Protein kinase C and an endogenous substrate associated with adenohypophyseal secretory granules

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Secretory granules isolated from anterior pituitary glands were examined for Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C) activity as well as the occurrence of granule-associated substrate proteins. Sheep adenohypophyses were fractionated by differential and sucrose-density-gradient centrifugation to yield a granule fraction enriched for luteinizing-hormone (lutropin)-containing secretory granules. Marker-enzyme analysis showed no detectable cytosolic contamination, although there were small amounts of plasma membranes (2–4%) and lysosomes (4–6%) associated with the preparation. As determined by histone-H1 phosphorylation after DEAE-cellulose DE-52 chromatography, protein kinase C activity with a marked dependence on Ca^{2+} and lipid (4-fold increase in their presence) was evident in the secretory-granule fraction. Phosphorylation *in vitro* of the secretory-granule fraction by endogenous and exogenous protein kinase C revealed a protein of M_r 36000, which by two-dimensional SDS/polyacrylamide-gel electrophoresis showed multiple sites of phosphorylation. The M_r -36000 protein was not found in cytosolic or plasma-membrane fractions and was not phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase. Several secretory-granule proteins served as substrates for the catalytic subunit, the most prominent of which were of M_r 63000, 23000 and 21000. From these data, we suggest that phosphorylation of secretory-granule-associated proteins by protein kinase C and by cyclic AMP-dependent protein kinase may be important in secretion regulation in the anterior pituitary gland.

INTRODUCTION

The steps in signal transduction following binding of a hypothalamic hormone to its receptor on pituitary cell membranes are unclear. The mediators for regulation of adenohypophyseal function have been suggested to involve changes in cyclic nucleotide metabolism, Ca^{2+} flux or membrane phospholipid turnover, but the specific subcellular events being modified or level of integration between these putative second messengers have yet to be established for any of the multiple cell types of the adenohypophysis. Protein phosphorylation is an important final common pathway in all cells, and at least three protein kinases have been identified for the anterior pituitary gland: cyclic AMP-dependent protein kinase (cyclic AMP-kinase) (Lemaire *et al.*, 1971), Ca^{2+} /calmodulin-dependent protein kinase (Hatada *et al.*, 1983) and Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C) (Turgeon *et al.*, 1984). Although each has been implicated as having a mediating role for one or all of the hypothalamic hormones, their substrates and association with subcellular compartments in non-tumorous pituitary cells have not been resolved.

The secretory granule is a likely focal point for hormonal regulation; not only is it a concentrating and storage vehicle for the pituitary hormones, but it provides the milieu for hormone processing and the final barrier to the extracellular fluid before fusion of granule and cell membranes. Regulation of these events through protein phosphorylation has been examined for secretory granules of chromaffin cells (Creutz *et al.*, 1983;

Summers & Creutz, 1985) and pancreatic islet cells (Brocklehurst & Hutton, 1984; Thams *et al.*, 1984), but little is known for anterior pituitary granules. Cyclic AMP-kinase is reported to be contained in pituitary dense-granule fractions (Lemaire *et al.*, 1971; Lewin *et al.*, 1983), but in an early study anterior pituitary granules were found to be phosphorylated by an endogenous protein kinase whose activity was independent of cyclic AMP (Labrie *et al.*, 1971).

In the present study we have isolated and characterized secretory granules from sheep anterior pituitary glands. Both protein kinase C and cyclic AMP-kinase are present in the granule fractions, and we provide evidence for secretory-granule-associated substrates for these enzymes.

EXPERIMENTAL

Preparation of subcellular fractions

Female sheep anterior pituitary glands were collected within 40 min of slaughter from heads transported on ice to the laboratory from the abattoir. Unless indicated otherwise, each experiment consisted of anterior pituitary glands (total weight approx. 1g) from two sheep. Tissue was minced and then homogenized at 4 °C in 10 vol. of homogenization buffer (0.25 M-sucrose, 10 mM-Tris/HCl, 0.1 mM-PMSF, 0.1 mM-Tos-Lys- CH_2Cl , pH 7.4). For preparation of secretory-granule fractions, all subsequent operations were at 4 °C; the homogenate was initially spun at 3000 g (45 s), yielding the P_1 pellet. The supernatant of P_1 was spun at 4000 g (15 min) to yield the

Abbreviation used: LH, luteinizing hormone (lutropin); PMSF, phenylmethanesulphonyl fluoride; Tos-Lys- CH_2Cl , tosyl-lysylchloromethane ('TLCK').

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P₂ pellet, and the supernatant of P₂ was centrifuged at 26000 *g* (15 min) to yield the crude granule fraction (P₃). The P₃ pellet was resuspended in homogenization buffer, and a sample was layered on top of a discontinuous sucrose density gradient (in 10 mM-Hepes, pH 7.0). The sucrose gradient (1.4 ml, 2 M; 0.7 ml, 1.4 M; 0.35 ml, 1.35 M; 0.35 ml, 1.30 M) was centrifuged at 150000 *g*_{av.} for 90 min (Beckman SW 60 rotor); serial 0.3 ml fractions were collected through the bottom of the gradient tube. The supernatant of P₃ was spun at 100000 *g*_{av.} (60 min) (Beckman Ty 65 rotor) to obtain cytosol (S₄).

