Ca²⁺-dependent binding of cytosolic components to insulin-secretory granules results in Ca²⁺-dependent protein phosphorylation

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1. Incubation of a rat islet cell tumour homogenate with [γ -³²P]ATP resulted in the Ca²⁺-dependent phosphorylation of 100 000-, 57 000-, 29 000-, 26 000- and 14 000-*M_r* proteins. The Ca²⁺ concentration required was in the low- μ M range. 2. Isolated insulin granules did not exhibit Ca²⁺-dependent protein phosphorylation, whereas a soluble protein fraction showed the Ca²⁺-dependent phosphorylation of a 57 000-*M_r* protein. Combination of insulin granules with the soluble protein fraction resulted in the additional Ca²⁺-dependent phosphorylations of 100 000-, 29 000- and 10 000-*M_r* proteins. The latter phosphorylations were not enhanced by exogenous calmodulin, but nevertheless were inhibited by trifluoperazine. Removal of endogenous calmodulin from the soluble protein fraction before incubation with insulin granules did not abolish the Ca²⁺-dependent phosphorylations of the 100 000-, 29 000- and 10 000-*M_r* proteins but rendered the Ca²⁺-dependent phosphorylation of the 57 000-*M_r* soluble protein dependent on exogenous calmodulin. 3. The components of the soluble protein fraction responsible for the interaction with insulin granules bound to intact granules in a Ca²⁺-dependent manner. 4. After phosphorylation, the 29 000-*M_r* protein remained attached to granules, whereas the 100 000- and 10 000-*M_r* proteins dissociated from granules. 5. These Ca²⁺-dependent phenomena may be of regulatory importance in the secretory mechanism.

An increase in the cytosolic Ca²⁺ concentration of the pancreatic B-cell appears to be necessary for the release of insulin in response to secretagogues (see Wollheim & Sharp, 1981). The effects of this increase in cytosolic [Ca²⁺] at the molecular level may include the activation of Ca²⁺-dependent enzymes involved in the exocytotic mechanism.

Ca²⁺-dependent protein phosphorylation is involved in the regulation of many cellular processes at the biochemical level. Examples include the control of glycogen metabolism (see Cohen, 1982), smooth-muscle contraction (see Hartshorne & Siemankowski, 1981) and ion transport (LePeuch *et al.*, 1979), and there is increasing evidence that the control of secretion may be mediated in this way.

The incorporation of ³²P derived from extracellular [³²P]P_i into cellular proteins accompanies histamine release from mast cells (Sieghart *et al.*, 1978), catecholamine release from the adrenal medulla (Amy & Kirshner, 1981) and the release reaction of platelets (Nishikawa *et al.*, 1980).

Similarly, glucose-induced insulin release from pancreatic islets (Suzuki *et al.*, 1981) and K⁺-induced insulin release from Syrian-hamster insulinoma cells (Schubart *et al.*, 1980b) are accompanied by endogenous protein phosphorylation reactions.

Tissue homogenates of pancreatic islets (Harrison & Ashcroft, 1982; Landt *et al.*, 1982) and a Syrian-hamster insulinoma (Schubart *et al.*, 1980a) catalyse the incorporation of radioactivity from [γ -³²P]ATP into endogenous protein substrates; in both systems the effects of Ca²⁺ are enhanced by the Ca²⁺-dependent regulatory protein, calmodulin.

The objectives of the present investigation were to study Ca²⁺-dependent protein phosphorylation reactions catalysed by homogenates of a rat islet-cell tumour, and to further characterize such reactions at the subcellular level. The tumour used in these studies secretes insulin by a mechanism indistinguishable from that of pancreatic islets (Sopwith *et al.*, 1981), and serves as a convenient source of gram-quantities of material for biochemical investigations of the B-cell at the subcellular and molecular level.

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Experimental

Preparation of subcellular fractions

The rat islet cell tumour was originally 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 (4-morpholine-ethanesulphonic acid), 0.27 M-sucrose and 1 mM-EGTA adjusted to pH 6.5 with Tris. Insulin granules were isolated from the homogenate using Percoll (Pharmacia, Uppsala, Sweden)-density-gradient centrifugation as described previously (Hutton *et al.*, 1982), and finally suspended in a solution containing 5 mM-Mes and 0.27 M-sucrose adjusted to pH 6.5 with Tris. These were either used immediately or stored at -70°C . The granules so isolated were essentially free of other subcellular organelles as evaluated by electron microscopy, electrophoretic analyses and their content of marker proteins (Hutton *et al.*, 1982).

