Correlation of Ca^{2+} - and calmodulin-dependent protein kinase activity with secretion of insulin from islets of Langerhans

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A Ca^{2+} -activated and calmodulin-dependent protein kinase activity which phosphorylates predominantly two endogenous proteins of 57 kDa and 54 kDa was found in a microsomal fraction from islet cells. Half-maximal activation of the protein kinase occurs at approx. $1.9 \mu\text{M}$ - Ca^{2+} and $4 \mu\text{g}$ of calmodulin/ml (250 nM) for phosphorylation of both protein substrates. Similar phosphoprotein bands (57 kDa and 54 kDa) were identified in intact islets that had been labelled with $[^{32}\text{P}]\text{P}_i$. Islets prelabelled with $[^{32}\text{P}]\text{P}_i$ and incubated with 28 mM-glucose secreted significantly more insulin and had greater incorporation of radioactivity into the 54 kDa protein than did islets incubated under basal conditions in the presence of 5 mM-glucose. Thus the potential importance of the phosphorylation of these proteins in the regulation of insulin secretion is indicated both by activation of the protein kinase activity by physiological concentrations of free Ca^{2+} and by correlation of the phosphorylation of the substrates with insulin secretion in intact islets. Experiments undertaken to identify the endogenous substrates indicated that this calmodulin-dependent protein kinase may phosphorylate the α - and β -subunits of tubulin. These findings suggest that Ca^{2+} -stimulated phosphorylation of islet-cell tubulin via a membrane-bound calmodulin-dependent protein kinase may represent a critical step in the initiation of insulin secretion from the islets of Langerhans.

Ca^{2+} is required for insulin secretion from the islets of Langerhans (Grodsky & Bennett, 1966). All known insulinogenic stimuli affect Ca^{2+} fluxes in intact islets, leading to an increase in the intracellular content of Ca^{2+} (Malaisse *et al.*, 1978). For these reasons, it is generally assumed that intracellular Ca^{2+} concentrations represent a critical regulator of the secretory process. However, the mechanism by which cellular Ca^{2+} exerts its effects is poorly understood.

A good candidate for the transduction of a signal reflecting changes in Ca^{2+} concentration is the Ca^{2+} -binding protein, calmodulin. Calmodulin binds Ca^{2+} ions at micromolar concentrations and subsequently alters the activities of several enzyme systems (Cheung, 1980). Calmodulin is present in high concentrations in the pancreatic islets (Sugden

et al., 1979; Landt *et al.*, 1982) and has been shown to alter such activities as adenylate cyclase (Valverde *et al.*, 1979), cyclic nucleotide phosphodiesterase (Sugden & Ashcroft, 1981), plasma-membrane ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase (Kotagal *et al.*, 1982), and a myosin light-chain kinase activity (MacDonald & Kowluru, 1982) in islet-cell homogenates. A Ca^{2+} -activated and calmodulin-dependent protein kinase has been described in an insulin-secreting tumour (Schubart *et al.*, 1980), and is similar to a membrane-bound protein kinase in adipose tissue (Landt & McDonald, 1980). We have demonstrated a Ca^{2+} -activated and calmodulin-dependent protein kinase activity in islets and have localized this activity to the microsomal fraction (Landt *et al.*, 1982). This protein kinase activity was shown to phosphorylate an unidentified endogenous protein substrate of 57 kDa. A similar calmodulin-dependent protein kinase activity has been reported by Ashcroft and associates (Gagliardino *et al.*, 1980; Harrison & Ashcroft, 1982).

Our present studies have focused on further

Abbreviations used: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Pipes, 1,4-piperazinediethanesulphonic acid.

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characterization of this Ca^{2+} -activated calmodulin-dependent protein kinase activity. We have also sought to correlate this protein kinase activity with insulin secretion in the intact islets, and to investigate the identity of the protein substrate.