Protein kinase assays

Protein kinase C assay. Before assay, the subcellular fractions were subjected to DEAE-cellulose chromatography. Sucrose-density-gradient fractions or cytosol, diluted with 10 mM-Tris/HCl (pH 7.4)/10 mM-2-mercaptoethanol/1 mM-EDTA, were applied to a 0.3 ml DE-52 DEAE-cellulose column previously equilibrated with homogenization buffer without PMSF. The column was eluted with 0.8 ml of 10 mM-Tris/HCl (pH 7.4)/15 mM-2-mercaptoethanol, followed by 1.2 ml and 2.0 ml of this buffer containing 65 mM- and 200 mM-NaCl respectively; 0.15 ml fractions were collected. Protein kinase C was assayed as described previously (Turgeon *et al.*, 1984). The reaction mixture contained 21.1 mM-Tris/HCl, pH 7.4, 5.3 mM-magnesium acetate, 190 µg of histone H1/ml, 21.3 µM-[γ-³²P]ATP (500–750 d.p.m./pmol), 52 µM-CaCl₂ or 520 µM-EGTA, with or without lipid (16.7 µg of phosphatidylserine/ml and 1.25 µg of dioleoin/ml). The reaction was initiated by addition of enzyme and was incubated for 15 min at 30 °C; termination was by precipitation on to ET31 paper and washing with 10% (v/v) trichloroacetic acid, followed by ethanol.

Cyclic AMP-kinase assay. Activity was determined by measuring the extent of ³²P incorporation into H1A histones by the procedure of Corbin & Reimann (1974), modified by the addition of 0.25% sodium tungstate to the trichloroacetic acid solutions. The reaction mixture contained 5.7 mM-MgCl₂, 31.5 mM-Mes, 8.6 mg of histones/ml, 140 µM-[γ-³²P]ATP (75–125 d.p.m./pmol), 0.6 mM-isobutylmethylxanthine, and either 0 or 1 µM-cyclic AMP. Reaction was started by the addition of fractions diluted in 10 mM-Mes (pH 6.8)/15 mM-2-mercaptoethanol; the reaction was terminated after 30 min at 30 °C by precipitation on to filter paper as described for the protein kinase C assay.

Protein kinase purifications

Protein kinase C was purified from rabbit cortical brain tissue (eight brains from New Zealand White rabbits obtained after sodium pentobarbital anaesthesia). The initial stages of purification by DEAE-cellulose DE-52 chromatography were based on the procedure of Kikkawa *et al.* (1983), modified by the inclusion of 0.1 mM-PMSF and 0.1 mM-Tos-Lys-CH₂Cl in the homogenization medium, and addition of 1 µM-cyclic AMP to the solution before DEAE-cellulose DE-52 chromatography and in the initial column wash buffer. Thereafter Sephacryl S200 chromatography was used; the column (5 cm × 80 cm) was previously equilibrated with 20 mM-Tris/HCl, pH 7.4, containing 2 mM-EDTA, 1 mM-EGTA, 50 mM-2-mercaptoethanol, 2 mM-benzamidine and 0.1 mM-PMSF. Protein kinase C purified in this

manner displayed a marked dependence on Ca²⁺ and lipid for activity (specific activity of 243, 26.4 and 6.5 nmol/min per mg measured with Ca²⁺+lipid, EGTA+lipid and Ca²⁺ alone respectively). Since the enzyme was free of cyclic AMP-kinase and did not appear to contain other proteins capable of undergoing phosphorylation, enzyme at this stage of purification was used extensively in the phosphorylation studies herein reported. Similar results were obtained with enzyme purified to near-homogeneity (specific activity with Ca²⁺+lipid of 2341 nmol/min per mg) by phosphatidylserine affinity chromatography as described by Uchida & Filburn (1984), although the enzyme at this stage of purification was considerably less stable. The protein kinase C purified from sheep cortical brain tissue (one brain/preparation) by affinity chromatography, and used where indicated, had a specific activity (nmol/min per mg) of 391, 271 and 75.9 measured with Ca²⁺+lipid, EGTA+lipid and Ca²⁺ respectively.

The catalytic subunit of cyclic AMP-kinase, prepared from bovine cardiac muscle as described previously (Beavo *et al.*, 1974), was generously given by Dr. Donal Walsh (University of California, Davis).

Phosphorylation of cell fractions

Phosphorylation studies were carried out at 30 °C in 20 mM-Tris/HCl, pH 7.5, containing 5 mM-magnesium acetate, 25 µM-[γ-³²P]ATP (300–400 d.p.m./pmol) and final concentrations of CaCl₂, EGTA, cyclic AMP, phosphatidylserine and dioleoin of 0.05 mM, 0.5 mM, 0.01 mM, 16 µg/ml and 1.2 µg/ml respectively (total volume 0.05 ml). Protein kinase C was diluted with 10 mM-Mes, pH 6.5, containing 1 mM-EGTA and 5 mM-dithiothreitol, and cyclic AMP-kinase (catalytic subunit) with 10 mM-Mes, pH 6.8, containing 0.5 mg of bovine serum albumin/ml, before addition to the phosphorylation assay as indicated in the text. Reaction was initiated by addition of [γ-³²P]ATP, and for one-dimensional SDS/polyacrylamide-gel electrophoresis terminated at the times indicated (2.5–10 min) by addition of SDS sample buffer (final concns. in assay of 2% glycerol, 1% 2-mercaptoethanol, 0.46% SDS, 12.5 mM-Tris/HCl, pH 6.8, and 6.7 mM-EDTA) and the samples were immediately placed in a boiling-water bath for 4 min. For two-dimensional SDS/polyacrylamide-gel electrophoresis, 20 µl of assay mixture was rapidly mixed with 80 µl of a solution containing 9.5 M-urea, 2% Ampholines (0.4%, pH 3–10; 1.6%, pH 5–7), 2% (w/v) Nonidet P40, 25 mM-dithiothreitol and 10 mM-EDTA and immediately frozen in liquid N₂ until processed further.