A soluble protein fraction was obtained by centrifugation of the initial homogenate at 40000 g for 60 min at 4°C . A soluble protein fraction depleted of calmodulin was prepared by passing the supernatant through a column (1 cm $\times$ 5 cm) of DEAE-Sephadex ion-exchange resin (Pharmacia, Uppsala, Sweden) equilibrated with 0.4 M- NH_4HCO_3 and 1 mM-EGTA at pH 7.9. This fraction contained less than 5% of the initial calmodulin content as evaluated from its ability to activate Ca^{2+} -calmodulin-dependent cyclic AMP phosphodiesterase (E. J. Penn, unpublished work).

Phosphorylation assay

Protein samples were incubated for 1 min at 30°C in 50 μl of a solution containing 50 mM-Mes, 1 mM- MgCl_2 , 0.1 mM-dithiothreitol, 5 mM-EGTA and 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ {0.5–2.0 Ci/mmol; prepared from carrier-free $^{32}\text{P}]\text{P}_i$ (Amersham International, Amersham, Bucks., U.K.) by the method of Walsh *et al.* (1971)} adjusted to pH 6.9 with Tris. Other additions included 0.25 M-sucrose in experiments involving freshly-prepared granules, 1.2 μM -bovine brain calmodulin [prepared as in Hutton *et al.* (1981)], trifluoperazine (Smith, Kline and French, Welwyn Garden City, Herts., U.K.) and 5 mM- CaCl_2 . The latter addition resulted in a free Ca^{2+} concentration estimated at 15 μM (Storer & Cornish-Bowden, 1976). The reaction was terminated by the addition of 30 μl 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, 0.02% Bromophenol Blue and heating at 100°C for 5 min. Samples (50 μl) were then electrophoresed on polyacrylamide slab gels (15 cm $\times$ 15 cm $\times$ 0.1 cm) polymerized from 20% (w/v) acrylamide and 0.067%

NN'-methylenebisacrylamide, using the buffer system of Laemmli (1970). A mixture of glycogen phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c* was used as a molecular-weight calibration standard. Gels were stained with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.), dried and autoradiographed using Cronex-4 X-ray film (Dupont U.K., St. Neots, Cambs., U.K.). Quantification of autoradiograms was achieved by densitometric scanning using a Joyce-Loebl Chromoscan-3 densitometer (Joyce-Loebl, Gateshead, Tyne & Wear, U.K.).

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) as standard.

Results

Ca^{2+} -dependent protein phosphorylation in a crude tumour homogenate

The Ca^{2+} -dependent phosphorylations of 100 000-, 57 000-, 29 000-, 26 000- and 14 000- M_r proteins were detected against a background of many Ca^{2+} -independent phosphorylations on incubation of a crude tumour homogenate with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 1). The Ca^{2+} -dependent phosphorylations were all sensitive to free Ca^{2+} in the low- μM concentration range, as shown for the 100 000-, 57 000- and 29 000- M_r proteins (Fig. 2).

Protein phosphorylation in insulin-granule and soluble-protein fractions

Insulin granules incubated in the phosphorylation assay incorporated little ^{32}P into proteins relative to an equivalent amount of homogenate protein. No effect of Ca^{2+} was observed on the pattern of protein phosphorylation when added alone (Fig. 3b) or together with bovine brain calmodulin (results not shown).

Incubation of the soluble protein fraction with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ resulted in the Ca^{2+} -dependent phosphorylation of a 57 000- M_r protein (Fig. 3a). No further enhancement of phosphorylation was seen on addition of bovine brain calmodulin (results not shown).

Incubation of the insulin-granule and soluble-protein fractions together resulted in the Ca^{2+} -dependent phosphorylations of 100 000-, 29 000- and 10 000- M_r proteins in addition to the 57 000- M_r protein seen with the soluble protein fraction alone (Fig. 3c). It was not possible to identify these ^{32}P -labelled proteins with stained proteins on the corresponding electrophoretograms. The 100 000-, 57 000- and 29 000- M_r proteins were indistinguishable by electrophoresis from those of the same molecular weights that underwent Ca^{2+} -dependent phosphorylation in the crude tumour homogenate.