Experimental

Materials

Male Wistar rats (180–250 g) were purchased from Charles Rivers Laboratories (Wilmington, MA, U.S.A.) or male Sprague–Dawley rats (180–200 g) were obtained from Sasco (O'Fallon, MO, U.S.A.). Calmodulin was prepared from rat brain (Charbonneau *et al.*, 1979) or purchased from Calbiochem (La Jolla, CA, U.S.A.). These calmodulin stocks were compared and found to be equivalent by assay of Ca^{2+} -stimulated phosphodiesterase activity (Watterson *et al.*, 1976). [γ - ^{32}P]ATP was purchased from Amersham (Chicago, IL, U.S.A.). Ionophore A23187 was purchased from Calbiochem. [^{32}P]P_i, Protosol and Aquasol II were from New England Nuclear (Boston, MA, U.S.A.). Sheep anti-tubulin antibody (affinity-chromatography-purified) was from CABCO (Houston, TX, U.S.A.), and all other chemicals were reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chromatographically purified bovine brain tubulin was a gift from Dr. L. A. Brown, St. Louis University.

Isolation of pancreatic islets and islet-cell membranes

Islets were isolated by collagenase digestion (Lacy & Kostianovsky, 1967) and purified on dialysed Ficoll gradients (Shibata *et al.*, 1976). The islets were then washed in Hanks solution and selected under a dissecting microscope. When subcellular fractionation of islets was performed, the islets were isolated under aseptic conditions and cultured overnight at 24°C (under air/CO₂, 19:1) in culture medium 1066 (GIBCO, Grand Island, NY, U.S.A.) containing 8 mM-glucose, 1% glutamine and 1% streptomycin and penicillin, with 10% (v/v) foetal-calf serum.

Subcellular fractionation of islets was performed by the technique of Naber *et al.* (1980), with modifications as described previously (Colca *et al.*, 1982). Where indicated, the protein kinase assays employed the microsomal fraction of the islet cells, which is enriched in protein kinase activity (Landt *et al.*, 1982). This microsomal fraction contains a 2–3-fold enrichment of the endoplasmic-reticulum marker enzymes glucose 6-phosphatase and NADH:cytochrome *c* reductase (Colca *et al.*, 1982). This fraction is devoid of mitochondria and secretory granules, as judged by both biochemical (succinate:cytochrome *c* reductase activity and extractable

insulin content) and morphological criteria (Colca *et al.*, 1982). Protein was assayed by using fluorescamine, with bovine serum albumin as standard (Orr *et al.*, 1976).

Protein kinase assays

Protein kinase assays were performed as described previously (Landt *et al.*, 1982). Briefly, incubations were performed in medium (50 μl) containing 20–50 μg of homogenate protein or 2–4 μg of microsomal protein, 50 mM-Pipes (pH 7.0 at 37°C), 10 mM-MgCl₂, 0.1 mM-dithiothreitol, 0.2 mM-EGTA, with or without 1 μM -calmodulin and with or without 1 mM-CaCl₂. In some experiments, the concentrations of calmodulin and Ca^{2+} were varied. The incubation medium containing the indicated tissue was preincubated for 2 min at 37°C, and the reaction was initiated by addition of 5–10 μCi of [γ - ^{32}P]ATP (final concn. 5–10 μM -ATP) and terminated after 5–120 s by the addition of 25 μl of 9% (w/v) SDS/6% (w/v) 2-mercaptoethanol/15% (v/v) glycerol (stop solution) and incubation in a boiling-water bath for 2 min. Samples were then separated on 10%-polyacrylamide/SDS slab gels (6–15 h at 100 V), stained for protein with Coomassie Blue, destained, and dried. After autoradiography, protein bands were cut from the gels, by using the exposed film as a guide. These bands were then hydrated (0.25 ml of water), heated at 50°C in Protosol (1 ml), and ^{32}P incorporation was quantified by liquid-scintillation counting.