One-dimensional SDS/polyacrylamide-gel electrophoresis was performed on either 15% or 8–15% polyacrylamide gels (8 cm × 8 cm; 0.5 mm thickness) with a 3% -acrylamide stacking gel, by the procedure of Laemmli (1970). Two-dimensional SDS/polyacrylamide-gel electrophoresis was carried out as described by O'Farrell (1975); samples were resolved by isoelectric focusing (11 cm × 3 mm-diam. gels) and in the second dimension on a 10% -polyacrylamide separating gel. *M_r* calibration was accomplished with the following standards (*M_r* in parentheses): myosin (205000), β-galactosidase (116000), phosphorylase *b* (97400), bovine serum albumin (66000), egg albumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), PMSF-treated trypsinogen (24000), soya-bean

trypsin inhibitor (20 100) and α -lactalbumin (14000). pI calibration was determined by running an additional sample-loaded isoelectric-focusing gel in each experiment. Gels were subsequently sliced into 0.5 cm sections, and soaked for 4 h in 0.5 ml of deionized degassed water for determination of the pH. Gels were stained with Coomassie Brilliant Blue and dried under vacuum on filter paper. Autoradiography was performed with Kodak X-Omat XAR-5 film.

Other assays

Luteinizing hormone (LH) (secretory-granule marker) was measured by radioimmunoassay with an ovine: ovine system using anti-oLH, GDN No. 15, purified oLH, LER 1056-C2, and ovine reference standard NIH-LH-S23; homogenization buffer did not interfere with the LH assay. Protein was measured by the method of Bradford (1976), with bovine serum albumin as standard. The cytosolic marker enzymes lactate dehydrogenase and phosphoglucosmutase were measured spectrophotometrically as described by Bergmeyer *et al.* (1963) and Bergmeyer & Klotzsch (1963) respectively. 5'-Nucleotidase activity (plasma-membrane marker) was measured spectrophotometrically by determination of adenosine released from AMP by the procedure of Ipata (1967). β -Glucuronidase (lysosomal marker) was assayed by the method of Russell (1981), with 4-methylumbelliferyl β -D-glucuronide as substrate, by measuring the liberation of the fluorescent compound 4-methylumbelliferone. Sucrose density was determined in a refractometer.

Electron microscopy

Fractions were dialysed against 10 mM-Tris/HCl, pH 7.4, at 4 °C for 3 h and spun at 15000 g (5 min) to remove sucrose; the pellet was washed once in 10 mM-Tris/HCl, pH 7.4, and then fixed in glutaraldehyde buffered with cacodylate and processed for electron microscopy.

RESULTS

Characterization of secretory-granule fraction

The anterior pituitary gland is composed of five or six different types of secretory cells with hormone-containing secretory granules which sediment at $d = 1.24$ – 1.19 on sucrose density gradients (Nansel *et al.*, 1979; Peng Loh

& Gainer, 1982; Lewin *et al.*, 1983). We chose one of these hormones, LH, as a marker for secretory granules. Compared with the homogenate, there is about a 2-fold enrichment of LH in the P_3 fraction; when this fraction is applied to a discontinuous sucrose density gradient, the LH enrichment in the peak fraction recovered at $d = 1.21$ – 1.22 (fraction 5) is increased to 5-fold (Table 1). This fraction, though containing most of the LH granules, is not homogeneous relative to the other hormones; there is overlap with prolactin and growth hormone, which are contained in the more-dense granules, and with thyroid-stimulating hormone and corticotropin, which are contained in the less-dense granules (Nansel *et al.*, 1979). Although LH was the hormone that we chose to measure quantitatively as a marker for secretory granules, the distribution of another secretory-granule hormone, prolactin, was determined qualitatively by its appearance after Coomassie Blue staining of polyacrylamide gels for each of the sucrose gradient fractions and verified by electrophoresis of authentic prolactin. As expected from other studies, this hormone was associated with the more-dense-granule fraction (predominantly fraction 4), but was also identified in fractions 5 and 6 from the sucrose gradient. In fractions 7–12, there was a marked diminution in Coomassie Blue staining of the band corresponding to prolactin, except for a modest increase in fraction 9.

A representative separation with sucrose density gradient centrifugation is shown in Fig. 1. There are two major protein peaks: fractions 5 and 9. Fraction 5 contains about 60% of the LH on the gradient and is composed of secretory granules, as shown by electron microscopy. In contrast, fraction 9 contains a mixture of membranes, lysosomes, mitochondria and secretory granules, as shown by electron microscopy. The purity of the secretory-granule preparation (fraction 5) was established by measurement of lactate dehydrogenase and phosphoglucosmutase for cytosolic markers, and 5'-nucleotidase and β -glucuronidase activities as markers for plasma membranes and lysosomes respectively. For lactate dehydrogenase activity in cytosol (S_4), the mean determination for three separate experiments was 886 ± 30 nmol/min per mg of protein; there was no detectable lactate dehydrogenase activity in the secretory-granule fractions of any of these preparations. Phosphoglucosmutase activity in cytosol was 1230 nmol/min

Table 1. Distribution of luteinizing hormone, 5'-nucleotidase and β -glucuronidase after subcellular fractionation

Abbreviation: n.d., not detectable. '% of total' is calculated on basis of activities in $P_1 + P_2 + P_3 + S_4$.

