Characterization of protein kinase C and its role in catecholamine secretion from bovine adrenal-medullary cells

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1. Protein kinase C activity towards exogenous histone was detected in a cytosolic fraction of bovine adrenal medulla. The enzyme was dependent on Ca^{2+} and phosphatidylserine for its activity, with half-maximal activation being achieved at approx. $18\text{ }\mu\text{M}$ free Ca^{2+} and $8\text{ }\mu\text{g}$ of phosphatidylserine/ml. Both diolein and 4β -phorbol 12-myristate 13-acetate (TPA) decreased the Ca^{2+} requirement of the enzyme, half-maximal activation being obtained at approx. $12\text{ }\mu\text{M}$ and $9\text{ }\mu\text{M}$ free Ca^{2+} respectively in the presence of these agents. 2. Many endogenous proteins in the adrenal-medullary cytosolic fraction were detected whose phosphorylation was dependent on the presence of both Ca^{2+} and phosphatidylserine. 3. TPA stimulated catecholamine release from cultured bovine adrenal-chromaffin cells in a Ca^{2+} -dependent manner. A23187 also stimulated catecholamine secretion, and at sub-optimal concentrations of TPA and A23187 a synergistic secretory response was obtained. 4. These results are consistent with protein kinase C having a regulatory role in exocytosis in bovine adrenal chromaffin cells.

An increase in the cytosolic Ca^{2+} concentration appears to be the primary intracellular signal for secretagogue-induced release of catecholamines from the adrenal-medullary chromaffin cell (for review see Baker & Knight, 1984). One Ca^{2+} -sensitive enzyme possibly involved in the transduction of this Ca^{2+} signal into an activation of the exocytotic process is protein kinase C.

Protein kinase C is dependent on both Ca^{2+} and phospholipid for its activity, and diacylglycerol increases the affinity of the enzyme for both these activators (for review see Nishizuka, 1984). It has thus been proposed that the receptor-mediated breakdown of phosphoinositides, which occurs in many cell types and results in the production of diacylglycerol (for review see Berridge, 1984), may be linked to the activation of protein kinase C *in vivo*. The physiological role of protein kinase C has been most studied in the platelet release reaction (see Nishizuka, 1984). Nishizuka (1984) has proposed that a physiological response to an agonist such as thrombin is the result of the activation of two synergistic pathways. One path-

way involves the activation of protein kinase C by diacylglycerol and can be artificially stimulated by permeant synthetic diacylglycerols or by tumour-promoting phorbol esters, such as TPA, which can serve as analogues of diacylglycerol in their capacity to activate protein kinase C (Castagna *et al.*, 1982). The second pathway involves an increase in the cytosolic Ca^{2+} concentration and can be artificially activated by the Ca^{2+} ionophore A23187.

TPA has also been found to induce secretion in pancreatic B-cells (Virji *et al.*, 1978; Malaisse *et al.*, 1980; Hutton *et al.*, 1984), pancreatic acini (Gunther, 1981; Wooten & Wrenn, 1984) and anterior pituitary cells (Smith & Vale, 1980). Furthermore, TPA and A23187 have been shown to have synergistic actions on secretion in pancreatic islets (Zawalich *et al.*, 1983), pancreatic acini (de Pont & Fleuren-Jakobs, 1984), mast cells (Katakami *et al.*, 1984) and adrenal glomerulosa cells (Kojima *et al.*, 1983).

Knight & Baker (1983a) reported that TPA decreased the Ca^{2+} -concentration-dependency of secretion from high-voltage permeabilized bovine adrenal chromaffin cells, and thus postulated a role for protein kinase C in the secretory mechanism of these cells. However, apart from the observation

Abbreviations used: TPA, 4β -phorbol 12-myristate 13-acetate.

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that a protein kinase C-like activity present in the soluble fraction of bovine adrenal medulla bound to chromaffin-granule membranes in a Ca^{2+} -dependent manner (Creutz *et al.*, 1983), the characterization of chromaffin-cell protein kinase C has not been documented.

The present paper describes the properties of protein kinase C from the adrenal medulla and further investigates the effect of TPA on the secretory process in chromaffin cells.