In some experiments, designed to determine the identity of the endogenous phosphorylated protein substrates, chromatographically purified tubulin was added to protein kinase assays where the microsomal fraction was used as a source of the protein kinase activity. Briefly, tubulin was purified from calf brain by (NH₄)₂ SO₄ fractionation, followed by DEAE-Sephadex batch elution and column chromatography with Sephadex G-25 (Lee, 1982). The purified tubulin was precipitated with MgCl₂, and extensively dialysed against 1 M-sucrose/10 mM-Na₂B₄O₇/0.1 mM-GTP/0.5 mM-MgCl₂, pH 7.0. Other experimental protocols involved preincubation of the microsomal fraction with sheep anti-tubulin antibody (30 min, 37°C) to bind endogenous tubulin. The anti-tubulin antibody was dialysed before use to remove the original borate buffer. Iodinated tubulin standards were used to verify that this preparation of antibody would bind tubulin under these experimental conditions.

Intact-islet experiments

Studies were performed with intact islets to determine if phosphorylation of these protein substrates occurred in intact cells, and whether this phosphorylation was associated with stimulated insulin secretion. In these studies, islets were isolated from 10–11 rats and preincubated in incubation medium

(pH 7.4) for 60 min at 37°C. The incubation medium contained 5 mM-Hepes, 0.1% bovine serum albumin, 5 mM-D-glucose, 115 mM-NaCl, 24 mM- NaHCO_3 , 5 mM-KCl, 1 mM- MgCl_2 and 2.5 mM- CaCl_2 . The preincubated islets were washed with fresh incubation medium and loaded with 400–600 μCi of [^{32}P]P_i in 400 μl of medium for 20 min at 37°C. In initial experiments, islets were pre-labelled with [^{32}P]P_i for 0–90 min. Longer loading periods increased background radioactivity, making it more difficult to demonstrate experimentally induced increases in phosphorylation of the protein bands of interest. Thus, for experiments presented, a 20 min [^{32}P]P_i-loading period was used. The radiolabelled islets were washed extensively with incubation medium at 4°C to remove residual [^{32}P]P_i, and transferred to silicone-treated Petri plates; 100 islets were individually selected with a Pasteur pipette and placed in randomized (10 mm $\times$ 75 mm) borosilicate culture tubes. The islet samples were maintained in a water bath at 4°C until the experiment was begun.

Experimental incubations were initiated by removal of the cold incubation medium and addition of 0.25 ml of medium at 37°C containing experimental additions. The islets were incubated at 37°C under an atmosphere of O_2/CO_2 (19:1) for the indicated times. The medium was then removed with the aid of a dissecting microscope and kept for insulin determinations. The islets were dissolved in 3% SDS/2% 2-mercaptoethanol/5% glycerol (70 μl), placed in a boiling-water bath for 2 min, and then subjected to electrophoresis and autoradiography as described above. Immunoreactive insulin was quantified by the method of Wright *et al.* (1971). In experiments utilizing shorter incubation times, insulin secretion was not measured. In these experiments, incubations were performed in 50 μl of medium and were terminated after the indicated times by addition of 25 μl of stop solution. The samples were then processed for liquid-scintillation counting as described above. All experiments were performed in triplicate.

Statistical analysis of the data

Statistical evaluation of the data was by randomized blocks design (intact-islet experiments), single classification analysis of variance, or Student's *t* test, as appropriate.

Ca^{2+} concentrations

Free Ca^{2+} concentrations were determined by calculation of the Ca^{2+} chelated by EGTA, which was added to buffer the final Ca^{2+} concentrations. Total Ca^{2+} concentration in stock solutions was confirmed by atomic-absorption spectrophotometry. The calculation of free Ca^{2+} concentration assumed an association constant for Ca-EGTA of $10^{6.84}$ at pH 7.0 and was determined by the method

of Schatzmann (1973). The calculated Ca^{2+} concentration was confirmed by measurement with an Orion 93–20 Ca^{2+} -selective electrode in conjunction with an Orion 811 microprocessor (Simons, 1982).

Results

Further characterization of protein kinase activity

Our previous studies described a Ca^{2+} -activated calmodulin-dependent protein kinase that phosphorylated a 57 kDa islet-cell protein band. Longer electrophoretic separation (15 h rather than 6 h) of the phosphoproteins demonstrated that this band was in fact at least two separate protein bands migrating at relative molecular weights of 57 000 and 54 000.