Fraction	Luteinizing hormone (μ g/mg of protein)*	5'-Nucleotidase		β -Glucuronidase	
		(nmol/min per mg)†	(% of total)	(pmol/min per mg)†	(% of total)
P_1	11.6 ± 1.9	10.9 ± 3.3	17 ± 4	243 ± 50	10 ± 1
P_2	12.7 ± 1.6	8.9 ± 2.5	29 ± 1	349 ± 44	31 ± 1
P_3	28.4 ± 6.4	20.6 ± 5.1	54 ± 3	444 ± 7	34 ± 2
P_3 {	Fraction 5	55.0 ± 6.4	3 ± 1	214 ± 58	5 ± 1
	Fraction 9	15.2 ± 2.4	39 ± 2	535 ± 152	4 ± 1
S_4	7.3 ± 0.8	n.d.	—	326 ± 50	25 ± 3

* $n = 13$ separate preparations.

† $n = 3$ separate preparations.

‡ P_3 : fractions 5 and 9 refer to those fractions obtained after purification of P_3 by sucrose-density-gradient fractionation.

Fig. 1. Sucrose-density-gradient fractionation of P_3 from sheep anterior-pituitary extract

The distribution of LH (Δ), total protein ($\square$), β -glucuronidase (∇) and 5'-nucleotidase ($\blacklozenge$) after gradient centrifugation is represented for each as a percentage of the total applied to the gradient. The sucrose density of each fraction is also shown ($\circ$). Fraction 1 indicates bottom of the gradient. Electron photomicrographs (magnification $\times 22500$) of fractions 5 and 9 are shown above.

per mg, but was not detectable in the secretory-granule fraction in the same experiment. For 5'-nucleotidase (plasma-membrane marker), 54% of total activity was obtained in P_3 (crude granule fraction); when this fraction was subjected to sucrose-density-gradient centrifugation, most of this activity was recovered in fraction 9 (Fig. 1). As shown in Table 1, the specific activity of 5'-nucleotidase was approx. 27-fold higher (as determined in three separate experiments) in fraction 9 than in the secretory-granule fraction (fraction 5). Table 1 shows that approx. 5% of β -glucuronidase activity in pituitary tissue was recovered in fraction 5, although this represented the lowest specific activity measured in any of the subcellular fractions. In summary, these data indicate that the secretory-granule fraction is free of cytosol, although there are small amounts of plasma membranes (2–4%) and lysosomes (4–6%) associated with the preparation, based on enzyme recovery measurements.

Protein kinase C activity

As we and others have shown, protein kinase C is present in anterior-pituitary cytosol, and we next

explored the possibility that protein kinase C may be associated with secretory granules. For these experiments, a sample of fraction 5 from the sucrose density gradient was chromatographed on DE-52 DEAE-cellulose, and protein kinase C activity was determined for each fraction in a histone-phosphorylation assay; DEAE-cellulose chromatography was necessary to demonstrate the requirement of the enzyme for Ca^{2+} and lipid, as reported for the membrane-associated enzyme in other tissues (Kikkawa *et al.*, 1983). As shown in Fig. 2, the enzyme is eluted similarly whether the starting material is a cytosolic or a secretory-granule fraction. In Table 2, data are summarized showing that protein kinase activity with a marked dependence on both Ca^{2+} and lipid is associated with anterior-pituitary secretory granules and that the Ca^{2+} /lipid-dependency is similar for the cytosolic and the secretory-granule enzyme (approx. 3–5-fold stimulation of activity measured with Ca^{2+} + lipid in each case). It is unlikely that the secretory-granule protein kinase C activity can be accounted for by cytosolic contamination, because no activity of the marker enzymes, lactate dehydrogenase and phosphoglucosmutase, could be detected in the granule fraction. In one

Fig. 2. Ca^{2+} /phospholipid-dependent protein kinase activity in sheep anterior-pituitary secretory granules

After sucrose-density-gradient centrifugation, a sample of fraction 5 (secretory granules; 2.4 mg of protein) was applied to a DE-52 DEAE-cellulose column and eluted with a stepped gradient of NaCl (0–200 mM) in 10 mM-Tris/HCl (pH 7.4)/15 mM-2-mercaptoethanol. The eluted fractions were assayed for protein kinase C activity by determining incorporation of ^{32}P into histone H1 in the presence of Ca^{2+} plus lipid (phosphatidylserine and diolein) ($\square$), EGTA plus lipid ($\circ$), or Ca^{2+} alone ($\triangle$). Protein ($\bullet$) and conductivity ($\blacklozenge$) for each fraction were also determined. The inset (same axes as in main Figure) shows the elution pattern and protein kinase C activity for cytosol (S_4) from the same preparation. Conditions were identical for cytosol (2.5 mg applied to column) and secretory-granule chromatography.

experiment, a comparison of protein kinase C activity in the secretory-granule fraction and in the plasma-membrane-rich fraction 9 was made; after elution from DEAE-cellulose DE-52, the activity of protein kinase C in the secretory-granule fraction was 15-fold greater than could be accounted for by the 4% contamination (as

measured in this experiment) of the fraction with plasma membrane.

