Involvement of protein kinase C in the phosphorylation of an insulin-granule membrane protein

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1. The involvement of protein kinase C in the Ca^{2+} -dependent phosphorylation of a 29000- M_r insulin-granule membrane protein prepared from a rat insulinoma was investigated. 2. Protein kinase C activity towards exogenous lysine-rich histone was detected in a cytosolic fraction prepared from an insulinoma homogenate in the presence of EGTA. This activity bound reversibly to insulin granules in a Ca^{2+} -dependent manner. 3. Phosphatidylserine liposomes removed both protein kinase C activity and the 29000- M_r protein-phosphorylating activity from the cytosolic fraction in a Ca^{2+} -dependent fashion. 4. Protein kinase C activity and the enzymic activity responsible for the phosphorylation of the 29000- M_r granule protein behaved identically on sucrose-density-gradient centrifugation, ion-exchange chromatography, $(\text{NH}_4)_2\text{SO}_4$ fractionation and gel filtration of the cytosolic fraction. 5. These results are consistent with protein kinase C being the enzyme responsible for the phosphorylation of the 29000- M_r insulin-granule membrane protein.

Given the apparent importance of Ca^{2+} as a second messenger in the pancreatic β -cell (see Wollheim & Sharp, 1981) and the key regulatory role of protein phosphorylation in many cellular processes (see Rosen & Krebs, 1981), the involvement of Ca^{2+} -dependent protein phosphorylation in the stimulus–secretion coupling mechanism of insulin release has been investigated. Using subcellular fractions isolated from a transplantable rat insulinoma, we have previously shown that the incubation of insulin granules with a cytosolic fraction in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ resulted in the Ca^{2+} -dependent phosphorylation of 100000-, 29000- and 10000- M_r proteins (Brocklehurst & Hutton, 1983). The presence of both subcellular fractions was required for these Ca^{2+} -dependent phosphorylations, and, although they were inhibited by the calmodulin antagonist trifluoperazine, they were still apparent when a calmodulin-depleted soluble-protein fraction was substituted for a normal cytosolic fraction. Attention was focused on the 29000- M_r protein, which appeared to be a granule-membrane substrate for a cytosolic protein kinase. Since trifluoperazine has been

shown to inhibit a Ca^{2+} -activated phospholipid-dependent protein kinase, termed protein kinase C (Schatzman *et al.*, 1981), the possible involvement of this enzyme in the phosphorylation of the 29000- M_r protein was investigated.

Experimental

Preparation of subcellular fractions

The rat insulinoma was induced by X-irradiation (Chick *et al.*, 1977) and was propagated by subcutaneous implantation in an inbred strain of rats (Hutton *et al.*, 1981).

A tumour homogenate was prepared in a solution containing 10 mM-Mes, 0.27 M-sucrose and 1 mM-EGTA adjusted to pH 6.5 with Tris. Insulin granules and a soluble-protein fraction were prepared from the homogenate as previously described (Hutton *et al.*, 1982; Brocklehurst & Hutton, 1983). A high-speed soluble-protein fraction was prepared by further centrifugation of the soluble-protein fraction at 230000g for 30 min at 4°C in a Beckman L5-65 ultracentrifuge (SW 50.1 rotor).

Protein kinase C assay

Protein kinase C was assayed by a modified version of the method of Minakuchi *et al.* (1981).

Abbreviation used: Mes, 4-morpholine-ethanesulphonic acid.

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Protein samples were incubated at 30°C for 1 min in 50 μ l of a solution containing 50 mM-Mes, 1 mM-MgCl₂, 0.1 mM-dithiothreitol, 5 mM-EGTA and 0.1 mM-[γ -³²P]ATP {0.5–2.0 Ci/mmol; prepared from carrier-free [³²P]P_i (Amersham International, Amersham, Bucks., U.K.) by the method of Glynn & Chappell (1964)} adjusted to pH 6.9 with Tris. Lysine-rich histone [calf thymus type III-S; Sigma (London) Chemical Co., Poole, Dorset, U.K.] at a final protein concentration of 0.3 mg/ml was used as the exogenous substrate. Other additions included 5 mM-CaCl₂ [resulting in a free Ca²⁺ concentration estimated at 15 μ M (Storer & Cornish-Bowden, 1976)] and 80 μ g of phosphatidylserine (bovine brain; Sigma)/ml. Phosphatidylserine was dissolved in chloroform, and, after evaporation of the solvent, was dispersed by sonication for 30 s at ambient temperature with a MSE sonifier in a solution containing 10 mM-Mes adjusted to pH 6.5 with Tris before addition to the assay.

