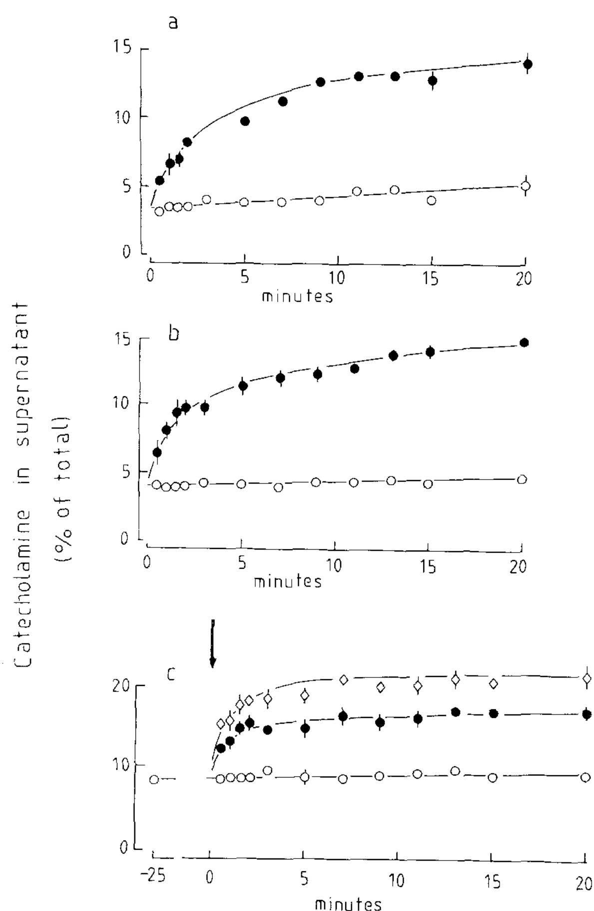

Fig. 3. The rate of Ca^{2+} and TPA-dependent exocytosis. (*a* & *b*) *Ca^{2+}-dependent rate*. Cells in buffer containing 5 mM MgATP and 1 mM EGTA giving close to 0.01 μM Ca^{2+} were rendered leaky and immediately diluted either without (*a*), or with (*b*) 113 nM TPA. After 7 min at 20°C the cells were cooled to 0°C and challenged with ice-cold CaEGTA buffers corresponding to 0.01 μM Ca^{2+} (○) and 10 μM Ca^{2+} (●). 15 min later the cells were rapidly warmed from 0 to 20°C—the warming up time taking 20 sec. The suspension was centrifuged at various times after this (abscissa). The time for the cells to be pelleted was less than 15 sec. Data points are mean of 3 determinations, the error bars being the SEM. (*c*) *TPA dependent rate*. Cells suspended as above were rendered leaky and challenged with CaEGTA buffer corresponding to 0.4 μM Ca^{2+} for 10 min at 20°C before being cooled to 0°C for a further 10 min. While still at this temperature CaEGTA was added to raise the Ca^{2+} to 2 μM (◇), or TPA was added (113 nM) at the constant Ca^{2+} concentration of 0.4 μM (●). Some of the cells were kept at 0.4 μM Ca^{2+} in the absence of TPA (○). After 15 min, i.e., at the time indicated by the arrow, the temperature was suddenly raised from 0 to 20°C as described above and the suspensions centrifuged at various times later. Data are mean of 3 determinations, the error bars being the SEM

had been removed. This seems not to be the case, however, as secretion ceases abruptly if the Ca^{2+} is suddenly reduced from 10 to 0.1 μM. The evidence therefore supports the idea that the slow rate of secretion reflects the rate at which granules fuse with the plasma membrane in response to a Ca^{2+} stimulus.

Table 1. Effect of various putative inhibitors on protein kinase C activity and Ca^{2+}-dependent exocytosis in electropermeabilized chromaffin cells[a]

Inhibitor	Protein kinase C activity (% of control) (SEM, $n = 3$)	Inhibition of Ca^{2+}-dependent exocytosis[b]
300 μM H7	20 (5)	0.91
200 μM W7	61 (2)	0.25
400 μ/ml polymixin B	12 (4)	0.0
30 μM trifluoperazine	55 (11)	0.24
10 μM calmidazolium	22 (4)	0.42
5 mM spermine	33 (16)	0.10
300 μM neomycin	52 (1)	0.15
1 μM mellitin	52 (9)	0.53[c]
300 μM sphingosine	18 (1)	0.94[d]
1 mM amiloride	28 (1)	0.20
5 mM spermidine	67 (10)	0.35
300 μM adriamycin	14 (11)	0.62
100 μM retinal	64 (8)	1.02
100 μM retinol	130 (13)	0.75

[a] Results are expressed as a % of control activity (SEM, $n = 3$).
[b] Expressed as a fraction of the control response to raising Ca^{2+} from 0.01 to 10 μM.
[c] Inhibition of Ca^{2+}-dependent exocytosis was tested at 0.3 μM as higher concentrations brought about a large release of catecholamine.
[d] 100 μM sphingosine.

The Effects of Some Putative Inhibitors of Protein Kinase C

If protein kinase C is involved in altering the sensitivity of exocytosis to Ca^{2+}, it is important to establish how this is brought about. There seem to be two major possibilities: (i) that activation of protein kinase C alters the Ca sensitivity of a second Ca-sensitive process that is essential for exocytosis or (ii) that protein kinase C is, itself, the Ca^{2+} receptor for exocytosis serving to integrate the activating effect of both Ca^{2+} and diacylglycerol. If the first possibility is correct, it should be possible to find inhibitors of protein kinase C that remove the leftward shift due to phorbol esters without affecting the underlying Ca-activation curve, whereas if the second possibility is correct, all inhibitors of protein kinase C will reduce the extent of secretion as well as the phorbol ester-induced shift.