Phosphorylation in secretory-granule fraction

Incubation of anterior-pituitary secretory granules with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ resulted in incorporation of label into proteins (Fig. 3). The presence of Ca^{2+} plus lipid markedly stimulated incorporation of ^{32}P into a protein band, with an estimated M_r value of 36000 (lane 4). Addition of exogenous protein kinase C in the presence of lipid and Ca^{2+} further enhanced the phosphorylation of this protein band (lane 5). Only very slight phosphorylation of this M_r -36000 protein was seen when either Ca^{2+} or lipid was omitted. When protein kinase C was run without the secretory granules but in the presence of lipid and Ca^{2+} , no phosphorylated proteins were discernible (lane 1). Phosphorylation of the M_r -36000 protein was observed in all five secretory-granule preparations used in the course of this study (mean M_r value of 36283 ± 556 for six gel determinations on the five preparations).

Co-incubation of cyclic AMP plus EGTA and secretory granules resulted in phosphorylation of several proteins as seen on the autoradiogram, suggesting the presence of cyclic AMP-kinase (lane 8, Fig. 3). When

Table 2. Comparison of protein kinase C activity in secretory granules and cytosol

Results are means $\pm$ S.E.M. for three separate preparations, and are derived from a summation of activity present in the peak fractions from DEAE-cellulose chromatography. Data are expressed per mg of secretory-granule- or cytosol-associated protein applied to the column.

Assay conditions	Protein kinase C activity (pmol of ^{32}P incorporated into histone/min per mg of protein)	
	Secretory granules	Cytosol
Ca^{2+} plus lipid	100 $\pm$ 4	241
EGTA plus lipid	38 $\pm$ 3	85
Ca^{2+} alone	25 $\pm$ 1	51

Fig. 3. Autoradiogram of phosphorylation of endogenous proteins associated with sheep anterior-pituitary secretory granules

Phosphorylation of secretory granules (fraction 5 obtained by sucrose-density-gradient centrifugation) was carried out for 10 min at a concentration of 0.4 mg/ml in the incubation mixture, and the ^{32}P -labelled proteins were resolved by electrophoresis on a 15% polyacrylamide gel at 4.2 μg of protein/lane. The lanes designated on the autoradiogram correspond to the following incubation conditions: (1) protein kinase C (PK-C) + Ca^{2+} + lipid (phosphatidylserine and diolein); (2) fraction 5 + Ca^{2+} ; (3) fraction 5 + PK-C + Ca^{2+} ; (4) fraction 5 + Ca^{2+} + lipid; (5) fraction 5 + PK-C + Ca^{2+} + lipid; (6) fraction 5 + EGTA + lipid; (7) fraction 5 + PK-C + EGTA + lipid; (8) fraction 5 + EGTA + cyclic AMP; (9) fraction 5 + EGTA + cyclic AMP-kinase catalytic subunit; (10) cyclic AMP-kinase catalytic subunit. PK-C, where added, was from sheep brain, purified by phosphatidylserine affinity chromatography. The activities of cyclic AMP-kinase and protein kinase C (measured with Ca^{2+} + lipid) added to the incubation mixture were 24.8 and 0.418 pmol/min respectively.

endogenous cyclic AMP-kinase activity in the granule fraction was measured by histone phosphorylation assay, the mean total activity in the presence of cyclic AMP in three experiments was 401 ± 8 pmol/min per mg of secretory-granule protein (basal activity 124 ± 13 pmol/min per mg). As shown in the autoradiogram in Fig. 3, if the granule fraction was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of exogenous catalytic subunit of cyclic AMP-kinase plus EGTA, the incorporation of label into the bands identified with cyclic AMP was markedly enhanced (cf. lanes 8 and 9). The cyclic AMP-kinase system increased the phosphorylation of M_r -63000, -28000, -23000, -21000 and -18000 proteins in the secretory-granule fractions. When the phosphorylation incubation experiment was repeated for fractions 3–12 from a sucrose density gradient, incorporation of ^{32}P into proteins of these M_r values was found predominantly in fractions 4–6 (results not shown). The most prominent phosphorylation stimulated by the exogenous catalytic subunit appeared to be associated with the M_r -63000, -23000 and -21000 proteins as determined in five preparations of secretory granules. When purified catalytic subunit of cyclic AMP-kinase was incubated in the absence of granule fraction, no phosphorylated

proteins were evident (lane 10, Fig. 3). We found no evidence for a M_r -36000 substrate for cyclic AMP-kinase in secretory granules similar to that seen with protein kinase C.

Association of M_r -36000 protein specific to secretory granules

To establish the granule association of the M_r -36000 protein distinct from cytosol, we carried out phosphorylation incubation studies with cytosolic fractions. Autoradiography of cytosol after incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ revealed proteins with M_r values of 22000, 30000, 32000, 50000 and approx. 200000, which were phosphorylated on addition of protein kinase C in the presence of Ca^{2+} and lipid (Fig. 4, lane 4). For comparison, shown in lane 2 is the autoradiogram of the secretory-granule fraction from the same preparation and for which the same total amount of protein per lane (4.2 μg) was used. Addition of protein kinase markedly enhanced phosphorylation of

Fig. 4. Autoradiogram of protein kinase C-stimulated phosphorylation of endogenous proteins associated with secretory granules, compared with cytosol

Phosphorylation incubations with protein kinase C and secretory granules (P_35 ; fraction 5 from sucrose-density-gradient centrifugation) or cytosol (S_4) from the same preparation were carried out for 4 min at a concentration of 0.4 mg of granule or cytosolic protein/ml in the incubation mixture. The ^{32}P -labelled proteins were resolved by electrophoresis on a gradient (8–15%) polyacrylamide gel at 4.2 μg of protein/lane. The amount of protein kinase C (PK-C; Sephacryl-purified from rabbit brain) used in each phosphorylation assay had an activity (measured with Ca^{2+} + lipid) of 180 pmol/min.