The reaction was terminated by the addition of 30 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 0.02% Bromophenol Blue, and heating at 100°C for 5 min. Samples were then subjected to electrophoresis on polyacrylamide slab gels and autoradiography as described previously (Brocklehurst & Hutton, 1983). The extent of histone phosphorylation was quantified by either densitometric scanning of the histone bands on autoradiograms or by cutting out the dried region of the gel containing the histone bands. These gel fragments were then added to 5 ml of water in a scintillation vial and the radioactivity present was determined by Cerenkov counting in a Packard Tri-Carb liquid-scintillation counter.

Insulin-granule phosphorylation assay

Protein samples were incubated with insulin granules (approx. 100 μ g of protein) in the phosphorylation assay described previously (Brocklehurst & Hutton, 1983) and the extent of phosphorylation of the 29000-*M_r* granule protein was determined by densitometric scanning of autoradiograms of electrophoretograms.

Binding of protein kinase C activity to insulin granules

Freshly prepared insulin granules (approx. 100 μ g of protein) were preincubated with the soluble-protein fraction (approx. 25 μ g of protein) at 30°C for 1 min in 50 μ l of a solution containing 0.1 M-Mes, 1 mM-MgCl₂, 0.25 M-sucrose, 0.1 mM-dithiothreitol, 1.2 mM-EGTA (taking into account the EGTA present in the soluble-protein fraction) and 1.3 mM-CaCl₂, adjusted to pH 6.5 with Tris. After centrifugation for 2 min in an Eppendorf Microfuge, the supernatants were removed and

either kept at 4°C or stored at –20°C before assay, and the pellets were resuspended in 50 μ l of preincubation medium, now with a final EGTA concentration of 1 mM, in either the absence or the presence of 1.1 mM-CaCl₂. Granules were again isolated by centrifugation and the supernatants removed for assay. A control experiment where both the original preincubation of insulin granules with the soluble protein fraction and the subsequent resuspension of the granule pellet after centrifugation were in the absence of CaCl₂ was also performed. Supernatants were assayed for protein kinase C activity as described in the legend to Table 2.

Preincubation of the soluble-protein fraction with phosphatidylserine liposomes

Phosphatidylserine liposomes were prepared by a modified version of the method of Gregoriadis (1976). A 0.15 ml portion of a 10 mg/ml solution of phosphatidylserine in chloroform was placed in a 10 ml glass conical flask, and the solvent evaporated by gentle swirling until a thin lipid layer was left on the walls of the flask. The lipid film was dispersed in 1.8 ml of a solution containing 50 mM-Mes, 1 mM-MgCl₂, 0.1 mM-dithiothreitol and 0.5 mM-EGTA adjusted to pH 6.5 with Tris, and the resulting suspension left on ice for 60 min.

A high-speed soluble-protein fraction (approx. 3 mg of protein) was incubated with phosphatidylserine liposomes (0.48 mg of phosphatidylserine) at 30°C for 5 min in a final volume of 6 ml of a solution containing 50 mM-Mes, 1 mM-MgCl₂, 0.1 mM-dithiothreitol and 0.7 mM-EGTA (taking into account the EGTA present in the soluble-protein fraction), adjusted to pH 6.5 with Tris, and in either the absence or the presence of 0.8 mM-CaCl₂. Control preincubations in the absence of phosphatidylserine were also performed. After centrifugation at 230000 *g* for 30 min at 4°C in a Beckman L5-65 ultracentrifuge (SW 50.1 rotor) to pellet the liposomes, samples (25 μ l) of the supernatants and controls were assayed for protein kinase C activity and the ability to phosphorylate the 29000-*M_r* insulin-granule protein as described in the legends to Figs. 1 and 2 respectively.