Figure 4 and Table 1 illustrate the effects of a variety of putative protein kinase C inhibitors on the Ca-dependent release of catecholamines and on the TPA-sensitive component of the Ca^{2+}-activa-

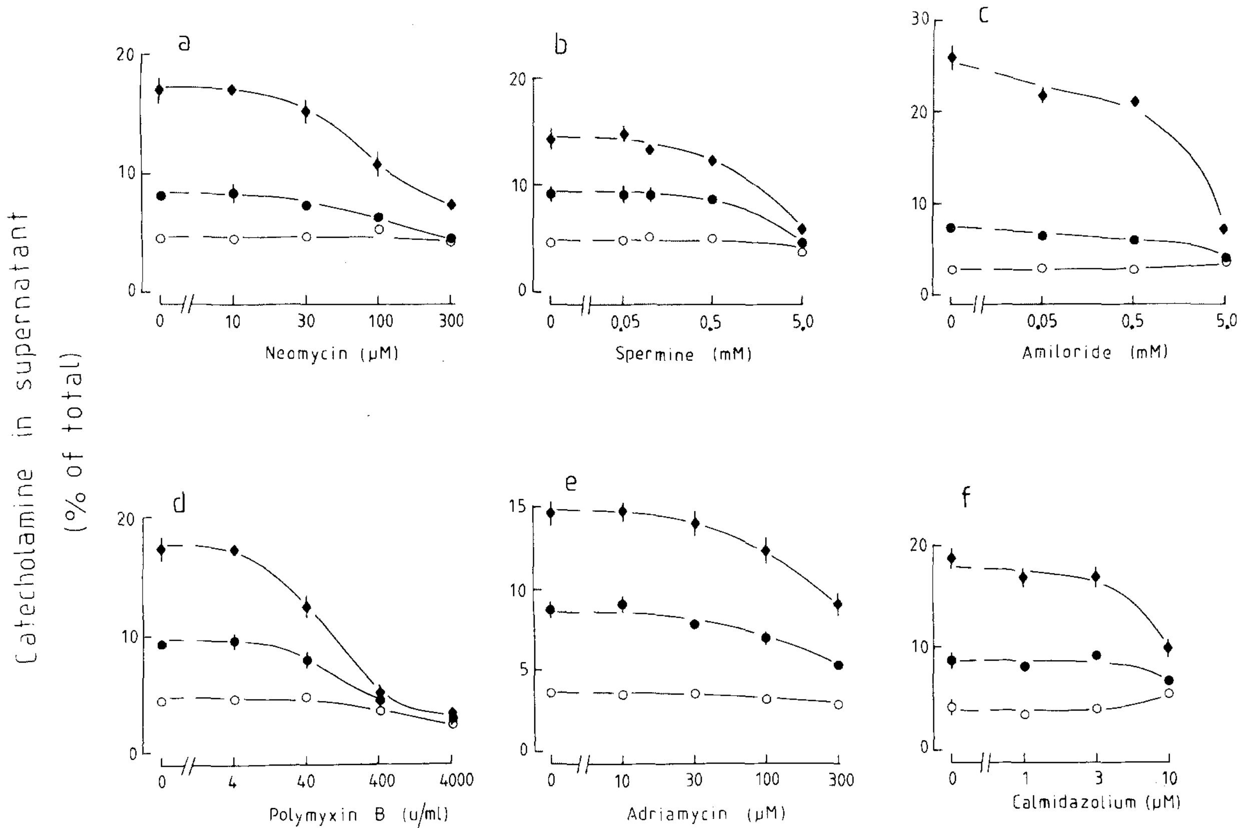

Fig. 4. The effect of putative inhibitors of protein kinase C on Ca-dependent secretion in the presence of TPA. Cells suspended in a medium containing 4 mM MgATP and 0.5 mM EGTA were rendered permeable and incubated for 15 min with 30 nM TPA together with either (*a*) neomycin, (*b*) spermine, (*c*) amiloride, (*d*) polymixin B, (*e*) adriamycin, or (*f*) calmidazolium before being challenged for a further 20 min with 15 mM CaEGTA buffers corresponding to 0.1 µM Ca^{2+} (○), 0.4 µM Ca^{2+} (●), 10 µM Ca^{2+} (♦). Data points are means of 3 determinations, the error bars being the SEM

tion curve. All the agents examined have been reported to inhibit protein kinase C, although to varying extends and with varying degrees of specificity (Nishizuka, 1980; Katoh et al., 1981; Mazzei, Katoh & Kuo, 1982; Takai, Kishimoto & Nishizuka, 1982). The striking finding is that none of the agents were capable of removing selectively the phorbol-ester component without at the same time inhibiting the underlying Ca^{+}-activation curve. These findings are most consistent with the second possibility. The most effective agents were amiloride, polymixin B, spermine and chlorpromazine (100 µM). Rather surprisingly H7, which is reputed to be a specific inhibitor of protein kinase C (Hidaka et al., 1984), had little effect even in the presence of low concentrations of ATP where the reported competition between H7 and ATP should be minimal.

A variety of other putative inhibitors of protein kinase C, calmodulin, phospholipase C and A_2, and also agents that have been implicated in other TPA-mediated cellular events (Weinstein, 1981; Slaga et al., 1982; Blackwell & Flower, 1983; Jetten et al., 1985) have been tested. These include difluoromethylornithine hydrochloride (2 mM), putrescine (5 mM), heparin (0.3 mg/ml), and R59022 diacylglycerol kinase inhibitor (30 µM), quinacrine (100 µM), dibromoacetophenone (100 µM)—all of which had no effect on Ca^{+} or TPA-sensitive components of secretion. A number of rather nonspecific calmodulin antagonists including trifluoperazine, W7, calmidazolium (R24571) and melittin (Hidaka et al., 1980; Wrenn et al., 1981; Katoh et al., 1982; Mazzei et al., 1984) inhibit Ca-dependent catecholamine release although as their concentration is increased this inhibition is obscured by a large calcium-independent release of catecholamines, probably because they also exert a detergent-like action promoting cell lysis. Of the other agents examined the most potent inhibitor was neomycin. Some inhibition was also seen at high concentrations of certain local anesthetics (Takai et al., 1982), e.g. procaine (1 mM), tetracaine (1 mM), propanolol (1 mM), again though it seemed not possible to selectively inhibit the phorbol ester component of secretion without at the

same time inhibiting the underlying Ca^+-activation curve.