Fig. 5. Autoradiogram showing distribution across a sucrose density gradient of proteins phosphorylated by protein kinase C

Fractions 3–12 from sucrose-density-gradient centrifugation of P_3 were each incubated for 2.5 min in the presence of [γ - 32 P]ATP and protein kinase C + Ca^{2+} + lipid. An equal volume from each fraction was added to the incubation and subsequently loaded on to a 15% polyacrylamide gel. As reference: for fraction 5, the incubation mixture contained 1.0 mg of protein/ml, and 6 μ g of protein was subjected to electrophoresis. The numbers above the lanes correspond to the fractions obtained from the sucrose density gradient. The amount of protein kinase C (Sephacryl-purified from rabbit brain) used in each phosphorylation assay had an activity (measured with Ca^{2+} + lipid) of 180 pmol/min.

a 36000- M_r protein and also a 22000- M_r band (lanes without added kinase not shown). The protein-kinase-C-dependent phosphorylation of the M_r -36000 protein was not observed in cytosol (Fig. 4). When protein kinase C was incubated without pituitary extract but in the presence of lipid and Ca^{2+} , there was stimulation of incorporation of 32 P into a M_r -78000–80000 protein, probably representing autophosphorylation (lane 1).

To determine whether the M_r -36000 substrate for protein kinase C was specifically associated with the secretory-granule fraction, we carried out phosphorylation incubations on fractions 3–12 from a sucrose-density-gradient separation of P_3 . For this experiment, an equal volume of sample from each sucrose-density-gradient fraction was added to the phosphorylation assay and subsequently loaded on to the gel. Incubation of each fraction with exogenous protein kinase C in the presence of Ca^{2+} plus lipid stimulated the phosphorylation of a M_r -36000 protein, which was most pronounced in fraction 5 (Fig. 5, lane 5); this fraction corresponds to the secretory-granule fraction used in the experiments described above. Moving across the gradient, from fraction 6 onward there is a gradual diminution in this protein kinase C substrate (Fig. 5), and the substantially lower amounts associated with fraction 9 appear to preclude any association of this protein with lysosomes. In contrast with the M_r -36000 protein, another protein

Fig. 6. Comparison, by two-dimensional SDS/polyacrylamide-gel electrophoresis, of phosphorylation of secretory-granule proteins catalysed by protein kinase C and cyclic AMP-kinase

Phosphorylation of secretory granules (fraction 5 obtained by sucrose-density-gradient centrifugation) was carried out for 7.5 min at a concentration of 0.5 mg/ml in the incubation mixture, and samples (75 μ l, containing 22.5 μ g of protein) were resolved by two-dimensional SDS/polyacrylamide-gel electrophoresis. The autoradiograms shown correspond to incubation of fraction 5 under the following conditions: (a) protein kinase C + Ca^{2+} + lipid; (b) protein kinase C + EGTA + lipid; (c) cyclic AMP-kinase catalytic subunit + EGTA. The activities of cyclic AMP-kinase and Sephadex-purified protein kinase C from rabbit brain (measured with Ca^{2+} + lipid) added to the incubation mixture were 133 and 180 pmol/min respectively.

kinase C substrate, of M_r 22000, appears to be evenly distributed across the gradient; this may be indicative of a relatively non-specific association with fraction P_3 . The distribution of the M_r -22000 protein could account for the variable amounts of this protein found to be associated with different secretory-granule preparations (Figs. 3 and 4). Other proteins whose phosphorylation is stimulated by the addition of exogenous protein kinase C have estimated M_r values of 16000 and 12000 and appear to be preferentially associated with the more-dense fractions from the sucrose gradient, and a M_r -50000 protein is associated with the less-dense upper end of the sucrose gradient (Fig. 5). When Ca^{2+} was replaced with EGTA in the incubation for the phosphorylation assay, there was only minimal incorporation of ^{32}P into these proteins (results not shown). Thus the distribution of the M_r -36000 substrate for protein kinase C corresponds to that found for secretory granules as determined with LH as a secretory-granule marker.

Two-dimensional gel electrophoresis of ^{32}P -labelled secretory granules

Figs. 6(a) and 6(b) depict autoradiographs obtained after two-dimensional gel electrophoresis of secretory granules incubated with $[\gamma\text{-}^{32}P]\text{ATP}$ and exogenous protein kinase C under conditions where protein kinase C is maximally active (a; Ca^{2+} +lipid) or exhibits low activity (b; EGTA+lipid). The protein of M_r 36000, previously shown to be a substrate for phosphorylation by protein kinase C (Figs. 3 and 4), is clearly resolved as a train of up to at least six spots (Fig. 6a), with pI values in the range 5.7–7.0. Phosphorylation of particular proteins has previously been shown to produce a charge shift to a more acidic form, generally in the region of 0.15–0.20 pH unit (Garrison & Wagner, 1982), and thus the pattern of behaviour observed in Fig. 6(a) would be consistent with phosphorylation occurring at multiple sites on the molecule. No protein corresponding to the train of ^{32}P -containing spots was evident by Coomassie Blue staining of the gel. In addition to the M_r -36000 protein, which exhibits multiple ^{32}P -containing spots, a protein of M_r 22000, appearing essentially as a single spot with some trailing (pI approx. 4.4), was also a substrate for protein kinase C in the secretory-granule fraction (Fig. 6a).