The Ca^{2+} -sensitivity of this protein kinase activity with regard to the phosphorylation of these proteins was investigated by using the islet-cell microsomal fraction. This subcellular fraction served as a source of both kinase and endogenous substrates. These assays were terminated after an incubation period of 5 s such that protein phosphorylation would reflect the rate of enzyme activity rather than the availability of the endogenous substrates (Landt *et al.*, 1982). In the presence of calmodulin (1 μM) the rate of phosphorylation of both the 57 kDa and 54 kDa protein bands was stimulated by free Ca^{2+} concentrations in the micromolar range (Fig. 1). However, in the absence of added calmodulin, the addition of Ca^{2+} (0.1 μM –1 mM) did not affect the rate of protein kinase activity (results not shown). The activation of protein kinase activity by Ca^{2+} was saturable with respect to Ca^{2+} concentration and similar for the phosphorylation of both protein bands. In three experiments, half-maximal activation of protein kinase activity occurred at $1.99 \pm 0.87 \mu\text{M}$ - and $1.92 \pm 0.78 \mu\text{M}$ - Ca^{2+} for the 57 kDa and 54 kDa protein bands respectively.

The calmodulin requirement for the phosphorylation of these protein bands by the islet-cell microsomal protein kinase activity was investigated in similarly designed experiments. In the absence of Ca^{2+} and in the presence of 200 μM -EGTA, calmodulin (2–40 $\mu\text{g}/\text{ml}$) did not affect the rate of phosphorylation of either protein band (results not shown). The addition of calmodulin in the presence of 800 μM free Ca^{2+} produced a concentration-dependent increase in protein kinase activity (Fig. 2). Half-maximal activation of the rate of phosphorylation occurred at 4.37 ± 1.09 and $4.05 \pm 0.40 \mu\text{g}$ of calmodulin/ml for the 57 kDa and 54 kDa protein bands respectively ($n = 3$). Maximal stimulation of the rate of phosphorylation occurred at approx. 10 μg of calmodulin/ml (590 nM).

Cyclic AMP did not affect the protein kinase activity present in the islet-cell microsomal fraction. In these studies, protein kinase assays were per-

Fig. 1. Dependence of islet-cell protein kinase activity on free Ca^{2+}

The rate of calmodulin-dependent protein kinase activity was measured in 5 s assays as described in the Experimental section. The islet-cell microsomal fraction was utilized as the source for both protein kinase and endogenous substrates. Free Ca^{2+} concentrations were maintained with 0.2 mM-EGTA as described in the Experimental section. Ca^{2+} -dependent P_i incorporation was determined by subtracting [^{32}P] P_i incorporated into the 57 kDa band (0.94 ± 0.1 pmol/mg per 5 s) and 54 kDa band (0.67 ± 0.07 pmol/mg per 5 s) in the absence of Ca^{2+} . The results are those obtained with 1 μM -calmodulin in a representative experiment ($n = 3$).

Fig. 2. Calmodulin-dependence of islet-cell protein kinase activity

The rate of protein kinase activity was measured as described in the Experimental section. The islet-cell microsomal fraction was used as a source of both protein kinase activity and endogenous substrates. Results show the rate of protein phosphorylation of the 57 kDa and 54 kDa substrates in the presence of 800 μM free Ca^{2+} for a representative experiment ($n = 3$).

formed with 10 μM - or 100 μM -cyclic AMP and the phosphodiesterase inhibitor theophylline (1 mM), in 5 s incubations (5 μM -ATP) and in 2 min incubations (20 μM -ATP). In no case ($n = 3$) did cyclic AMP affect the phosphorylation of the 57 kDa or 54 kDa protein bands (results not shown).