Experiments in the Presence of a Low Molecular Weight Substrate of Protein Kinase C

An alternative strategy to inhibiting protein kinase C is to flood the permeable cell with a low molecular weight substrate for the kinase that might be expected to compete for the kinase with its endogenous substrates (Ferrari et al., 1985). Once such synthetic peptide is GSRRRRG (P. Parker, *personal communication*). Millimolar levels of the peptide appear to inhibit both the underlying Ca^+-activation curve and also the TPA-sensitive component. However, this high concentration of the peptide could be having an effect in in some way other than as an alternative substrate for C-kinase. It may be significant, for example, that similar concentrations of other basic polypeptides, e.g., poly L lysine and poly L arginine, inhibit secretion in much the same was as GSRRRG.

The Effect of TPA under Conditions where Ca-Dependent Secretion is Inhibited by Agents that Are Not Known to be Inhibitors of Protein Kinase C

Ca-dependent catecholamine release from electropermeabilized cells can also be inhibited under a variety of conditions other than those known to inhibit protein kinase C. Accordingly, we have examined the effect of TPA under conditions where the extent of secretion is inhibited by exposure to high chloride (Knight & Baker, 1982), high osmotic pressure (Knight & Baker, 1982), GTPγS (Knight & Baker, 1985), and botulinum toxins (Knight, Tonge & Baker, 1985; Knight, 1986). In all instances TPA still elicited a leftward shift in the Ca-activation curve with little or not effect on the extent of exocytosis (Fig. 5). These experiments shed no direct light on the mechanism by which these disparate agents inhibit secretion; but they do show that despite a reduction in the extent of exocytosis, in no case is inhibition associated with a loss in sensitivity to TPA.

Irreversible Inhibition by High Levels of Calcium

If permeable cells are exposed to Ca^{2+} levels much in excess of 50 μM, the extent of secretion is reduced and the degree of inhibition increases progressively as the Ca^{2+} concentration is raised (Fig. 6*a*; Knight & Baker, 1982). Whereas TPA increases the apparent affinity for Ca^{2+} in the micromolar range of concentrations, it has much less effect on the inhibitory arm of the curve. There are two striking aspects of this inhibition: (i) Its onset is more rapid than that of exocytosis. Thus when permeable cells are transferred from 10^{-8} to 10^{-3} M Ca^{2+}, despite the fact that free Ca must rise through the micromolar range, little exocytosis is activated. (ii) Once established, the inhibition is irreversible. In cells that have been exposed to high Ca^{2+} a secretory response cannot be recovered by return to 10^{-8} M Ca^{2+} as subsequent re-exposure to micromolar free calcium fails to elicit any catecholamine secretion. Inhibition is still seen even after exposure to high calcium in the absence of ATP (Fig. 6*b*).

As protein kinase C is subject to Ca-dependent proteolytic cleavage (Takai et al., 1982; Kishimoto et al., 1983; Murray, Fournier & Hardy, 1987) we examined whether the irreversible inactivation of exocytosis induced by Ca^{2+} could be avoided by pre-exposure to a variety of protease inhibitors, but inclusion of leupeptin, TLCK and PMSF in the incubation medium left the Ca-response curve essentially unchanged (Fig. 6).

Divalent Cation Specificity of Catecholamine Release

Figure 7 provides information on the effectiveness of a number of divalent and trivalent cations as activators of exocytosis in permeabilized adrenal medullary cells. Essentially similar results were obtained in media based on glutamate or sucrose. Sr^{2+} and Ba^{2+} and, to a lesser extent, Pb^{2+}, Mn^{2+} and La^{3+} promote catecholamine release, whereas Co^{2+} and Zn^{2+} (up to 1 mM) are without effect. Points to note are: (i) the extent of secretion is greater in Sr^{2+} and Ba^{2+} than with Ca^{2+} and (ii) in the presence of Ca^{2+}, Sr^{2+}, Ba^{2+}, or Mn^{2+} secretion falls at high divalent cation concentrations, whereas that in response to Pb^{2+} and possibly La^{3+} does not. The experiments were, of necessity, done in the absence of Ca buffers, and so it could be argued that some of the activation may be due to displacement of Ca by the 'activating' cation. However, if this was the case it might be expected that all the divalent ions would have a similar displacing effect. The findings that Ba^{2+} and Sr^{2+} are potent activators, whereas Co^{2+} and Zn^{2+} do not trigger secretion at all even though Ca^{2+} can still activate exocytosis when added in the presence of Co^{2+} or Zn^{2+}, argue strongly against this possibility.