Sucrose-density-gradient centrifugation

A high-speed soluble-protein fraction was diluted with 3 vol. of a solution containing 10 mM-Mes, 0.1 mM-dithiothreitol and 1 mM-EGTA adjusted to pH 6.5 with Tris, to give a final protein concentration of 2.5 mg/ml. A 0.5 ml portion of this diluted sample was then layered on top of 4.8 ml of a 5–20% (w/w) linear sucrose gradient dissolved in 10 mM-Mes/0.1 mM-dithiothreitol/1 mM-EGTA adjusted to pH 6.5 with Tris. The gradient was centrifuged at 140000 *g* for 16 h at 4°C in a Beckman L5-

65 ultracentrifuge (SW 50.1 rotor), and then fractionated into 0.2 ml fractions. Portions (5 μ l) of alternate fractions were then assayed for protein kinase C activity and the ability to phosphorylate the 29000- M_r insulin-granule protein. The protein content of each fraction was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) as standard.

Ion-exchange chromatography

A high-speed soluble-protein fraction was made 50 mM with respect to NH_4HCO_3 and 1 mM with respect to 2-mercaptoethanol. A 40 ml portion of this solution (250 mg of protein) was pumped at 30 ml/h with a peristaltic pump (LKB, Bromma, Sweden) on to a column (10 cm $\times$ 3 cm) of DEAE-cellulose (DE52; Whatman, Maidstone, Kent, U.K.) at 4°C which had been equilibrated with 50 mM- NH_4HCO_3 /1 mM-2-mercaptoethanol. The column was washed with equilibration buffer overnight, and protein was then eluted from the resin by applying a 50–300 mM linear gradient of NH_4HCO_3 (total vol. 350 ml). Fractions (6 ml) were collected, and 1 ml samples of alternate fractions were freeze-dried and then redissolved in 0.1 ml of 10 mM-Mes adjusted to pH 6.5 with Tris. Samples (10 μ l) were then assayed for protein kinase C activity and the ability to phosphorylate the 29000- M_r protein.

(NH_4) $_2\text{SO}_4$ precipitation

A 20 ml sample of high-speed soluble-protein fraction (200 mg of protein) was made 0.1 mM with respect to dithiothreitol, 1 mM with respect to EDTA and 1 mM with respect to phenylmethanesulphonyl fluoride. Sufficient solid (NH_4) $_2\text{SO}_4$ was then added to give a 30%-saturated solution at 0°C. The resulting precipitate was separated after 30 min by centrifugation at 16000 g for 20 min at 4°C in a Sorvall RC-5 Superspeed centrifuge (SS-34 rotor). Solid (NH_4) $_2\text{SO}_4$ was added to the supernatant to give a 50%-saturated solution, and subsequently 65%- and 80%-saturated solutions, the precipitated material being recovered at each stage by centrifugation.

Each pellet was redissolved in 10 ml of 10 mM-Mes/0.1 mM-dithiothreitol/1 mM-EGTA adjusted to pH 6.5 with Tris, and samples (5 μ l) were assayed for protein kinase C activity and the ability to cause phosphorylation of the 29000- M_r insulin-granule protein.

Gel-filtration chromatography

A 5 ml sample (75 mg of protein) of the redissolved pellet from a 30–50%-satd.-(NH_4) $_2\text{SO}_4$ fraction of the soluble-protein fraction, prepared as described above, was loaded on to a column

(90 cm $\times$ 3.5 cm) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) which had been equilibrated with 50 mM- NH_4HCO_3 /1 mM-2-mercaptoethanol. Proteins were eluted with equilibration buffer at a flow rate of 24 ml/h, and 4 ml fractions were collected after the void volume (100 ml) was passed. A 1 ml sample of every third fraction was freeze-dried and then redissolved in 0.1 ml of 10 mM-Mes adjusted to pH 6.5 with Tris. Samples (10 μ l) were then assayed for protein kinase C activity and the ability to phosphorylate the 29000- M_r insulin-granule protein.