Intact-islet experiments

To determine whether phosphorylation of the 57 kDa and 54 kDa protein bands occurs in intact islets and if such phosphorylation could be correlated with insulin secretion, intact islets were incubated with [^{32}P] P_i to label endogenous pools of ATP. After experimental incubations to stimulate the rate of insulin secretion, the islets were dissolved in SDS and subjected to electrophoresis as described in the Experimental section. Parallel lanes on the polyacrylamide slab gels contained cell-free protein kinase incubations with or without Ca^{2+} and calmodulin as an internal marker for these protein substrates. The intact islets were found to phosphorylate proteins that had the same electrophoretic mobilities as did the 57 kDa and 54 kDa protein substrates (Fig. 3). In experiments using intact islets, the 54 kDa protein band was more heavily phosphorylated than was the 57 kDa band, whereas in the cell-free protein kinase assays phosphorylation of the 57 kDa band was more prominent (Fig. 3).

Islets that were prelabelled with [^{32}P] P_i and incubated for 5 min in the presence of 28 mM-D-glucose secreted significantly more insulin into the medium ($P < 0.05$) and had a significantly greater incorporation of [^{32}P] P_i into the 54 kDa protein band than did islets incubated with 5 mM-D-glucose ($P < 0.05$; Fig. 4). Although there was a trend toward an increase in [^{32}P] P_i incorporation into the 57 kDa protein band at the higher glucose concentration, this effect was not statistically significant ($P > 0.05$). The effect of 28 mM-glucose was more pronounced in the presence of 5 mM-theophylline, a known potentiator of glucose-induced insulin secretion. In the absence of extracellular Ca^{2+} , glucose-induced insulin secretion did not occur, and 28 mM-glucose decreased, rather than increased, the phosphorylation of the 54 kDa protein band ($P < 0.05$).

In contrast with results obtained in 5 min experiments, incubation of prelabelled islets for 30 s in medium containing 28 mM-glucose did not affect phosphorylation of the protein bands (results not shown). When the islets were incubated for various times with 28 mM-glucose rather than 5 mM-glucose (without theophylline) the maximum stimulation of the phosphorylation of the 54 kDa protein band occurred at 3 min (Fig. 5). Under these conditions and incubation times, there was no detectable stimulation of [^{32}P] P_i incorporation into the 57 kDa band.

A 5 min incubation of prelabelled islets with the

Fig. 3. Autoradiogram of phosphorylation of 57 kDa and 54 kDa proteins in intact islets and protein kinase assays

Protein kinase assays with islet-cell homogenates and intact-islet phosphorylation experiments were performed as described in the Experimental section. Lanes 1 and 2 show the effect of Ca^{2+} and calmodulin (1 mM and 1 μM respectively) on protein kinase activity. Lanes 3–5 show the phosphorylation of proteins in this molecular-weight region that occur in intact islets. The 57 kDa and 54 kDa protein bands were marked with pin holes by using the exposed film as a guide, and then cut from the gel for measurement of radioactivity.

Ca^{2+} -ionophore A23187 (10 μM) under otherwise standard conditions as described in the Experimental section also produced a modest increase in the [^{32}P]P_i incorporated into the islet-cell proteins. In each of four experiments, 10 μM -A23187 increased the phosphorylation of the 57 kDa ($14 \pm 3\%$) and 54 kDa ($16 \pm 8\%$) protein bands ($P < 0.05$).

Identification of the protein substrates

The islet-cell protein bands that were phosphorylated by the Ca^{2+} - and calmodulin-dependent protein kinase had electrophoretic mobilities on 10%-polyacrylamide gels that were indistinguishable from those of the α - and β -subunits of chromatographically purified brain tubulin. To determine if the tubulin subunits could serve as substrates for the islet-cell protein kinase, tubulin was added to protein kinase assays that used the islet-cell microsomal fraction as a source of the

Fig. 4. Effect of glucose and theophylline on phosphorylation of 57 kDa and 54 kDa protein bands and on insulin secretion in intact islets