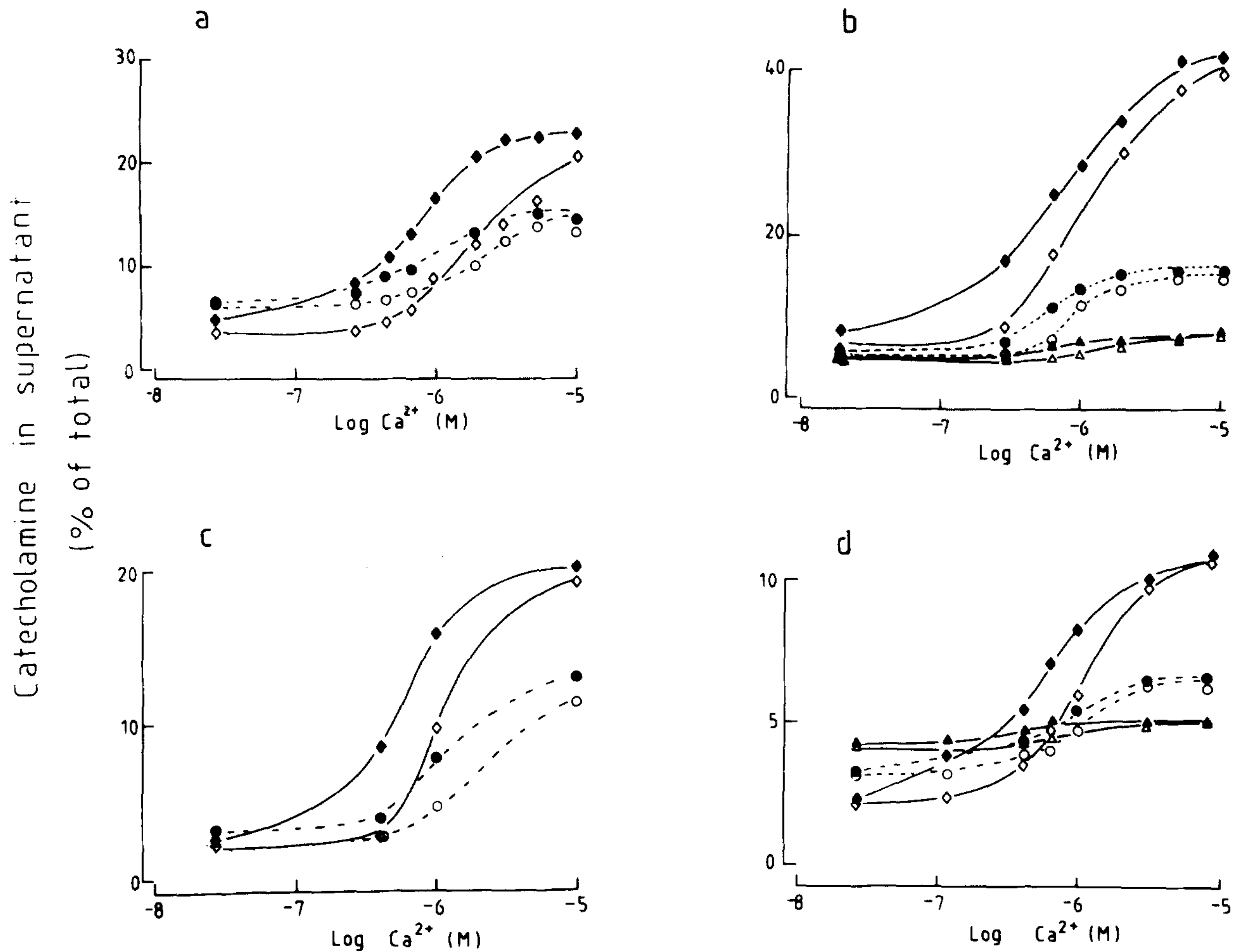

Fig. 5. TPA does protect against the effect of various inhibitors of Ca-dependent secretion. Cells were incubated in the presence (filled symbols) or absence (open symbols) of 60 nM TPA. (*a*) Inhibitory effect of Chloride. Cells in medium containing 4 mM MgATP and 0.5 mM EGTA were rendered leaky and diluted into either a similar solution with and without TPA (◇, ◆) or one in which the glutamate had been replaced isosmotically with chloride (the final concentration in the cell suspension being 120 mM Cl^-) and also in the presence of TPA (○, ●). After 15 min the cells were challenged with 15 mM CaEGTA buffers, and the catecholamine in the supernatant was determined 15 min later. (*b*) Inhibitory effect of high osmotic pressure. Cells in a sucrose based medium (calculated 330 mosM) containing 2 mM MgATP and 0.5 mM EGTA were rendered leaky and diluted into media with and without TPA and containing additional sucrose to increase the osmolarity to 660 mosM (○, ●) or containing glucose to increase the osmolarity to 1330 mosM (△, ▲). Cells in buffer in which the osmolarity remained unaltered at 330 mosM (◇, ◆). After 25 min incubation, the cells were challenged with 15 mM CaEGTA buffers and the catecholamine was determined 25 min later. (*c*) Inhibitory effect of GTPγS. Cells in a medium containing 2 mM MgATP and 1 mM BAPTA were rendered leaky and incubated either with 10 μM GTPγS (○, ●) or in the absence of guanine nucleotide (◇, ◆) and both in the presence or absence of TPA. After 6 min the cells were challenged with the Ca^{2+} shown for a further 15 min. (*d*) Inhibitory effect of botulinum toxin type D. Cells were incubated for 48 hr in culture containing either 5 μg/ml of botulinum toxin type D (○, ●), or 50 μg/ml botulinum toxin D (△, ▲), or in the absence of toxin (◇, ◆) before being washed into potassium glutamate based medium containing 3 mM MgATP and 0.4 mM EGTA and rendered leaky. Cells were then incubated with (filled symbols) or without (open symbols) 60 nM TPA for 10 min and challenged with 15 mM CaEGTA buffers for a further 20 min

Some Properties of a Protein Kinase C Isolated from the Bovine Adrenal Medulla

The activity of protein kinase C isolated from bovine adrenal medullary tissue was found to be 2029 (SEM 213; pmol/min/mg of enzyme, measured in triplicate from seven different preparations). In bovine adrenal medullary cells the majority of protein kinase C activity was found in the cytosol (cytosolic 86%, membrane-associated 14%, mean of 3 cell aliquots). Permeabilization of the cells and resuspension in potassium glutamate solution did not affect the total protein kinase C activity or its cellular distribution (cytosolic 90%, membrane associated 10%, mean of 3 cell aliquots). Adrenal medulla protein kinase C was not activated appreciably by Ca^{2+}