Experimental

Preparation of adrenal-medulla soluble fraction

Medullae from 8–12 fresh bovine adrenal glands (20–30g wet wt. of tissue) were disrupted with a 6s burst of a Waring blender and then homogenized with a loose-fitting Teflon pestle and glass homogenizer in approx. 80ml of an ice-cold solution containing 10mM-Mes, 0.3M-sucrose, 1mM-EGTA and 1mM-phenylmethanesulphonyl fluoride (added from a 0.1M stock solution prepared in ethanol), adjusted to pH6.5 with Tris. The homogenate was filtered through four layers of surgical gauze and the filtrate centrifuged at 480g for 10min at 4°C. The supernatant was centrifuged at 20000g for 30min at 4°C, and the resulting supernatant finally centrifuged at 257000g for 30min at 4°C. The yellow lipid film found floating on the top of the centrifuge tubes after the ultracentrifugation was removed with tissue paper, and the remaining supernatant constituted the soluble fraction used in subsequent experiments.

Protein was measured by the method of Bradford (1976), with bovine serum albumin (Calbiochem-Behring, San Diego, CA, U.S.A.) as standard.

Preparation of chromaffin cells

Bovine adrenal chromaffin cells were isolated from medullae by collagenase digestion as described by Greenberg & Zinder (1982) and maintained as monolayer cultures in basal Eagle's medium (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 5% (w/v) foetal-calf serum (Gibco), 2.93mM-glutamine, penicillin (100units/ml; Gibco), streptomycin (100µg/ml; Gibco), gentamicin (5µg/ml; Gibco) and cytosine arabinoside (10µg/ml; Gibco). Cells were plated at a density of 10^6 cells/1.5ml per 2cm² in 24-well cluster plates (Costar, Cambridge, MA, U.S.A.) and maintained at 37°C in a CO₂ incubator for 3 days before experiments.

Protein kinase C assay

Protein kinase C was assayed in Eppendorf microfuge tubes (Brinkmann Instrument Co., Westbury, NY, U.S.A.) by a modification of the

method of Minakuchi *et al.* (1981). Adrenal-medullary soluble fraction (approx. 2–3µg of protein) was incubated at 37°C for 3min in 50µl of a solution containing 50mM-Mes, 1mM-MgCl₂, 0.1mM-dithiothreitol, 5mM-EGTA and 0.1mM-[γ-³²P]ATP (approx. 0.5Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) adjusted to pH6.65 with Tris. Lysine-rich histone (calf thymus type III-S; Sigma Chemical Co., St. Louis, MO, U.S.A.) at a final concentration of 0.3mg/ml was used as the exogenous substrate. Other additions included CaCl₂, phosphatidylserine (Sigma), diolein (Sigma) and TPA (Sigma) at concentrations specified in the Figure legends. Free Ca^{2+} concentrations were calculated by the iterative procedure of Storer & Cornish-Bowden (1976) and checked with a Ca^{2+} electrode (WPI, New Haven, CT, U.S.A.); dissociation constants obtained in the presence of 0.1M-KCl were used in these calculations and were taken from Sillen & Martell (1964).

Phosphatidylserine and diolein were dissolved in chloroform and, after evaporation of the solvent, were dispersed by vigorous vortex-mixing in a solution containing 10mM-Mes adjusted to pH6.5 with Tris, before addition to the assay. In incubations requiring the addition of both phosphatidylserine and diolein, these two agents were mixed together while dissolved in chloroform. TPA was stored as a 1mM stock solution in dimethyl sulphoxide and subsequently diluted in a solution of 10mM-Mes adjusted to pH6.5 with Tris before addition to the assay. In incubations requiring the addition of both phosphatidylserine and TPA, the TPA was added to the phosphatidylserine just before dispersion of the lipid film into the aqueous buffer. Under the assay conditions used, the rate of incorporation of ³²P into histone was linear with respect to time and enzyme concentration.

The reaction was terminated by the addition of 0.5ml of an ice-cold solution of 25% (w/v) trichloroacetic acid. After standing on ice for 30min, samples were centrifuged for 5min at 4°C in an Eppendorf microfuge (Eppendorf Gerätebau, Hamburg, Germany). The supernatants were removed and the tubes rinsed in 0.75ml of ice-cold 25% (w/v) trichloroacetic acid. The precipitated protein was dissolved in 0.1ml of 0.1M-NaOH, and the radioactivity present determined by Čerenkov counting in a liquid-scintillation counter.