Fig. 1. Autoradiogram showing the effect of Ca^{2+} on protein phosphorylation in a crude tumour homogenate. Crude tumour homogenate (approx. $100\mu\text{g}$ of protein) was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of (i) 5 mM-EGTA or (ii) 5 mM-EGTA and 5 mM- CaCl_2 (free Ca^{2+} concentration of $15\mu\text{M}$). Phosphorylated proteins were detected by using polyacrylamide-gel electrophoresis and autoradiography (for further details see the Experimental section).

The Ca^{2+} -dependent phosphorylations of the 29 000- and 10 000- M_r proteins were not observed when fractions enriched in mitochondria, endoplasmic reticulum, plasma membrane or Golgi were incubated with the soluble protein fraction in the phosphorylation assay. A 100 000- M_r protein, however, underwent Ca^{2+} -dependent phosphorylation when these particulate fractions were assayed in this way (results not shown).

Fig. 2. Effect of free Ca^{2+} concentration on the phosphorylation of homogenate proteins

Crude tumour homogenate (approx. $30\mu\text{g}$ of protein) was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at different free Ca^{2+} concentrations, which were achieved by varying the concentration of CaCl_2 in the presence of 5 mM-EGTA. ^{32}P -labelled proteins were detected by electrophoresis and autoradiography and the extent of phosphorylation determined by densitometry. Data are shown for 100 000- ($\square$), 57 000- ($\bullet$) and 29 000- M_r ($\circ$) proteins, and results are expressed as percentages of the maximum peak height measured for each protein band.

The Ca^{2+} -dependent phosphorylations of the 100 000-, 29 000- and 10 000- M_r proteins observed on incubating insulin granules with the soluble protein fraction each showed a characteristic time course (Fig. 4). The extent of phosphorylation of the 10 000- M_r protein reached a maximum value within 10 min and thereafter declined, suggesting the presence of an active dephosphorylating mechanism for this phosphoprotein.

Investigation of the involvement of calmodulin in the interaction between insulin granules and the soluble protein fraction

The addition of bovine brain calmodulin to the combined insulin-granule and soluble-protein fractions had no effect on the Ca^{2+} -dependent phosphorylations of the 100 000-, 29 000- and 10 000- M_r proteins (results not shown). However, the calmodulin antagonist, trifluoperazine, inhibited these phosphorylations in a concentration-dependent fashion (Fig. 5).

The possible involvement of endogenous cal-

Fig. 3. Autoradiogram showing the effect of Ca^{2+} on protein phosphorylation in soluble-protein and insulin-granule fractions

(a) Soluble-protein fraction (approx. $25\mu\text{g}$ of protein); (b) insulin granules (approx. $100\mu\text{g}$ of protein); and (c) soluble-protein fraction plus insulin granules were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Track (i) incubation in each case was in the presence of 5mM-EGTA and track (ii) in the presence of 5mM-EGTA and 5mM-CaCl_2 (free Ca^{2+} concentration of $15\mu\text{M}$). Proteins were separated by electrophoresis and ^{32}P -labelled proteins detected by autoradiography.

modulin present in the soluble-protein fraction in mediating the phosphorylations of these proteins was investigated further by incubating a calmodulin-depleted soluble-protein fraction with insulin granules in the phosphorylation assay. The Ca^{2+} -dependent phosphorylations of the 100000 -, 29000 - and 10000 - M_r proteins were still evident under these conditions and not enhanced by the addition of bovine brain calmodulin. In contrast, the Ca^{2+} -dependent phosphorylation of the 57000 - M_r soluble protein was not observed unless bovine brain calmodulin was present in the assay (Table 1).

Ca^{2+} -dependent binding of components of the soluble protein fraction to insulin granules

Freshly-prepared osmotically-protected insulin granules that had been pre-incubated with the

Fig. 4. Time course of phosphorylation of the 10000 -(a), 29000 -(b) and 100000 - M_r (c) proteins

Soluble-protein (approx. $25\mu\text{g}$ of protein) and insulin-granule (approx. $100\mu\text{g}$ of protein) fractions were incubated together with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 5mM-EGTA in the absence (●) or presence (O) of 5mM-CaCl_2 for the designated time intervals. Samples were analysed by electrophoresis and autoradiography and the extent of phosphorylation of the 100000 -, 29000 - and 10000 - M_r proteins measured by densitometry. Each result is expressed as a percentage of the maximum peak height observed for the respective protein bands during the time courses.