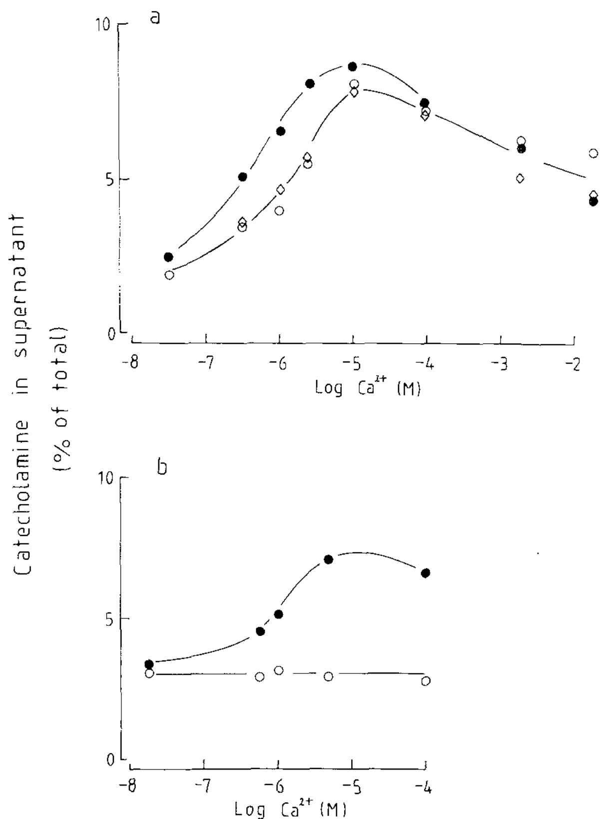

Fig. 6. Protease inhibitors do not prevent the inactivation of secretion by high Ca^{2+}. (*a*) Cells in solution containing 2.3 mM MgATP and 0.5 mM EGTA were electropermeabilized and incubated for 20 min either alone (○), or with 1 mM TLCK + 1 mM leupeptin + 0.2 mM PMSF (◇), or with 69 nM TPA (●) before being challenged with 15 mM CaEGTA buffers for a further 20 min. (*b*) Exposure to high Ca^{2+} under conditions that do not trigger secretion inactivates the secretory mechanism. Cells in solution containing 0.4 mM EGTA, but with no added MgATP, were rendered permeable, washed over 15 min to remove enogenous MgATP, and were then incubated with protease inhibitors (1 mM TLCK + 1 mM leupeptin + 0.2 mM PMSF) for 5 min. Half the cell suspension was then exposed to 2.5 mM Ca^{2+} for 10 min. The amount of catecholamine secreted as a result of this challenge was less than 1% of the total cellular content. The Ca^{2+} levels were then lowered by washing the challenged and unchallenged cell suspensions in buffers containing 10 mM EGTA and twice more in buffers containing 0.4 mM EGTA (wash time taking 15 min) before being finally resuspended in buffers containing 5 mM MgATP and 0.5 mM EGTA. Aliquots of the cell suspensions were then challenged with 15 mM CaEGTA buffers for 15 min and the catecholamine secreted in response to this Ca^{2+} challenge measured. Cells not previously exposed to Ca^{2+} (●); cells previously exposed to 2.5 mM Ca^{2+} (○)

in the absence or presence of phosphatidylserine (Fig. 8) but, consistent with results of Brocklehurst, Lee and Pollard (1986) a marked activation at micromolar concentrations of Ca^{2+} was seen when both diacylglycerol and phosphatidylserine were present. Interestingly, higher Ca^{2+} concentrations

Table 2. Effect of high Ca^{2+} and preincubation with Ca^{2+} on protein kinase C activity[a]

Pretreatment for 10 min at 30°C	Assay Ca^{2+}	Activity % of control
No preincubation	20 μM	100 (5)
	5 mM	20 (10)
EGTA	20 μM	98 (9)
20 μM Ca^{2+}	20 μM	50 (4)
5 mM Ca^{2+}	20 μM	0 (0)

[a] Results are expressed as a % of the enzyme activity obtained with 20 μM Ca^{2+} when the enzyme is not preincubated with Ca^{2+}. (SEM. $n = 3$). (Enzyme was incubated with Ca^{2+} in the presence of 1 mM leupeptin + 0.2 mM PMSF.)

(100 μM–10 mM) gave progressively less activity. This Ca^{2+}-dependent decline in activity was irreversible (Table 2). Preincubation of the enzyme with Ca^{2+} reduced kinase activity even when leupeptin, an inhibitor of the Ca^{2+}-dependent protease known to cleave protein kinase C, was present. This irreversible inhibition of kinase activity in vitro parallels closely the irreversible inhibition of secretion produced by preincubation of permeable cells with Ca^{2+} (Fig. 6).

Of the cations tested only Sr^{2+} and Ba^{2+} were able to substitute for Ca^{2+} in the activation of protein kinase C (Fig. 9*a*); Mn^{2+} had a small effect, and Co^{2+} and Zn^{2+} were ineffective. This is very similar to the order of effectiveness of cations to elicit catecholamine secretion.

The ability of various nucleotides to compete with [^{32}P]-ATP for protein kinase C is shown in Fig. 9*b*. Relative to ATP, GTP and CTP gave little or no inhibition of activity; however, ADP and ATPγS did inhibit [^{32}P] incorporation into histone. The sensitivity of the secretory process to these nucleotides is shown for comparison.