Fig. 6(c) shows the effect of phosphorylation of the granule fraction by the catalytic subunit of cyclic AMP-kinase in the presence of EGTA. It is clear from these data that neither of the protein kinase C substrates was phosphorylated under these conditions, although three other proteins (M_r 63000, 23000 and 21000) exhibited marked increases in ^{32}P labelling, and smaller increases were noted in several other proteins.

DISCUSSION

The intracellular location and possible functional compartmentalization of protein kinase C has been investigated for several tissues. For example, in brain Kikkawa *et al.* (1982) reported that the enzymes purified from cytosol or from membranes were indistinguishable from each other in their physical, kinetic and catalytic properties; approximately half of the total protein kinase C activity was recovered in the soluble fraction and half in the particulate fraction. In studies of redistribution of the enzyme after stimulation of intact cells, subsequent

cell fractionations have been limited to generating cytosol and particulate fractions; the specific membrane compartments have not been determined. In cells exposed to phorbol esters, a rapid increase in protein kinase C associated with the particulate fraction and a decrease in cytosolic enzyme activity has been reported for parietal yolk-sac cells (Kraft & Anderson, 1983) and GH_3 pituitary cells (Drust & Martin, 1985). Stimulation of pituitary cells with LH-releasing hormone (luliberin, the physiological secretagogue for LH) has been shown to result in a translocation of cytosolic protein kinase C to a particulate-associated form (Hirota *et al.*, 1985). Specific association with subcellular fractions after isolation has been tested, and there is some evidence that protein kinase C binds to insulin granules (Brocklehurst & Hutton, 1984) and to chromaffin-granule membranes (Summers & Creutz, 1985).

In the current study, we provide evidence for endogenous protein kinase C association with secretory granules isolated from sheep anterior-pituitary glands. We were not testing for factors which promote redistribution of the enzyme or which influence association with subcellular compartments, but the absence of a Ca^{2+} chelator during homogenization in the present study may have resulted in increased membrane association of protein kinase C. There is variable evidence regarding the role of Ca^{2+} in promoting such an association. For example, Melloni *et al.* (1985) suggest that Ca^{2+} added during homogenization of neutrophils and platelets influences the distribution of protein kinase C to a particulate fraction, and Wolf *et al.* (1985) report a Ca^{2+} -concentration-dependent binding of the enzyme to inside-out erythrocyte vesicles; however, Drust & Martin (1985) indicate that, for intact GH_3 pituitary cells, an increase in cytosolic Ca^{2+} plays, at most, a minor role in promoting enzyme redistribution. Whichever is the case, the purpose of the current studies was not to establish the absolute activity of the granule-associated enzyme but to determine if protein kinase C activity could be detected specific to the secretory-granule fraction, and this we were able to show. The potential physiological significance of such an association remains to be ascertained.

Understanding the role of protein kinase C in signal transduction requires, as an initial step, identification of its substrates for phosphorylation. Several specific proteins have been identified, such as the M_r -40000 substrate in platelets (Nishizuka, 1984), vinculin (Werth *et al.*, 1983), myosin light chain (Naka *et al.*, 1983), epidermal-growth-factor receptor (Cochet *et al.*, 1984) and erythrocyte glucose-transporter protein (Witters *et al.*, 1985). Of particular relevance to the present study, protein kinase C has been reported to phosphorylate a M_r -29000 insulin-secretory-granule membrane protein (Brocklehurst & Hutton, 1984) and a M_r -37000 chromaffin-granule membrane protein (Summers & Creutz, 1985). The latter protein is of special interest; in addition to similarity in M_r to the substrate that we have identified in anterior pituitary secretory granules, the M_r -37000 protein in chromaffin-granule membranes appears to be phosphorylated at multiple sites by protein kinase C, as does the M_r -36000 granule-associated protein from pituitary tissue, and both proteins have similar isoelectric points. The functional role of these granule-associated proteins has yet to be established. The possibility that the protein identified as binding to

chromaffin-granule membranes is related to the light chains of clathrin, which have M_r values varying from 30000 to 40000 depending on species, was shown not to be likely by Creutz & Harrison (1984). Another possible correlation is with the calelectrins (M_r 32500, 35000 and 67000), which bind to membrane fractions in adrenal medulla, liver and brain and which cause isolated chromaffin granules to aggregate in a Ca^{2+} -dependent manner (Südhof *et al.*, 1984).

To define a possible role in the regulation of secretory granules of anterior pituitary cells, we must establish if the 36000- M_r granule-associated protein is capable of undergoing phosphorylation in intact cells. Also at issue will be whether the substrate is a common component of the exocytotic process or whether it is specific to a particular hormone cell type in the adenohypophysis. In the current study, we used the hormone LH as a marker for secretory-granule enrichment, but the aim of the sucrose-gradient fractionation procedure was to produce a relatively pure secretory-granule fraction, not to discriminate for each granule type.