Results were corrected for the incorporation of radioactivity into trichloroacetic acid-precipitable material seen in control incubations. The corrected values in c.p.m. were then expressed as a percentage of the maximum value or a reference value obtained in each experiment (typically maximum values ranged from 10000 to 25000 c.p.m., with a total blank correction of less than 10%).

Assay for endogenous substrates of protein kinase C

Adrenal-medullary soluble fraction (approx. 10 μ g of protein) was incubated at 37°C for 1 min in the protein kinase C assay medium, except that the histone was omitted. The effects of 5 mM- CaCl_2 and 40 μ g of phosphatidylserine/ml on the phosphorylation pattern were investigated. The reaction was terminated by the addition of 50 μ l of a solution containing 0.24 M-Tris/HCl (pH 6.8), 0.86 M-sucrose, 3.8% (w/v) sodium dodecyl sulphate, 0.2 M-dithiothreitol and 0.2% Bromophenol Blue, and heating at 100°C for 5 min. Samples (50 μ l) were then electrophoresed on 15–20% (w/v) polyacrylamide gradient slab gels (15 cm $\times$ 15 cm $\times$ 0.1 cm) in the buffer system of Laemmli (1970). A mixture of glycogen phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and lysozyme (Bio-Rad, Richmond, CA, U.S.A.) was used to calibrate protein M_r values. Gels were stained with Coomassie Blue (Bio-Rad), dried, and autoradiographed by using Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY, U.S.A.).

Assay of catecholamine release

All release experiments were performed at 37°C. Culture medium was removed from each well and the cells were washed with 1 ml of standard release medium, consisting of 118 mM-NaCl, 4.7 mM-KCl, 1.2 mM- MgSO_4 , 2.2 mM- CaCl_2 , 10 mM-glucose and 25 mM-Hepes/NaOH (pH 7.32). Cells were preincubated in 0.5 ml of standard release medium for 10 min. The preincubation medium was then removed and the cells were incubated with 0.25 ml of secretagogue dissolved in standard release medium for 15 min. This medium was then removed and centrifuged at 4°C for 2 min in an Eppendorf microfuge to remove any cells, and the supernatant assayed for catecholamines. To each well was added 0.25 ml of 10% (v/v) acetic acid, and the plate was kept at -70°C for 30 min. After thawing, a sample from each well was assayed for catecholamines. The secretagogues used, TPA and ionophore A23187 (Sigma), were stored as 1 mM stock solutions in dimethyl sulphoxide and diluted in standard release medium before addition to the cells. The final concentration of dimethyl sulphoxide presented to the cells was 1% (v/v), which did not affect the rate of catecholamine release. The basal catecholamine release observed with these cells in a 15 min incubation was usually 4–6% of the total cellular catecholamine.

Catecholamines were measured by the trihydroxyindole method performed on ice as described by Kelner *et al.* (1985). Dopamine β -hydroxylase was measured by the method of Creveling *et al.*, (1962).

Results

Characterization of protein kinase C

The activation of bovine adrenal-medullary protein kinase C by phospholipid is shown in Fig. 1. Phosphatidylserine had no effect on enzyme activity in the absence of Ca^{2+} , but in the presence of approx. 70 μ M free Ca^{2+} phosphatidylserine increased the activity, with half-maximal activation occurring at approx. 8 μ g of phosphatidylserine/ml and maximal activation at approx. 80 μ g/ml.

In the absence of phosphatidylserine, Ca^{2+} had no effect on protein kinase activity, but in the presence of 40 μ g of phosphatidylserine/ml Ca^{2+} increased the activity, with half-maximal activation occurring at approx. 18 μ M and maximal activation at approx. 40 μ M free Ca^{2+} (Fig. 2). The diacylglycerol diolein (2.5 μ g/ml) had no effect on protein kinase activity in the absence of phosphatidylserine, but in the presence of phosphatidylserine diolein increased the Ca^{2+} -dependent enzyme activity, resulting in a shift to the left of the Ca^{2+} dose-response curve. In the presence of diolein, free Ca^{2+} caused half-maximal activation of protein kinase C at approx. 12 μ M and maximal activation at approx. 40 μ M. In the presence of