Islets were loaded with [^{32}P]P_i to label endogenous ATP pools as described in the Experimental section. Then 5 min incubations with islets were performed in incubation medium containing 5 mM- or 28 mM-glucose with or without 5 mM-theophylline as described in the Experimental section. Glucose (28 mM) significantly stimulated phosphorylation of the 54 kDa protein ($P < 0.05$). Incubations were also performed in the absence of Ca^{2+} (▨). Radioactivity (c.p.m.) in each band is normalized by consideration of the total radioactivity contained in the 100 islets in each sample ($n = 9$).

enzyme. Assays were performed at time periods greater than 15 s such that the substrate concentration would become rate-limiting. Addition of tubulin to these kinase assays resulted in a significant increase in [^{32}P]P_i incorporated into the 57 kDa and 54 kDa protein bands, whereas tubulin added in the absence of islet-cell protein was not phos-

Fig. 5. Effect of glucose on phosphorylation of 57 kDa and 54 kDa protein bands in intact islets incubated for various times

Islets were loaded with [${}^{32}\text{P}$]ATP to label endogenous ATP pools as described in the Experimental section. Experimental incubations with islets containing (□) 5 mM- or (▨) 28 mM-glucose were terminated after 1, 3 or 5 min [28 mM-glucose increased phosphorylation of the 54 kDa band ($P < 0.05$)]. Data represent means $\pm$ S.E.M. for four experiments performed in triplicate. Radioactivity (c.p.m.) per band was normalized as described in Fig. 4.

Fig. 6. Autoradiogram of protein kinase assays containing 1 μg of exogenous tubulin

Protein kinase assays were performed with the islet-cell microsomal fraction as described in the Experimental section. Assays were performed at 37°C for an extended period of 30 s so that the endogenous protein substrates become rate-limiting. Tubulin (1 μg) was added to assays with or without Ca^{2+} (800 μM) and calmodulin (1 μM). Lanes 5 and 6 contained tubulin in the absence of islet-cell protein, as a control for auto-phosphorylation.

phosphorylated (Fig. 6). Furthermore, phosphorylation of exogenous tubulin was stimulated by the addition of Ca^{2+} and calmodulin. In 30 s assays, the Ca^{2+} - and calmodulin-dependent phosphorylation of the subunit of tubulin migrating with the 57 kDa band was more pronounced ($P < 0.05$) than that for the tubulin subunit migrating with the 54 kDa band ($P > 0.05$). These results indicated that the tubulin subunits could serve as substrates for the islet-cell protein kinase, and that phosphorylated tubulin (as well as non-phosphorylated tubulin) had the same electrophoretic mobility as the endogenous 57 kDa and 54 kDa substrates.

To determine if the endogenous substrates for the islet-cell protein kinase activity may in fact be α - and β -tubulin, the islet-cell microsomal fraction was pretreated with anti-tubulin antibody before the assay of protein kinase activity. Pretreatment of the islet-cell microsomal fraction (4–5 μg of microsomal protein; 13–15 μg of antibody) for 30 min at 37°C decreased the subsequent Ca^{2+} - and calmodulin-dependent phosphorylation of the 57 kDa and 54 kDa protein bands by 79 ± 6 and $71 \pm 7\%$ respectively, as measured in 30 s assays ($n = 3$). This

effect of anti-tubulin antibody was reversed by pretreatment of the antibody at 100°C for 10 min (Fig. 7). Although heat treatment under these conditions did not totally destroy the antibody, this treatment served to decrease the antibody-induced inhibition of the phosphorylation of the 57 kDa band by 50–70% and that of the 54 kDa band by 80–100%. Control sheep immunoglobulin G was without effect (result not shown).

Experiments were undertaken to determine whether the phosphorylated endogenous substrates could be precipitated with tubulin antibody. After the standard protein kinase assay, the islet-cell microsomal fraction was treated with 1% Triton X-100 and 1% deoxycholate and then incubated with sheep anti-tubulin and goat anti-(sheep immunoglobulin) serum. To minimize dephosphorylation during the antibody treatment, incubations were performed in the presence of 10 mM-fluoride and 10 mM-EGTA for 30 min at 37°C. In four experiments the anti-tubulin antibody precipitated 555 ± 100 and 142 ± 30 c.p.m. of Ca^{2+} - and calmodulin-stimulated ${}^{32}\text{P}$ incorporated into the 57 kDa and 54 kDa protein bands respectively. This