Various treatments previously shown to inhibit secretion of catecholamines were tested for their effect on medulla protein kinase C activity. Kinase activity was unaltered (89–104% of control) by addition of glutamate, Cl^-, Br^- or acetate (all 150 mM) to the assay medium, although SCN^- (150 mM) completely abolished activity. Increasing osmotic pressure by addition of sucrose to the assay mixture (up to 1 osmolar) was also without effect on protein kinase C activity (86–99% of control). Pretreatment of adrenal medullary cells with botulinum toxin (40 μg/ml each of toxin A and D) for 66 hr inhibited Ca^{2+}-induced exocytosis by approximately 80% but did not reduce the protein kinase C activity in these cells (control 409, SEM 57, pmol/min; botulinum treated 526, SEM 36, pmol/min, $n = 3$). These results suggest that these inhibitors of secretion from elec-

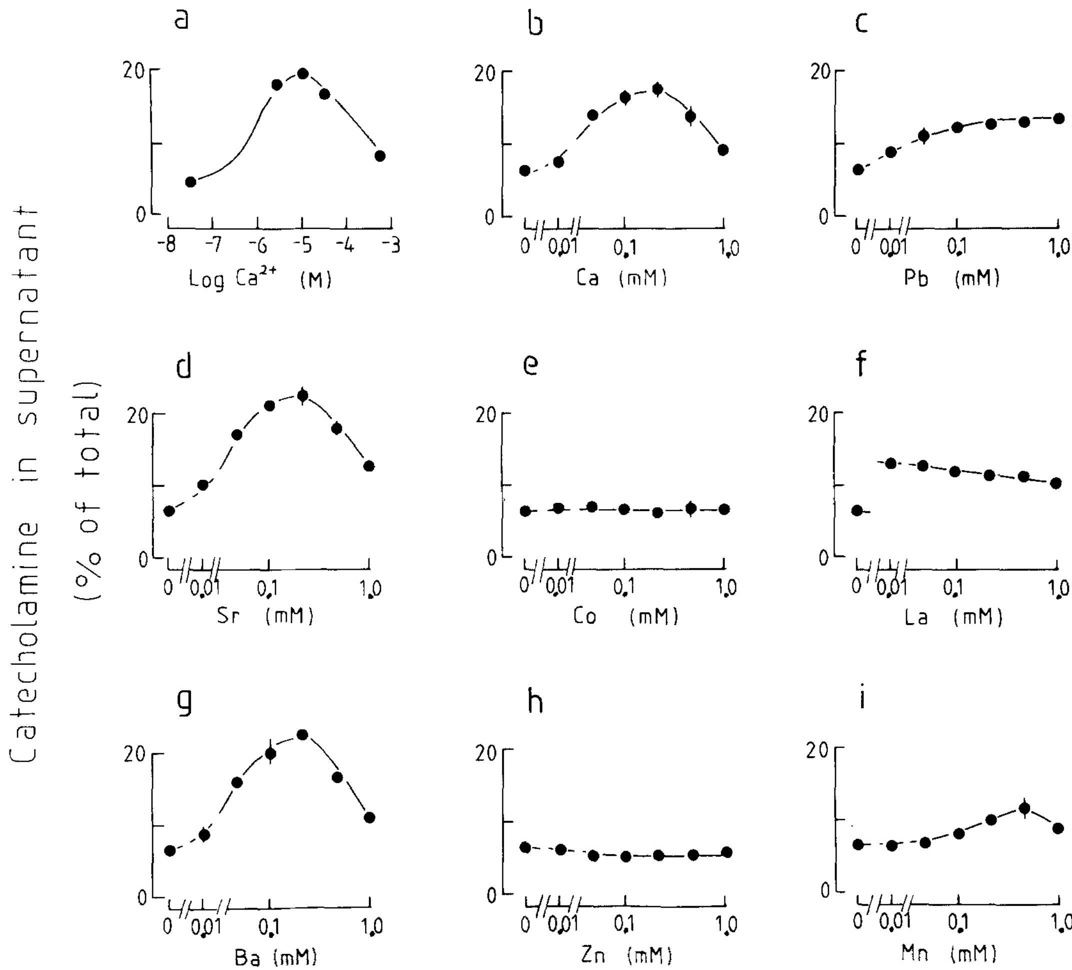

Fig. 7. Cation selectivity for triggering exocytosis. Cells suspended in a sucrose based medium containing 2 mM MgATP and 0.4 mM EGTA were rendered permeable and washed into a medium lacking EGTA. After 10 min, aliquots of the cell suspension were challenged either with (*a*) 7 mM CaEGTA buffers corresponding to the Ca^{2+} shown, or (*b*–*i*) with various concentrations of unbuffered cations as shown, i.e., (*b*) calcium, (*c*) lead, (*d*) strontium, (*e*) cobalt, (*f*) lanthanum, (*g*) barium, (*h*) zinc, (*i*) manganese. The catecholamine in the supernatant was determined 15 min later. Data are mean (SEM of 3 determinations)

tropermeabilized cells (anions, osmotic pressure, botulinum toxins) do not act by inhibiting protein kinase C. Various other putative kinase C inhibitors were tested on the medulla enzyme (Table 1).

Unlike the case with other preparations (Mathies et al., 1987) TPA treatment of intact cultured medullary cells (1 μM for 48 hr) did not result in a substantial downregulation of protein kinase C activity (control 350, SEM 35, pmol/min; TPA-treated 365, SEM 17, pmol/min, $n = 3$) nor did it have any effect on the secretory responses from intact cells to potassium or acetylcholine challenges or from leaky cells to Ca^{2+} challenges.

Endogenous Sources of Diacylglycerol

If protein kinase C is involved in Ca-dependent catecholamine release, there should be an endogenous source of diacylglycerol or some other molecule that can replace it as an activator of the enzyme. We have very little direct information on this point, but the following observations may be pertinent:

(i) Exposure of intact bovine cells to TPA, 1,2-dioctanoylglycerol or mezerein has little or no detectable effect on catecholamine release from intact cells in response to carbachol or high potassium, and measured at a range of different extracellular Ca^{2+} concentrations (*data not shown*).