soluble protein fraction in the presence of Ca^{2+} , separated by centrifugation, and subsequently incubated in the phosphorylation assay, displayed the Ca^{2+} -dependent phosphorylations of the 100000 -, 29000 - and 10000 - M_r proteins (Fig. 6b). When the preincubation was performed in the absence of Ca^{2+} , no subsequent Ca^{2+} -dependent protein phosphorylation was evident (Fig. 6a). This suggested that the components of the soluble protein fraction

Table 1. *Effect of bovine brain calmodulin on the phosphorylation of the 100000-, 57000-, 29000- and 10000-M_r proteins after removal of endogenous calmodulin from the soluble protein fraction*

A soluble protein fraction depleted of calmodulin by DEAE-Sephadex ion-exchange chromatography (approx. 10 µg of protein) was incubated with insulin granules (approx. 100 µg of protein) in the presence of [γ -³²P]ATP and 5 mM-EGTA. The effect of addition of 5 mM-CaCl₂ and 1.2 µM-bovine brain calmodulin on the phosphorylation of the 100000-, 57000-, 29000- and 10000-M_r proteins was measured by densitometry after electrophoresis and autoradiography. Results are expressed as a percentage of the maximum peak height observed for each protein band.

Assay conditions	Protein ...	Peak height (% of maximum)			
		100000 M _r	57000 M _r	29000 M _r	10000M _r
5 mM-EGTA		40	26	9	9
5 mM-EGTA + 1.2 µM-calmodulin		28	21	9	14
5 mM-EGTA + 5 mM-CaCl ₂		88	33	100	98
5 mM-EGTA + 5 mM-CaCl ₂ + 1.2 µM-calmodulin		100	100	86	100

Fig. 5. *Effect of trifluoperazine concentration on the phosphorylation of the 100000- (□), 29000- (○) and 10000-M_r (■) proteins*

Soluble-protein (approx. 25 µg of protein) and insulin-granule (approx. 100 µg of protein) fractions were incubated together with [γ -³²P]ATP in the presence of 5 mM-EGTA, 5 mM-CaCl₂ and the concentrations of trifluoperazine indicated. After electrophoresis and autoradiography, the extent of phosphorylation of the 100000-, 29000- and 10000-M_r proteins was determined by densitometry. For each protein band, results are corrected for the basal phosphorylation observed in the absence of CaCl₂ and then expressed as a percentage of the maximum peak height observed.

centrifuged to separate the granules. The labelled 29000-M_r protein was found to be in the granule pellet, whereas the 100000- and 10000-M_r phosphoproteins were in the supernatant (Fig. 7). This suggested that the 100000 and 10000-M_r proteins dissociated from the granules after their phosphorylation.

Discussion

Protein phosphorylation is an important regulatory mechanism in the control of many cellular events, and may be involved in mediating the effects of Ca²⁺ in stimulus–secretion coupling, possibly at the level of the secretory vesicle. The membrane of the adrenal-medullary chromaffin granule, for example, has been shown to possess Ca²⁺/calmodulin-dependent protein kinase activity that phosphorylates granule-membrane proteins (Burgoyne & Geisow, 1981).

We have observed that free Ca²⁺ in the low-µM concentration range stimulates the phosphorylation of several islet-cell tumour homogenate proteins. The Ca²⁺-dependent phosphorylation of a 57000-M_r protein observed with a crude tumour homogenate and a soluble protein fraction appears to be mediated by calmodulin. Removal of endogenous calmodulin abolishes this effect, which is restored on addition of bovine brain calmodulin. A protein of similar molecular weight has been reported to undergo Ca²⁺/calmodulin-dependent phosphorylation in islets of Langerhans by Harrison & Ashcroft (1982) and Landt *et al.* (1982). The latter group suggest, however, that it may be localized to a membrane fraction.

Insulin granules alone did not exhibit either Ca²⁺- or Ca²⁺/calmodulin-dependent protein phosphorylation. However, when incubated with a soluble protein fraction, the Ca²⁺-dependent phosphorylations of 100000- and 29000-M_r proteins were observed; these appeared to be identical with those seen with the crude homogenate. In addition

responsible for the interaction with insulin granules bound to intact granules in a Ca²⁺-dependent fashion.