Fig. 1. Effect of phosphatidylserine concentration on protein kinase C activity

Adrenal-medullary soluble fraction was incubated with [γ - 32 P]ATP, histone, 5 mM-EGTA and in the absence (○) or presence (●) of 5 mM- CaCl_2 (resulting in a free Ca^{2+} concentration of approx. 70 μ M) at the indicated concentrations of phosphatidylserine. The reaction was terminated by the addition of trichloroacetic acid, and the radioactivity incorporated into the histone proteins determined by Čerenkov counting. Results are expressed as means $\pm$ S.E.M. ($n = 6$), as a percentage of the maximum activity observed. For further details see the Experimental section.

Fig. 2. Effect of diolein on the Ca^{2+} -concentration-dependency of protein kinase C activity

Adrenal-medullary soluble fraction was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and histone at different free Ca^{2+} concentrations, obtained by varying the concentration of CaCl_2 in the presence of 5 mM-EGTA (○). The effects of 40 μg of phosphatidylserine/ml (□), 2.5 μg of diolein/ml (●) and 40 μg of phosphatidylserine/ml plus 2.5 μg of diolein/ml (■) on the Ca^{2+} -concentration-dependency are shown. Results are expressed as means $\pm$ S.E.M. ($n = 6$), as a percentage of the activity observed in the presence of phosphatidylserine and 5 mM- CaCl_2 . For further details see the Experimental section.

40 μg of phosphatidylserine/ml and a sub-optimal free Ca^{2+} concentration (16 μM), where the effect of diolein was most marked, half-maximal activation by diolein was obtained at a concentration of approx. 1 $\mu\text{g}/\text{ml}$ and maximal activation at approx. 10 $\mu\text{g}/\text{ml}$ (Fig. 3).

A similar series of experiments to those performed with diolein were carried out with TPA. In the absence of phosphatidylserine, TPA (0.1 μM) slightly increased enzyme activity in a Ca^{2+} -dependent fashion, but the effect of TPA was most marked in the presence of phosphatidylserine (Fig. 4). Again there was a shift to the left of the Ca^{2+} dose-response curve, with free Ca^{2+} giving half-maximal activation at approx. 9 μM and maximal activation at approx. 16 μM in the presence of TPA. In the presence of 16 μM free Ca^{2+} and 40 μg of phosphatidylserine/ml, TPA caused half-maximal activation at approx. 1 nM-TPA and maximal activation at approx. 10 nM-TPA (Fig. 5).

Detection of cytosolic substrates of protein kinase C

Ca^{2+} (approx. 70 μM) or phosphatidylserine (40 $\mu\text{g}/\text{ml}$) alone had no effect on the pattern of endogenous protein phosphorylation, but together they caused an increase in the phosphorylation of many protein bands (Fig. 6). Several proteins were

Fig. 3. Effect of diolein concentration on protein kinase C activity

Adrenal-medullary soluble fraction was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, histone and 5 mM-EGTA at the indicated diolein concentrations (○). The effects of 4.7 mM- CaCl_2 (resulting in a free Ca^{2+} concentration of approx. 16 μM) (●), 40 μg of phosphatidylserine/ml (□) and 4.7 mM- CaCl_2 plus 40 μg of phosphatidylserine/ml (■) on the diolein concentration-dependency are shown. Results are expressed as means $\pm$ S.E.M. ($n = 6$), as a percentage of the maximum activity observed. For further details see the Experimental section.

phosphorylated under control conditions, but this is not apparent from Fig. 6 because of the time of exposure of the autoradiogram chosen to illustrate best the combined effect of Ca^{2+} and phosphatidylserine on the phosphorylation pattern. The Ca^{2+} - and phosphatidylserine-dependent protein phosphorylation reactions were specifically inhibited by trifluoperazine (results not shown).

Effects of TPA and A23187 on catecholamine secretion

TPA stimulated catecholamine release from cultured bovine adrenal chromaffin cells (Fig. 7). The maximal secretory response obtained over a 15 min incubation was approx. 170% of the basal catecholamine release, and this was achieved with 1 μM -TPA; the half-maximal secretory response was observed at approx. 10 nM-TPA.