Results

Assay of protein kinase C activity in an insulinoma soluble-protein fraction

The addition of phosphatidylserine and Ca^{2+} to an insulinoma soluble-protein fraction markedly stimulated its capacity to phosphorylate exogenous histone type III-S, indicating the presence of a protein kinase C-like enzyme. Histone phosphorylation in the absence of either effector represented $12.7 \pm 2.3\%$ (mean $\pm$ S.E.M.; $n = 3$) of the stimulated value. Phosphatidylserine alone was essentially without effect ($14.0 \pm 2.0\%$ of stimulated rate), and Ca^{2+} alone also did not affect basal activity significantly ($17.3 \pm 2.3\%$). The Ca^{2+} /phosphatidylserine-dependent histone kinase activity was inhibited by 0.1 mM-trifluoperazine (results not shown).

Binding of protein kinase C activity to insulin granules

Preincubation of insulin granules with the soluble-protein fraction in the presence of Ca^{2+} decreased the histone kinase activity of the supernatant measured in the presence of Ca^{2+} and phosphatidylserine to the basal value of an untreated sample (Table 1). When the preincubation was performed in the absence of Ca^{2+} , histone kinase activity of the supernatant measured in the presence of Ca^{2+} and phosphatidylserine was not decreased, but was found to have a decreased phosphatidylserine-dependence. This was possibly because of phospholipid present in the supernatant in the form of lysed granules which did not sediment on centrifugation. Resuspension, in a solution not containing Ca^{2+} , of the 'insulin-granule/soluble-protein complex' formed in the presence of Ca^{2+} resulted in the release into the supernatant of histone kinase activity. This activity measured in the presence of Ca^{2+} and phosphatidylserine was greater than that of the original soluble-protein fraction. This increase in activity may be a reflection of the selective elimination by this process of competing soluble ATP-hydrolysing enzymes or endogenous soluble substrates or inhibitors of protein kinase C. When the 'insulin-

Table 1. *Effect of Ca^{2+} on the binding of protein kinase C to and subsequent elution from insulin granules*

Freshly prepared insulin granules (approx. 100 μg of protein) were preincubated with the soluble-protein fraction (approx. 25 μg of protein) in the presence of 1.2 mM-EGTA plus 1.3 mM- CaCl_2 as described in the Experimental section. After centrifugation, the granule pellets were resuspended in the presence of 1 mM-EGTA in either the absence or the presence of 1.1 mM- CaCl_2 , and the granules were again isolated by centrifugation. The supernatants from both centrifugations, and also those from a control experiment in which both the preincubation of insulin granules with the soluble-protein fraction and the subsequent resuspension of the granule pellet were performed in the absence of CaCl_2 , were assayed for protein kinase C activity. The original soluble-protein fraction diluted in preincubation buffer in the absence of CaCl_2 to the same extent as in the preincubation with granules was also assayed for protein kinase C activity. The assay was conducted as follows: samples (25 μl) were incubated at 30°C for 1 min in a final volume of 50 μl of a solution containing 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.5–2.0 Ci/mmol) and 0.3 mg of lysine-rich histone/ml, with other additions as shown. Histone proteins were separated by electrophoresis, and the radioactivity present in the bands was determined by Cerenkov counting. Results are expressed as c.p.m. incorporated into the histone bands. Values in parentheses represent c.p.m. incorporated into the same area of the gel when the assay was performed in the absence of histone. Qualitatively similar results were obtained in three separate experiments.