Fig. 7. Effect of anti-tubulin serum on protein kinase activity

Islet-cell microsomal protein was pretreated with control buffer, sheep anti-tubulin serum, or heat-treated anti-tubulin serum for 30 min at 37°C . Protein kinase assays were then initiated after addition of pretreated membranes and terminated after 30 s as described in the Experimental section. Results are from a representative experiment ($n = 4$).

represented a significant increase of the precipitation of ^{32}P relative to that which occurred in control tubes that were incubated with non-specific antibody ($P < 0.05$, paired t test). However, blank tubes incubated in the absence of specific antibody did contain significant radioactivity above background (274 ± 72 and 91 ± 32 c.p.m. in 57 kDa and 54 kDa regions respectively), indicating that there was incomplete solubilization or reaggregation of the membranes during the antibody treatment.

Discussion

These studies have expanded our previous observation that pancreatic islets contain a membrane-bound calmodulin-dependent protein kinase activity (Landt *et al.*, 1982), and support the hypothesis that the Ca^{2+} -activated phosphorylation of endogenous proteins by this kinase activity may play a role in the regulation of insulin secretion.

The concentration of Ca^{2+} required for half-maximal activation of the islet-cell protein kinase ($1\text{--}3\ \mu\text{M}$) is consistent with the possibility that this protein kinase is activated by increased Ca^{2+} concentrations that occur during the stimulation of insulin secretion. Other evidence indicates that Ca^{2+} concentrations in islets increase after treatment with insulinotropic agents (Wollheim & Sharp, 1981) and may attain micromolar values. In permeabilized islets (Yaseen *et al.*, 1982), micromolar free Ca^{2+} concentrations are required to stimulate insulin secretion. We have shown that the islet-cell microsomal fraction, in which this protein kinase activity is located, also contains an active Ca^{2+} -sequestering

activity that has an affinity for Ca^{2+} in this concentration range, i.e. $1\text{--}3\ \mu\text{M}$ (Colca *et al.*, 1982). The calmodulin requirement for this enzyme activity is similar to the calmodulin requirement for a similar membrane-bound protein kinase that was discovered in adipocytes (Landt & McDonald, 1980).

It appears that this calmodulin-dependent protein kinase activity in the islet-cell microsomal fraction is not regulated by cyclic AMP. Ashcroft and colleagues (Gagliardino *et al.*, 1980; Harrison & Ashcroft, 1982) have also described a calmodulin-stimulated phosphorylation of an endogenous protein band (53–55 kDa) in an islet-cell homogenate. These investigators have reported that the phosphorylation of this protein band in islet-cell homogenates was increased by cyclic AMP (Harrison & Ashcroft, 1982). We have also found that cyclic AMP enhanced the phosphorylation of the 57 kDa protein band in assays which used the whole islet homogenate. Thus $10\ \mu\text{M}$ -cyclic AMP and $5\ \text{mM}$ -theophylline produced a $78 \pm 24\%$ increase in the rate of phosphorylation of the 57 kDa protein band ($P < 0.05$) and a $35 \pm 4\%$ decrease in the rate of phosphorylation of the 54 kDa protein band ($P < 0.05$; $n = 3$, 5 s assays). Similar results were obtained in protein kinase assays that were incubated for 2 min, but the magnitude of the effects was less (results not shown). At present, it is not clear whether the differential effect of cyclic AMP on protein phosphorylation patterns in the homogenate as compared with the microsomal fraction indicates the presence of an additional protein kinase activity and/or substrates that is/are eliminated in the latter fraction.