After pre-incubation of insulin granules with the soluble protein fraction in the presence of Ca²⁺, and the subsequent resuspension of the granules with [γ -³²P]ATP and Ca²⁺, the final mixture was again

Fig. 6. Autoradiogram illustrating the Ca^{2+} -dependent binding of components of the soluble protein fraction to insulin granules and the resulting Ca^{2+} -dependent protein phosphorylation

(a) Freshly prepared insulin granules (approx. $100\mu\text{g}$ of protein) were pre-incubated with the soluble protein fraction (approx. $25\mu\text{g}$ of protein) for 1 min at 30°C in $50\mu\text{l}$ of a solution containing 50mM -Mes, 1mM - MgCl_2 , 0.25M -sucrose, 0.1mM -dithiothreitol and 5mM -EGTA adjusted to pH 6.9 with Tris. The mixture was then centrifuged in an Eppendorf microfuge (Eppendorf Geratebau, Netheler & Hinz G.m.b.H., Hamburg, Germany) for 2 min and the granule pellet resuspended in $50\mu\text{l}$ of the same solution. Granules were again isolated by centrifugation and then resuspended with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the phosphorylation assay medium containing (i) 5mM -EGTA or (ii) 5mM -EGTA plus 5mM - CaCl_2 . Phosphorylated proteins were detected by electrophoresis and autoradiography (for further details see the Experimental section). (b) As in (a), except that the pre-incubation of granules with the soluble protein fraction and subsequent washing of the granule pellet were performed with the additional presence of 5mM - CaCl_2 .

Fig. 7. Autoradiogram illustrating the localization of the $100\,000$ -, $29\,000$ - and $10\,000$ - M_r phosphoproteins

Freshly prepared insulin granules were pre-incubated with the soluble protein fraction in the presence of CaCl_2 . After centrifugation, the granule pellet was washed and then resuspended with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in phosphorylation assay medium in the presence of CaCl_2 as in Fig. 6(b) (ii). After incubation for 1 min at 30°C the mixture was again centrifuged in an Eppendorf microfuge. The pellet (i) and the supernatant (ii) of this final centrifugation were analysed by electrophoresis and autoradiography.

the Ca^{2+} -dependent phosphorylation of a $10\,000$ - M_r protein was now apparent.

Although exogenous calmodulin did not enhance the Ca^{2+} -dependent phosphorylations of the $100\,000$ -, $29\,000$ - and $10\,000$ - M_r proteins, their inhibition by the calmodulin antagonist, trifluoperazine, suggested a role for endogenous calmodulin. However, the removal of endogenous calmodulin from the soluble protein fraction before incubation with insulin granules did not abolish these Ca^{2+} -dependent protein phosphorylations, which

still remained insensitive to the addition of bovine brain calmodulin. These apparently contradictory results perhaps reflect the involvement of granule-bound calmodulin in the phosphorylation reactions, or the involvement of a kinase that has calmodulin as an integral subunit, as is the case for phosphorylase kinase (Cohen *et al.*, 1978). Tri-fluoperazine has also been shown to inhibit a Ca^{2+} -activated protein kinase that is not dependent on calmodulin, but on phospholipid for its activity (Schatzman *et al.*, 1981). One further possibility is that the relatively high concentrations of tri-fluoperazine required to inhibit the Ca^{2+} -dependent protein phosphorylations may reflect a non-specific interaction with the granule membranes due to the hydrophobicity of the molecule.

The Ca^{2+} -dependent phosphorylations of the 100000-, 29000- and 10000- M_r proteins could be dissociated into two distinct Ca^{2+} -dependent steps; namely the binding of soluble protein fraction components to insulin granules and the actual phosphorylation reactions. The 29000- M_r protein remained attached to granules, whereas the 100000- and 10000- M_r proteins dissociated from granules after their phosphorylation. These results could be accounted for if the 29000- M_r protein was a granule protein that becomes phosphorylated on binding a cytosolic kinase, and if the 100000- and 10000- M_r proteins were cytosolic proteins that bind to granules and upon phosphorylation are released.

The present findings are consistent with a hypothesis that the increase in cytosolic Ca^{2+} concentration of the B-cell during the stimulation of insulin secretion results in an interaction between components of the cytosol and insulin-granule membranes. These Ca^{2+} -dependent interactions, which result in protein phosphorylation, may play an important regulatory role in the exocytotic mechanism.

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