The recovery of cyclic AMP-kinase activity in the secretory-granule fraction has been reported previously for bovine (Lemaire *et al.*, 1971) and pig (Lewin *et al.*, 1983) anterior-pituitary cells. We now confirm this observation for the sheep adenohypophysis and show that the substrates for phosphorylation by cyclic AMP-kinase and by protein kinase C in the secretory granules appear to be distinct from each other. A role for cyclic AMP-kinase in the mechanism of hypothalamic hormone action in anterior pituitary cells has been the subject of many studies for each hormone; the results are as diverse as the secretory cells of the adenohypophysis. For the LH-secreting cell, cyclic AMP does not mediate the immediate exocytotic response to luliberin, but cyclic AMP does mimic the self-priming action of luliberin whereby the LH-secretory response is augmented by prior exposure to luliberin (Turgeon & Waring, 1986). This is independent of luliberin-receptor changes and appears to involve secretory-granule recruitment, thus making our observation of cyclic AMP-kinase specific substrates associated with secretory granules of special interest for future investigations of luliberin action.

In summary, we show that protein kinase C activity can be detected specific to the secretory-granule fraction of sheep anterior pituitary glands and that protein kinase C stimulates phosphorylation of a M_r -36000 protein associated with these pituitary granules. This protein was not a substrate for cyclic AMP-kinase, although the enzyme stimulated the phosphorylation of other secretory-granule proteins. Thus protein phosphorylation, which is a suggested regulator of granule function for other secretory cells, may participate in the regulation of secretory granules in some anterior pituitary cell types.

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REFERENCES

- Beavo, J., Bechtel, P. & Krebs, E. (1974) *Methods Enzymol.* **38**, 299–308
- Bergmeyer, H.-U. & Klotzsch, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 131–133, Academic Press, New York and London
- Bergmeyer, H.-U., Bernt, E. & Hess, B. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 736–741, Academic Press, New York and London
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Brocklehurst, K. & Hutton, J. (1984) *Biochem. J.* **220**, 283–290
- Cochet, C., Gill, G., Meisenhelder, J., Cooper, J. & Hunter, T. (1984) *J. Biol. Chem.* **259**, 2553–2558
- Corbin, J. & Reimann, E. (1974) *Methods Enzymol.* **38**, 287–290
- Creutz, C. & Harrison, J. (1984) *Nature (London)* **308**, 208–210
- Creutz, C., Dowling, L., Sando, J., Villar-Palasi, C., Whipple, J. & Zaks, W. (1983) *J. Biol. Chem.* **258**, 14664–14674
- Drust, D. & Martin, T. (1985) *Biochem. Biophys. Res. Commun.* **128**, 531–537
- Garrison, J. & Wagner, J. (1982) *J. Biol. Chem.* **257**, 13135–13143
- Hatada, Y., Munemura, M., Fukunaga, K., Yamamoto, H., Maeyama, M. & Miyamoto, E. (1983) *J. Neurochem.* **40**, 1082–1089
- Hirota, K., Hirota, T., Aguilera, G. & Catt, K. (1985) *J. Biol. Chem.* **260**, 3243–3246
- Ipata, P. (1967) *Anal. Biochem.* **20**, 30–36
- Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 13341–13348
- Kikkawa, U., Minakuchi, R., Takai, Y. & Nishizuka, Y. (1983) *Methods Enzymol.* **99**, 288–298
- Kraft, A. & Anderson, W. (1983) *Nature (London)* **301**, 621–623
- Labrie, F., Lemaire, S., Poirier, G., Pelletier, G. & Boucher, R. (1971) *J. Biol. Chem.* **246**, 7311–7317
- Laemmli, U. (1970) *Nature (London)* **227**, 680–685
- Lemaire, S., Pelletier, G. & Labrie, F. (1971) *J. Biol. Chem.* **246**, 7303–7310
- Lewin, M., Reyl-Desmars, F. & Ling, N. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6538–6541
- Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. & B. Horecker (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6435–6439
- Naka, M., Nishikawa, M., Adelstein, R. & Hidaka, H. (1983) *Nature (London)* **306**, 490–492
- Nansel, D., Gudelsky, G. & Porter, J. (1979) *Endocrinology (Baltimore)* **105**, 1073–1077
- Nishizuka, Y. (1984) *Science* **225**, 1365–1370
- O'Farrell, P. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Peng Loh, Y. & Gainer, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 108–112
- Russell, J. (1981) *Anal. Biochem.* **113**, 229–238
- Südhof, T., Ebbecke, M., Walker, J., Fritsche, U. & Boustead, C. (1984) *Biochemistry* **23**, 1103–1109
- Summers, T. & Creutz, C. (1985) *J. Biol. Chem.* **260**, 2437–2443
- Thams, P., Capito, K. & Hedekov, C. (1984) *Biochem. J.* **221**, 247–253
- Turgeon, J. & Waring, D. (1986) *Am. J. Physiol.* **250**, E62–E68
- Turgeon, J., Ashcroft, S., Waring, D., Milewski, M. & Walsh, D. (1984) *Mol. Cell. Endocrinol.* **34**, 107–112
- Uchida, T., & Filburn, C. (1984) *J. Biol. Chem.* **259**, 12311–12314
- Werth, D., Nidel, J. & Pastan, I. (1983) *J. Biol. Chem.* **258**, 11423–11426
- Witters, L., Vater, C. & Lienhard, G. (1985) *Nature (London)* **315**, 777–778
- Wolf, M., LeVine, H., May, W., Cuatrecasas, P. & Sahyoun, N. (1985) *Nature (London)* **317**, 546–549