Both the 57 kDa and 54 kDa phosphoproteins were found to occur in intact islets. The increase in phosphorylation of the 54 kDa protein produced by glucose in intact-cell studies was correlated with stimulation of insulin secretion and supports the contention that the calmodulin-dependent protein kinase activity is involved in the insulin secretory response. As expected, removal of extracellular Ca^{2+} prevented glucose-induced insulin secretion and the glucose-induced increase in the phosphorylation of this protein. The paradoxical decrease in phosphorylation of the 54 kDa protein band produced by glucose in the absence of extracellular Ca^{2+} may result from the redistribution of remaining cellular Ca^{2+} . Glucose is thought to affect Ca^{2+} metabolism at several β -cell loci (Wollheim & Sharp, 1981). The involvement of Ca^{2+} in this cellular response is also supported by the increase in phosphorylation of these proteins caused by the Ca^{2+} ionophore A23187.

Suzuki *et al.* (1981) have shown that elevated concentrations of glucose increased the steady-state incorporation of $[^{32}\text{P}]\text{P}_i$ into at least six islet-cell phosphoproteins. However, it is not clear whether

these results reflect an increase in protein kinase activity or an increase in [^{32}P]P_i uptake from the incubation medium, since these studies were done in the continued presence of [^{32}P]P_i in the medium. The present studies were performed with islets that were extensively washed and free of external radiolabel. In the present experiments, total ^{32}P content was not affected detectably by the experimental treatments (results not shown). Although the present technique rules out the participation of [^{32}P]P_i uptake in the interpretation of the results, an increase in ATP synthesis by the experimental glucose concentration (Ashcroft *et al.*, 1973) would decrease the specific radioactivity of the ATP pools and impart a negative bias to the results. These experiments with intact islets are also limited technically by the high background radioactivity that may obscure small changes in [^{32}P]P_i incorporation into specific protein bands. This problem may be circumvented in future experiments by immunoprecipitation of the endogenously phosphorylated substrates with anti-tubulin antibody.

Cell-free experiments favour the hypothesis that the major substrates for the Ca^{2+} - and calmodulin-dependent protein kinase may be the α - and β -subunits of tubulin. This conclusion is supported, since: (1) the electrophoretic mobilities of the 57 kDa and 54 kDa protein substrates are identical with those of the subunits of tubulin; (2) tubulin added to the islet-cell protein kinase assays augmented the phosphorylation of the 57 kDa and 54 kDa bands, in a Ca^{2+} - and calmodulin-sensitive manner; (3) pre-treatment of the islet-cell microsomal fraction with anti-tubulin antibody greatly diminished the subsequent protein kinase activity; and (4) the endogenous phosphorylated substrates could be immunoprecipitated by incubation with anti-tubulin antibody. Also, incubation of the endogenously phosphorylated protein substrates with anti-tubulin antibody at 37°C significantly decreased the rate of dephosphorylation of these substrates in the absence of fluoride and EGTA (results not shown).

Burke & DeLorenzo (1981) have also presented evidence that brain cytosol contains a Ca^{2+} -calmodulin-dependent protein kinase activity that can phosphorylate the α - and β -subunits of tubulin. They have further postulated that this calmodulin-dependent protein kinase system may be directly involved in neurotransmitter release (DeLorenzo, 1981). The characteristics of the brain enzyme with regard to Ca^{2+} requirement (half-maximal, 0.8 μM) and calmodulin-sensitivity (half-maximal, 4 $\mu\text{g}/\text{ml}$) (Burke & DeLorenzo, 1981) are similar to those described for the islet enzyme in the present study.

Although the significance of our findings with regard to microtubule function in islets is not clear, calmodulin is known to have effects on microtubule assembly *in vitro*. Calmodulin has been reported

to inhibit microtubule assembly (Marcum *et al.*, 1978; Nishida *et al.*, 1979), and under certain conditions may increase microtubule assembly (Lee & Wolff, 1982). Microtubules are thought to play a critical role in the release process for insulin secretory granules from the β -cell (Lacy *et al.*, 1968, 1972; Malaisse *et al.*, 1971). Thus, if increases in cellular Ca^{2+} affect microtubule function in the β -cell by phosphorylation of tubulin via calmodulin-dependent protein kinase, this may represent an important transduction of the intracellular Ca^{2+} signal produced by insulinotropic agents such as glucose.

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