Sample	Additions to assay	Protein kinase activity (c.p.m.)
(1) Soluble-protein fraction	No addition	2685
	0.65 mM- CaCl_2	3596
	Phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	2863
	0.65 mM- CaCl_2 plus phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	7970 (2614)
(2) Supernatant from preincubation of insulin granules with the soluble-protein fraction in the absence of CaCl_2	No addition	2247
	0.65 mM- CaCl_2	7403
	Phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	2228
	0.65 mM- CaCl_2 plus phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	9802 (2348)
(3) Supernatant from preincubation of insulin granules with the soluble-protein fraction in the presence of CaCl_2	No addition	2386
	Phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	2916
(4) Supernatant after resuspension of the granule pellet from (2) in the absence of CaCl_2	0.55 mM- CaCl_2 plus phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	1212
(5) Supernatant after resuspension of the granule pellet from (3) in the absence of CaCl_2	No addition	1394
	0.55 mM- CaCl_2	3553
	Phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	1773
	0.55 mM- CaCl_2 plus phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	20 100 (498)
(6) Supernatant after resuspension of the granule pellet from (3) in the presence of CaCl_2	Phosphatidylserine (80 $\mu\text{g}/\text{ml}$)	1430

granule/soluble-protein complex', formed in the presence of Ca^{2+} , was resuspended in the presence of Ca^{2+} , there was no release of protein kinase C activity into the supernatant. Also, resuspension in the absence of Ca^{2+} of the granule pellet from the preincubation of insulin granules with the soluble-protein fraction in the absence of Ca^{2+} did not release protein kinase C activity into the supernatant.

Preincubation of the soluble-protein fraction with phosphatidylserine liposomes

After preincubation of the soluble-protein fraction with phosphatidylserine liposomes in the presence of Ca^{2+} , protein kinase C activity was no longer detectable in the supernatant; when the preincubation was performed in the absence of Ca^{2+} , protein kinase C activity was still present in the

supernatant (Fig. 1). Similarly, the supernatant after the preincubation of the soluble-protein fraction with liposomes in the presence of Ca^{2+} no longer showed the capacity to mediate the phosphorylation of the 29000- M_r insulin-granule protein; when the preincubation was performed in the absence of Ca^{2+} , the supernatant exhibited the Ca^{2+} -dependent phosphorylation of the 29000- M_r granule protein (Fig. 2). There was a general decrease in the extent of phosphorylation of all soluble-protein fraction proteins after treatment with liposomes compared with the control fractions. This effect was not accompanied by a general loss of protein into the liposome pellet, as judged by the Kenacid Blue R-stained electrophoretogram, and was independent of the presence of Ca^{2+} in the preincubation.

It was difficult to redissolve the liposome pellet

Fig. 1. Autoradiogram showing the effect of preincubation of the soluble-protein fraction with phosphatidylserine liposomes on protein kinase C activity

High-speed soluble-protein fraction was preincubated with phosphatidylserine liposomes in the presence of 0.7 mM-EGTA and in the absence or presence of 0.8 mM- CaCl_2 . The liposomes were then removed by centrifugation as described in the Experimental section. Control preincubations in the absence of liposomes were also performed. Samples (25 μl) of the supernatants and controls were incubated at 30°C for 1 min in a final volume of 50 μl of a solution containing 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.5–2.0 Ci/mmol), phosphatidylserine (80 $\mu\text{g}/\text{ml}$) and in the absence or presence of lysine-rich histone (15 μg of protein). CaCl_2 was also added to a final concentration of 0.4 mM in incubations (1), (2), (6), (8) and (10) so that the Ca^{2+} concentration was the same in all incubations except (5), (7) and (9). Incubations were as follows: (1) EGTA preincubation control; (2) EGTA preincubation supernatant; (3) EGTA/ Ca^{2+} preincubation control; (4) EGTA/ Ca^{2+} preincubation supernatant; (5) and (6) histone; (7) and (8) EGTA preincubation control plus histone; (9) and (10) EGTA preincubation supernatant plus histone; (11) EGTA/ Ca^{2+} preincubation control plus histone; (12) EGTA/ Ca^{2+} preincubation supernatant plus histone. Proteins were separated by electrophoresis and labelled proteins detected by autoradiography.