A23187 also stimulated catecholamine release, but in this case the effect had not reached a plateau by 10 μM -A23187 (Fig. 8). Concentrations of A23187 higher than 10 μM were not tested because of possible cytotoxic effects (Knight & Baker, 1983b). In the presence of 0.1 μM -TPA, however, there was a shift to the left of the A23187 dose-

Fig. 4. Effect of TPA on the Ca^{2+} -concentration-dependency of protein kinase C activity

Adrenal-medullary soluble fraction was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and histone at different free Ca^{2+} concentrations, obtained by varying the concentration of Ca^{2+} in the presence of 5 mM-EGTA (○). The effects of 40 μg of phosphatidylserine/ml (□), 0.1 μM -TPA (●) and 40 μg of phosphatidylserine/ml plus 0.1 μM -TPA (■) on the Ca^{2+} -concentration-dependency are shown. Results are expressed as means $\pm$ S.E.M. ($n = 6$), as a percentage of the activity observed in the presence of phosphatidylserine and 5 mM- CaCl_2 . For further details see the Experimental section.

Fig. 6. Autoradiogram showing the effect of Ca^{2+} and phosphatidylserine on protein phosphorylation in an adrenal-medullary soluble fraction

Adrenal-medullary soluble fraction was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 5 mM-EGTA in the presence of: (i) no further addition; (ii) 5 mM- CaCl_2 (resulting in a free Ca^{2+} concentration of approx. 70 μM); (iii) 40 μg of phosphatidylserine/ml; and (iv) 5 mM- CaCl_2 plus 40 μg of phosphatidylserine/ml. Phosphorylated proteins were detected by polyacrylamide-gel electrophoresis and autoradiography. For further details see the Experimental section.

Fig. 5. Effect of TPA concentration on protein kinase C activity

Adrenal-medullary soluble fraction was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, histone and 5 mM-EGTA at the indicated TPA concentrations (○). The effects of 4.7 mM- CaCl_2 (resulting in a free Ca^{2+} concentration of approx. 16 μM) (●), 40 μg of phosphatidylserine/ml (□) and 4.7 mM- CaCl_2 plus 40 μg of phosphatidylserine/ml (■) on the TPA-concentration-dependency are shown. Results are expressed as means $\pm$ S.E.M. ($n = 6$) and a percentage of the maximum activity observed. For further details see the Experimental section.

response curve, and a maximal effect of A23187 was observed at 0.3 μM -A23187 (Fig. 8). At concentrations of A23187 of 0.1–0.3 μM the combined effect of A23187 and 0.1 μM -TPA on secretion was significantly greater ($P < 0.005$) than that which would be expected if the effects of the secretagogues were additive.

The time courses of catecholamine release evoked by 0.1 μM -TPA and 0.3 μM -A23187, separa-

Fig. 7. *Effect of TPA concentration on catecholamine release from cultured adrenal chromaffin cells*
Cultured chromaffin cells were incubated at 37°C for 15 min with various concentrations of TPA as described in the Experimental section. Results were calculated as the percentage of total cellular catecholamine released from the cells into the medium for each condition and then expressed as a percentage of the basal release. Values shown are means $\pm$ S.E.M. ($n = 9$).

Fig. 8. *Effect of TPA on the A23187-concentration-dependency of catecholamine release from cultured adrenal chromaffin cells*
Cultured chromaffin cells were incubated at 37°C for 15 min with various concentrations of A23187 in either the absence (○) or the presence (●) of 0.1 μM-TPA as described in the Experimental section. Results were calculated as the percentage of total cellular catecholamine released from the cells into the medium for each condition and then expressed as a percentage of the basal release. Values shown are means $\pm$ S.E.M. ($n = 9$). The differences between the secretory responses obtained in the presence of both secretagogues and those expected if their combined actions were additive were assessed by Student's t test ($*P < 0.005$).