(ii) Exposure to Ca^{2+} concentrations that trigger exocytosis does not seem to cause significant Ca-dependent breakdown of those endogenous phospholipids that can be labeled during a 1-hr exposure of permeable cells to [^{14}C]-arachidonate, [^{3}H]-inositol (loaded in the presence of 1 mM CTP) or [^{32}P]-ATP. After extensive washing of the permeable cells to remove free label, subsequent exposure to

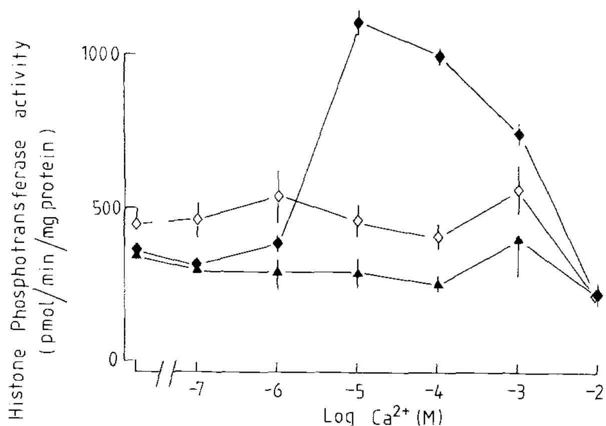

Fig. 8. Ca^{2+} dependence of protein kinase C activity isolated from bovine adrenal medulla. Protein kinase activity (*see* Materials and Methods) was measured at pH 6.6 over the range of Ca^{2+} concentrations shown using CaEGTA buffers. Protein kinase C in the presence of diolein and phosphatidylserine (♦), in the presence of phosphatidylserine (◇), and in the absence of any added phospholipids (▲). Data points are the means of 3 determinations, the error bars being the SEM

10 μM Ca^{2+} released less than 1% of the incorporated label (*data not shown*).

(iii) Exposure of permeable cells to the nonhydrolyzable GTP analogue, GTP-γ-S, that has been shown in a number of tissues to activate phospholipase C-induced hydrolysis of phosphatidylinositides to release diacylglycerol and inositol phosphates (Haslam & Davidson, 1984; Cockroft & Gomperts, 1985; Merritt et al., 1986), inhibits Ca-dependent exocytosis in electropermeabilized bovine adrenal medullary cells rather than stimulating it (Knight & Baker, 1985).

These observations strongly suggest that in bovine adrenal medullary cells: (i) a supply of diacylglycerol or a suitable replacement is not normally rate limiting for the activation of protein kinase C; (ii) Ca-induced breakdown of phosphatidylinositides is not an important source of diacylglycerol for activating exocytosis; and (iii) irrespective of any requirement for GTP in the production of diacylglycerol, exocytosis may be subject to a fairly powerful, GTP-dependent inhibitory control (*see also* Knight & Baker, 1985).

The simplest hypothesis consistent with this data is that in bovine adrenal medullary cells protein kinase C is close to saturation either with diacylglycerol or some diacylglycerol analogue. Electropermeabilization may bring about a reduction in the level of this putative endogenous activator, permitting the modest leftward shifts in the Ca-activation curve observed with TPA, mezerein and dioctanoylglycerol.

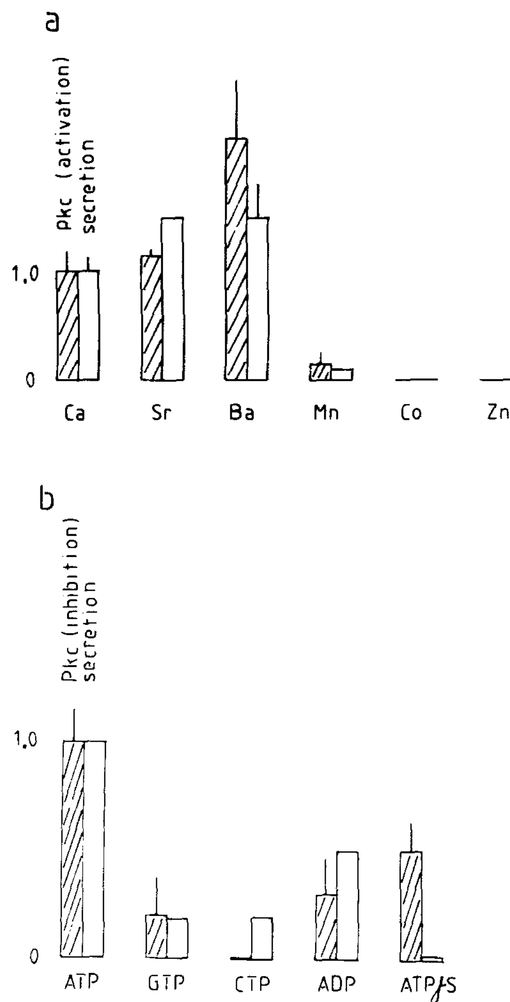

Fig. 9. Cation and nucleotide specificity for activating protein kinase C and exocytosis. Hatched bars are the protein kinase C activity, and the open bars are catecholamine secretion. (*a*) Cation dependence. The diolein/phosphatidylserine-sensitive protein kinase C activity (Materials and Methods) was measured in the presence of 100 μM each of the various cations shown. The diolein/phosphatidylserine-independent level of [^{32}P] incorporation into histone was constant over the range of cations. The activities are normalized to that seen in the presence of 100 μM Ca^{2+}. Data points are the means of 4 determinations, the error bars being the SEM. The catecholamine secreted from electropermeabilized cells in response to 100 μM each of these cations is shown relative to that secreted in the presence of 100 μM Ca^{2+}. (*b*) Nucleotide dependence. Protein kinase C activity was measured as described in Materials and Methods except that only labeled ATP (2 nM, 2×10^6 cpm) was used and the activity measured in the presence and absence of 100 μM of each unlabeled nucleotide determined. The decrease in the [^{32}P] incorporated in histone as a result of these unlabeled nucleotides is a measure of their competition with [^{32}P] ATP for protein kinase C. This inhibition of counts is expressed as a fraction of the decrease seen with 100 μM ATP. The presence of 100 μM CTP, for example, did not reduce the amount of [^{32}P] label incorporated, and so, relative to ATP, is shown not to inhibit the enzyme. Catecholamine secreted from electropermeabilized cells incubated with 5 mM of the nucleotides shown in response to a 20 μM Ca^{2+} challenge

Discussion

The properties of leaky chromaffin cells seem to depend considerably on the method of rendering them permeable, and thus presumably on the extent of their leakiness. Thus, although there are many similarities between digitonin treated cultured cells and electropermeabilized cells in suspension, there are also considerable differences (Bittner, Holz & Neubig, Holz, 1986; Holz & Senter, 1986).