Fig. 2. Autoradiogram showing the effect of preincubation of the soluble-protein fraction with phosphatidylserine liposomes on the phosphorylation of the 29000- M_r granule protein

High-speed soluble-protein fraction was preincubated with phosphatidylserine liposomes as described in the legend of Fig. 1. Samples (25 μl) of the supernatants and controls were incubated at 30°C for 1 min in a final volume of 50 μl of a solution containing 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.5–2.0 Ci/mmol) and in the absence or presence of insulin granules (approx. 100 μg of protein). CaCl_2 was also added to a final concentration of 0.4 mM in incubations (1), (2), (6), (8) and (10) so that the Ca^{2+} concentration was the same in all incubations except (5), (7) and (9). Incubations were as follows: (1) EGTA preincubation control; (2) EGTA preincubation supernatant; (3) EGTA/ Ca^{2+} preincubation control; (4) EGTA/ Ca^{2+} preincubation supernatant; (5) and (6) insulin granules; (7) and (8) EGTA preincubation control plus insulin granules; (9) and (10) EGTA preincubation supernatant plus insulin granules; (11) EGTA/ Ca^{2+} preincubation control plus insulin granules; (12) EGTA/ Ca^{2+} preincubation supernatant plus insulin granules. Proteins were separated by electrophoresis and labelled proteins detected by autoradiography.

Protein fractionation of the soluble-protein fraction

Rate-zonal sucrose-density-gradient centrifugation of the soluble protein fraction resolved protein kinase C activity as a single symmetrical zone (Fig. 3). The phosphorylating activity directed towards the 29000- M_r insulin-granule protein also sedimented as a single peak of activity, in the same position of the gradient.

obtained after preincubation of the soluble-protein fraction with phosphatidylserine and Ca^{2+} . Consequently it was technically not feasible to elute enzyme activity from the liposomes by lowering the Ca^{2+} concentration.

Fig. 3. Protein and sucrose concentrations (a) and protein kinase C and 29000-M_r-protein kinase activities (b) of sucrose-density-gradient fractions of the soluble-protein fraction

(a) Protein concentrations (mg/ml) and sucrose concentrations (% w/v) of fractions resulting from sucrose-density-gradient centrifugation of the soluble-protein fraction (see the Experimental section). (b) The sucrose-density-gradient fractions were assayed for histone kinase activity in the presence of Ca²⁺ and phosphatidylserine (protein kinase C activity) and for the ability to phosphorylate the 29000-M_r insulin-granule protein in the presence of Ca²⁺. Results in both cases are expressed as a percentage of the maximum activity observed.

Similar activity profiles of protein kinase C and the 29000-M_r-protein-phosphorylating activity were obtained on assay of freeze-dried fractions resulting from ion-exchange chromatography of the soluble-protein fraction (Fig. 4). In each case at least two peaks of activity were apparent. Other workers have also obtained two peaks of protein kinase C activity after DEAE-cellulose chromatography during the preparation of the enzyme from rat brain (Kikkawa *et al.*, 1982) and pig spleen (Schatzman *et al.*, 1983).

The 30–50%-satd.-(NH₄)₂SO₄ fraction of the soluble-protein fraction was found to contain most of the protein kinase C activity and the 29000-M_r-protein-phosphorylating activity (Fig. 5). After gel

Fig. 4. Protein concentration and conductivity (a) and protein kinase C and 29000-M_r-protein kinase activities (b) of fractions resulting from ion-exchange chromatography of the soluble-protein fraction

(a) Protein concentration (mg/ml) and conductivity (mmho) of fractions resulting from ion-exchange chromatography of the soluble-protein fraction on DEAE-cellulose (see the Experimental section). (b) After freeze-drying, the fractions resulting from ion-exchange chromatography of the soluble-protein fraction were assayed for histone kinase activity in the presence of Ca²⁺ and phosphatidylserine (protein kinase C activity) and for the ability to phosphorylate the 29000-M_r insulin-granule protein in the presence of Ca²⁺. Results in both cases are expressed as a percentage of the maximum activity observed. Qualitatively similar results were obtained with three different preparations.

filtration of this (NH₄)₂SO₄ fraction, a single peak of protein kinase C activity and a single peak of 29000-M_r-protein-phosphorylating activity were obtained, with maximal activities being observed in the same fraction (Fig. 6).