Fig. 9. *Time course of catecholamine release induced by TPA, A23187 and TPA plus A23187 from cultured adrenal chromaffin cells*
Cultured chromaffin cells were incubated at 37°C for the designated time intervals in the presence of 0.1 μM-TPA (○), 0.3 μM-A23187 (●) or 0.1 μM-TPA plus 0.3 μM-A23187 (□). Results were calculated as the percentage of the total cellular catecholamine released from the cells into the medium for each condition and the corresponding basal secretion at each time point was then subtracted. Values shown are means $\pm$ S.E.M. ($n = 6$). The differences between the secretory responses obtained in the presence of both secretagogues and those expected if their combined actions were additive were assessed by Student's t test ($*P < 0.01$).

Table 1. *Effect of removal of extracellular Ca²⁺ on TPA- and A23187-induced catecholamine release from cultured adrenal chromaffin cells*

Cultured chromaffin cells were incubated at 37°C for 15 min with 0.1 μM-TPA, 0.3 μM-A23187 or 0.1 μM-TPA plus 0.3 μM-A23187 in standard release medium containing 2.2 mM-CaCl₂ or in medium from which CaCl₂ was omitted. When incubations were performed in medium containing no added CaCl₂, the cells were also washed and preincubated in this medium as described in the Experimental section. Results were calculated as the percentage of total cellular catecholamine released from the cells into the medium for each condition and then expressed as a percentage of the basal secretory rate in the presence of 2.2 mM-CaCl₂. Values shown are means $\pm$ S.E.M. ($n = 6$).

Secretagogue	Catecholamine release (% of + Ca ²⁺ value)	
	+ Ca ²⁺	- Ca ²⁺
None	100 $\pm$ 0	101 $\pm$ 4
0.1 μM-TPA	136 $\pm$ 4	108 $\pm$ 4
0.3 μM-A23187	129 $\pm$ 3	109 $\pm$ 4
0.1 μM-TPA + 0.3 μM-A23187	190 $\pm$ 5	113 $\pm$ 3

tely and in combination (these concentrations of secretagogues were chosen to illustrate their synergistic action best), are shown in Fig. 9. The synergistic action of these secretagogues was apparent at time points between 6 and 15 min.

Both the separate and the combined actions of $0.1\text{ }\mu\text{M}$ -TPA and $0.3\text{ }\mu\text{M}$ -A23187 were dependent on extracellular Ca^{2+} (Table 1). Also, the catecholamine-secretory response to these agents was paralleled by a release of dopamine β -hydroxylase when results were expressed as a percentage of the respective basal release: for catecholamines the secretory responses to $0.1\text{ }\mu\text{M}$ -TPA, $0.3\text{ }\mu\text{M}$ -A23187 and $0.1\text{ }\mu\text{M}$ -TPA plus $0.3\text{ }\mu\text{M}$ -A23187 were 150 ± 2 , 123 ± 6 and 199 ± 4 respectively, and for dopamine β -hydroxylase the corresponding values were 160 ± 12 , 125 ± 5 and 203 ± 11 respectively over a 15 min incubation period (values are means $\pm$ S.E.M., and $n = 3$ in both cases).

Discussion

The results presented confirm that a protein kinase C-like enzyme is present in bovine adrenal medulla. At Ca^{2+} concentrations that support activity in the presence of phosphatidylserine, diolein and TPA increased both maximal enzyme activity and the apparent affinity of the enzyme for Ca^{2+} . These properties are consistent with those reported for the brain enzyme (Takai *et al.*, 1979a,b; Castagna *et al.*, 1982), but the shifts of the Ca^{2+} dose-response curve to lower Ca^{2+} concentrations induced by diolein and TPA were not as marked for the adrenal-medullary enzyme. Indeed, the degree to which protein kinase C can be activated by diolein appears to vary, depending on the tissue from which the enzyme is isolated. Thus diolein had only a very slight effect on protein kinase C purified from pig spleen (Schatzman *et al.*, 1983). Protein kinase C has also been characterized in other secretory tissues, including the pancreatic B-cell (Tanigawa *et al.*, 1982; Lord & Ashcroft, 1984; Hutton *et al.*, 1984), adenohypophysis (Turgeon *et al.*, 1984) and pancreatic acinar cells (Wrenn, 1983).