The most striking feature of these experiments on electrically-permeabilized bovine adrenal medullary cells is that, under a wide variety of conditions, exposure to the phorbol ester TPA always increases the apparent affinity of exocytosis for calcium. This effect cannot be blocked by aspirin or indomethacin and is mimicked by a number of activators of protein kinase C including mezerein, 1,2-dioctanoylglycerol and other phorbol esters. Their order of effectiveness on exocytosis roughly parallels their effectiveness as activators of protein kinase C. In addition, the actions of 1,2-dioctanoylglycerol, mezerein and TPA are additive at low concentrations but not at high concentrations, which suggests that they act at the same site. One anomalous finding is that 1-oleoyl,2-acetylglycerol is without effect on the Ca-activation curve, although it can activate the adrenal medulla enzyme in vitro. A negative result of this kind could have many explanations including: the rather hydrophobic analogue may fail to enter the cell, or once inside it may be inactivated rapidly. Further experiments are required to answer these questions.

The simplest working hypothesis arising out of these experiments is that protein kinase C is intimately involved with exocytosis in bovine chromaffin cells (Knight & Baker, 1983). This proposal does, however, rely heavily on the identification of protein kinase C in terms of its sensitivity to phorbol esters and other agents. This is obviously very indirect and could be highly misleading especially if other kinases with similar activator specificity are discovered. However, until that is the case, it seems reasonable to work on the assumption that protein kinase C is the most likely mediator of the phorbol ester effect on exocytosis and to attempt to elucidate the mode of action of the kinase.

Although there is a small and variable effect of TPA on the extent of exocytosis, its major action is to induce a leftward shift of the Ca-activation curve with no effect on exocytosis at very low levels of free calcium. The absence of response to TPA at very low Ca^{2+} and minimal response at high Ca^{2+} argues strongly against TPA acting completely independently of calcium as such an action should increase exocytosis by an equal amount at all Ca^{2+} concentrations. The two most likely ways in which protein kinase C may be involved are either as a modulator of another Ca-dependent process or as an integral part of the machinery controlling exocytosis. If the former is true, it ought to be possible to find inhibitors that do not affect the Ca-activation curve but remove the TPA-induced shift, whereas if the latter is true it should not be possible to disentangle the two. We have examined a large number of agents that have been reported to inhibit protein kinase C as well as other known inhibitors of exocytosis. In no case was the TPA-induced shift removed selectively and in all instances where the extent of exocytosis was reduced, the TPA-shift persisted. The extent of exocytosis was reduced by a number of agents that are known to inhibit protein kinase C but none selectively removed the TPA-induced shift. These included adriamycin, polymixin B, amiloride, spermine, and spermidine, all of which inhibited medulla protein kinase C at a concentration causing a marked reduction in secretion. Inhibition of secretion and medulla protein kinase C was also seen with trifluoperazine, W7, calmidazolium, melittin—all of which are rather nonspecific inhibitors of calmodulin and protein kinase C.

Taken together, these data strongly support the second possibility, that protein kinase C is intimately involved in the control machinery of exocytosis in permeabilized cells. The response observed in the bovine adrenal medullary cells (*see* Fig. 1*a*) can be modelled very closely if it is assumed that the kinase must bind diacylglycerol before it can bind calcium (Baker, 1986). As diacylglycerol is probably always within membranes, this reaction sequence would imply that in bovine chromaffin cells protein kinase C must first become membrane associated before it can react with calcium to promote exocytosis. Indeed, the binding of protein kinase C to membrane phospholipids, often termed the 'translocation' of the enzyme, is considered to be an essential step in the activation of the kinase by physiological stimuli (Nishizuka, 1980; Kraft & Anderson, 1983*b*; Gopalakrishna et al., 1986).

In addition, if protein kinase C is involved in exocytosis it ought to be possible to find other parallels between exocytosis and known properties of the kinase. Our preliminary data suggest that both exocytosis in permeable bovine chromaffin cells and protein kinase C isolated from bovine adrenal medulla have a rather specific requirement for MgATP and both have similar divalent cation specificity. Furthermore, protein kinase C from the bovine adrenal medulla is irreversibly inhibited by exposure to high Ca^{2+}, and it is of some interest that exposure of permeable cells to high levels of Ca^{2+}

rapidly results in irreversible inhibition of exocytosis, inhibition occuring more rapidly than the initiation of exocytosis. The inhibitory effects of high Ca^{2+} on exocytosis are not prevented by protease inhibitors, which would be expected to inhibit Ca^{2+}-dependent cleavage of protein kinase C. While none of these observations provides particularly strong evidence for the involvement of protein kinase C in exocytosis, none are incompatible with such a role. The finding that medulla protein kinase C is resistant to downregulation, is almost entirely phospholipid dependent and can be activated by either Sr^{2+} or Ba^{2+} at least as well as Ca^{2+} is surprising considering the properties of C kinases isolated from other tissues reported elsewhere (Nishizuka, 1980; Matthies et al., 1987). However, there is increasing evidence that even within a particular tissue there may be several classes of protein kinase C, each possibly having quite different properties (Knopf et al., 1986; Ido et al., 1987; Kariya & Takai, 1987).

If protein kinase C is intimately involved in the control of exocytosis, it is important to discover from where it normally obtains its diacylglycerol. An answer to this question will have to await measurements of diacylglycerol levels in electro-permeabilized cells; but the relatively modest leftward shift in the Ca-activation curve and the virtual insensitivity of intact cells to both TPA, 1,2-dioctanoylglycerol and mezerein suggest that the secretory system may be close to saturation with either diacylglycerol or another endogenous molecule with similar actions. If this is the case, initiation of exocytosis from the bovine adrenal medulla under physiological conditions will be largely dependent on calcium.

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