Discussion

Protein kinase C has been shown to be present in a wide variety of tissues (Kuo *et al.*, 1980; Minakuchi *et al.*, 1981), including rat pancreatic islets (Tanigawa *et al.*, 1982). We now report

Fig. 5. Protein concentration (a), 29000- M_r -protein kinase activity (b) and protein kinase C activity (c) of $(\text{NH}_4)_2\text{SO}_4$ fractions of the soluble-protein fraction

The soluble-protein fraction was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation as described in the Experimental section, and the protein concentration (mg/ml) of each fraction is shown in (a). The fractions were assayed for the ability to phosphorylate the 29000- M_r insulin-granule protein in the presence of Ca^{2+} (b) and for histone kinase activity in the presence of Ca^{2+} and phosphatidylserine (protein kinase C activity) (c). Results in (b) and (c) are expressed as a percentage of the maximum activity observed in each case.

the presence of Ca^{2+} -activated phospholipid-dependent protein kinase activity towards lysine-rich histone in the soluble-protein fraction of an insulinoma homogenate. The insulinoma protein kinase C activity was inhibited by trifluoperazine, as has been reported for the activity present in other tissues (Schatzman *et al.*, 1981).

Fig. 6. Protein concentration (a) and protein kinase C and 29000- M_r -protein kinase activities (b) of fractions resulting from gel-filtration chromatography of a 30-50%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction of the soluble-protein fraction

(a) Protein concentration (mg/ml) of fractions resulting from gel filtration on Sephadex G-100 of a 30-50%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction of the soluble-protein fraction (see the Experimental section). (b) After freeze-drying, the gel-filtration fractions were assayed for histone kinase activity in the presence of Ca^{2+} and phosphatidylserine (protein kinase C activity) and for the ability to phosphorylate the 29000- M_r insulin-granule protein in the presence of Ca^{2+} . Results in both cases are expressed as a percentage of the maximum activity observed.

We have previously shown that the component of the insulinoma soluble-protein fraction responsible for the Ca^{2+} -dependent phosphorylation of the 29000- M_r insulin-granule protein will bind to insulin granules in the presence of Ca^{2+} (Brocklehurst & Hutton, 1983) and can be subsequently eluted by lowering the Ca^{2+} concentration (K. W. Brocklehurst & J. C. Hutton, unpublished work). Protein kinase C activity has now been shown to behave in an identical way. Similar capacity of protein kinase C to associate with secretory-granule membranes is evidenced from the reported binding of the enzyme to an affinity column of adrenal chromaffin-granule membranes (Creutz *et al.*, 1982).

Phosphatidylserine liposomes extracted both protein kinase C activity and the component responsible for the phosphorylation of the 29000- M_r granule protein, in a Ca^{2+} -dependent fashion, indicating that the initial interaction between the

29000- M_r protein kinase and the insulin granule may be dependent on granule phospholipid.

Further evidence that protein kinase C is the agent in the soluble-protein fraction responsible for the phosphorylation of the insulin-granule 29000- M_r protein was obtained after analysis of fractions resulting from protein fractionation of the soluble-protein fraction for these two activities. Thus, after sucrose-density-gradient centrifugation, ion-exchange chromatography, $(\text{NH}_4)_2\text{SO}_4$ fractionation and gel filtration, similar activity profiles for protein kinase C and 29000- M_r -protein-phosphorylating activities were obtained.

Protein kinase C may play an important role in stimulus-secretion coupling in platelets, as it catalyses the phosphorylation *in vitro* of a 40000- M_r protein, which has been shown to undergo phosphorylation on stimulation of intact platelets (see Nishizuka, 1983). The evidence presented here suggests that, on stimulation of the β -cell, protein kinase C may attach to insulin granules and phosphorylate the 29000- M_r granule-membrane protein. The phosphorylation of this protein may alter the functional properties of the granule which render it capable of undergoing exocytosis.

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