Several endogenous cytosolic substrate proteins for protein kinase C were shown to be present in the adrenal medulla. At this point it is not possible to say whether any of these proteins may be related to those detected in other secretory tissues (Lord & Ashcroft, 1984; Thams *et al.*, 1984; Hutton *et al.*, 1984; Turgeon *et al.*, 1984; Wrenn, 1983) or to the proteins that have been shown to undergo secretagogue-induced changes in their phosphorylation state in intact bovine adrenal chromaffin cells (Amy & Kirshner, 1981).

Although Knight & Baker (1983a) stated that TPA had no effect on catecholamine secretion

from intact bovine adrenal-medullary cells, we found that TPA increased the basal secretory response by a maximum of approx. 170%. A23187 also stimulated catecholamine release from these cells, and at certain concentrations of TPA and A23187 the combined action of these secretagogues was synergistic. The concentration range over which TPA stimulated protein kinase C activity was lower than the range in which TPA induced catecholamine secretion, which possibly reflects the difference in the way that TPA is presented to protein kinase C in the two situations. Evidence that TPA exerts its effects on cells via protein kinase C is that the cellular TPA receptor is co-purified with protein kinase C from brain tissue (Niedel *et al.*, 1983).

The observations that TPA-induced catecholamine secretion was saturable, and that the induced response in both the presence and the absence of A23187 was Ca^{2+} -dependent and accompanied by an equivalent release of dopamine β -hydroxylase, are evidence that the induced secretion occurs by exocytosis. TPA-induced secretion has also been shown to be dependent on extracellular Ca^{2+} in pancreatic β -cells (Malaisse *et al.*, 1980; Hutton *et al.*, 1984) and pancreatic acini (Gunther, 1981), but the TPA-induced response in platelets is independent of the extracellular Ca^{2+} concentration (Rink *et al.*, 1983). The Ca^{2+} -dependence of TPA-induced secretion from a particular tissue will possibly reflect the degree to which TPA increases the affinity of protein kinase C for Ca^{2+} in that tissue.

It is of particular interest that the effect of TPA on the A23187 dose-response curve reported here was most marked at concentrations of A23187 just above the threshold concentration that elicits secretion, and similarly that the effect of TPA on protein kinase C activity was most marked at concentrations of free Ca^{2+} just above the threshold concentration that stimulated activity in the presence of phosphatidylserine (cf. Fig. 8 with Fig. 4). Although it has been proposed that TPA and Ca^{2+} affect two different pathways in the cell (Nishizuka, 1984), it is also possible that part of the synergism observed between these two effectors is actually mediated on protein kinase C in cells where protein kinase C cannot be fully activated by diacylglycerol at resting cytosolic Ca^{2+} concentrations.

It has been proposed that the activation of protein kinase C by diolein is linked to the receptor-mediated breakdown of phosphoinositides at the plasma membrane (Takai *et al.*, 1979b). However using cultured bovine adrenal chromaffin cells, Fisher *et al.* (1981) showed that, whereas the cholinergic stimulation of catecholamine release was mediated by nicotinic receptors, phos-

phatidylinositol turnover was mediated by muscarinic receptors. They concluded that phosphatidylinositol turnover was neither a prerequisite nor a consequence of secretion. Therefore, if protein kinase C has a role to play in the secretory process in these cells, then the activation of the enzyme by secretagogues may be mediated only by an increase in the cytosolic Ca^{2+} concentration. Knight & Baker (1983a) showed that TPA increased the apparent affinity of exocytosis from high-voltage permeabilized adrenal-medullary cells from $1.2\text{ }\mu\text{M}$ - to $0.5\text{ }\mu\text{M}$ - Ca^{2+} . Although these Ca^{2+} concentrations are an order of magnitude lower than those found to give half-maximal activation of adrenal-medullary protein kinase C in the absence and the presence of TPA respectively, it is possible that in the cell the activity of the enzyme is further modulated by other phospholipids, as has been shown for the brain enzyme *in vitro* (Kaibuchi *et al.*, 1982). Thus, although the adrenal-medullary protein kinase C is sensitive to diacylglycerol (and phorbol esters), diacylglycerol may have no role in the secretory process of bovine adrenal chromaffin cells.

The present findings are thus consistent with the hypothesis that activation of protein kinase C by an increase in the cytosolic Ca^{2+} concentration and the subsequent phosphorylation of substrate proteins are involved in the stimulus-secretion coupling mechanism of bovine adrenal chromaffin cells.

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