Substrates for cyclic AMP-dependent protein kinase in islets of Langerhans

Studies with forskolin and catalytic subunit

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To investigate substrates for cyclic AMP-dependent protein kinase in intact islets of Langerhans, batches of islets were incubated with [32 P]P_i for 1 h in the presence of 10 mM-glucose; the adenylate cyclase activator forskolin, which in parallel experiments was shown to increase islet cyclic AMP content and insulin release, was then added. Islets were homogenized and subcellular fractions prepared by differential centrifugation. Phosphopeptides were electrophoresed on sodium dodecyl sulphate/polyacrylamide gels and quantified by autoradiography and densitometry. Within 5 min forskolin caused increased labelling of M_r -25 000 and -30 000 cytosolic and M_r -23 000 and -32 000 particulate peptides; a rapid decrease in phosphorylation of M_r -18 000 and -34 000 cytosolic peptides was also observed. In addition, rather slower phosphorylation occurred of the M_r -15 000 peptide previously identified as histone H3 [Christie & Ashcroft (1984) *Biochem. J.* **218**, 87–99]. When similar subcellular fractions were incubated with [γ - 32 P]ATP and purified catalytic subunit of cyclic AMP-dependent protein kinase, peptides phosphorylated included cytosolic species of M_r 25 000 and 30 000 and particulate species of M_r 23 000 and 32 000. The distribution of RNA in the subcellular fractions suggested that the M_r -32 000 species could be a ribosomal protein. The 24 000 g pellet was heterogeneous, as judged by marker assays, and was therefore fractionated further by Percoll-density-gradient centrifugation. The peak containing the M_r -23 000 peptide was resolved from marker enzymes for plasma membranes, mitochondria and endoplasmic reticulum and coincided with a peak for insulin: hence the M_r -23 000 peptide is likely to be a secretory-granule component. The study demonstrates that the potentiation of insulin release that occurs when islet cyclic AMP is increased is accompanied by rapid phosphorylation of specific islet substrates for cyclic AMP-dependent protein kinase. The data are consistent with the hypothesis that protein phosphorylation is involved in the regulation of insulin secretion.

Changes in the intracellular concentration of cyclic AMP have been shown to be involved in the regulation of pancreatic B-cell function (reviewed by Sharp, 1979). Although the way in which cyclic AMP acts on insulin secretion is not known, evidence has been presented for effects of cyclic AMP on intracellular Ca²⁺ handling (Hahn *et al.*, 1980), Ca²⁺ influx (Henquin & Meissner, 1984), tubulin synthesis (Pipeleers *et al.*, 1976), microtubule assembly (Montague *et al.*, 1976) and secretory-granule interaction with microtubules (Suprenant & Dentler, 1982).

It is generally accepted that in eukaryotes cyclic AMP acts via phosphorylation of specific proteins catalysed by cyclic AMP-dependent protein kinase. Elucidation of the exact mechanism whereby cyclic AMP exerts its control on the secretory process thus requires a knowledge of the substrates for cyclic AMP-dependent protein kinase in islets of Langerhans. Several investigators have shown changes in the phosphorylation state of proteins in insulin-secreting tissues incubated with agents known to increase intracellular cyclic AMP concentration (Schubart *et al.*, 1980; Schubart, 1982; Suzuki *et al.*, 1983; Christie & Ashcroft, 1984), but a direct role for cyclic AMP-dependent protein

Abbreviation used: SDS, sodium dodecyl sulphate.

kinase in the phosphorylation of such peptides has not been demonstrated. In the present study we have used two approaches to study protein phosphorylation in islets; in intact cell studies, islets incubated with [32 P] P_i to label cellular ATP were stimulated with the adenylate cyclase activator forskolin. In studies using extracts of islets, islet fractions were incubated with [γ - 32 P]ATP and purified catalytic subunit of cyclic AMP-dependent protein kinase. Subcellular fractionation and SDS/polyacrylamide-gel electrophoresis were used for analysis of proteins phosphorylated.

Materials and methods

Materials

Collagenase was from Serva, Heidelberg, Germany. Forskolin was from Calbiochem, CP Laboratories, Bishop's Stortford, Herts., U.K. [32 P] P_i and cyclic AMP radioimmunoassay kits were from NEN Chemicals, Dreieich, W. Germany. [γ - 32 P]ATP, [125 I]-insulin and [2- 3 H]5'-AMP were from Amersham International, Amersham, Bucks., U.K. Catalytic subunit of bovine cyclic AMP-dependent protein kinase was a gift from Professor D.A. Walsh, Department of Biological Chemistry, University of California, Davis, CA, U.S.A. Rat insulin standard was a gift from Dr. A. J. Moody, Novo Research Laboratories, Copenhagen, Denmark. Guinea-pig anti-insulin serum was from Wellcome Laboratories, Beckenham, Kent, U.K. Quaternary aminoethyl (QAE)-Sephadex and Percoll were from Pharmacia, Uppsala, Sweden. X-OMAT AR and S films were from Kodak, Hemel Hempstead, Herts., U.K. Acryl-Aide was from Miles Laboratories, Slough, Bucks., U.K. Hepes, bovine serum albumin, dithiothreitol and cytochrome *c* were from Sigma. NADH, 5'-AMP and adenosine were from Boehringer, Lewes, Sussex, U.K. All other reagents were from BDH Chemicals, Poole, Dorset, U.K.

Preparation of islets of Langerhans

Islets were prepared by collagenase digestion (Coll-Garcia & Gill, 1969) of pancreases from male Wistar rats fed *ad libitum* on a standard laboratory diet. Free islets were collected from the digest with a wire loop.

Islet incubation medium

The medium used for incubation of intact islets of Langerhans had the following ionic composition (mM): Na⁺, 141; K⁺, 5.9; Ca²⁺, 2.5; Mg²⁺, 1.2; SO₄²⁻, 1.2; Cl⁻, 105; HCO₃⁻, 5; Hepes, 20. In some experiments the medium also contained 1.2 mM- P_i . The medium also contained bovine serum albumin (2 g/l) and was supplemented with

glucose, forskolin or [32 P] P_i at the concentrations indicated in the text.

Insulin release and islet cyclic AMP content

Batches of five islets were preincubated for 10 min at 37°C in 50 μ l of medium containing glucose (2 mM) in the absence or presence of 1.2 mM- P_i . A further 50 μ l of medium containing glucose alone or glucose plus forskolin was added and incubation continued for 60 min. The final concentration of glucose was 2 or 10 mM and that of forskolin (diluted from a 25 mM stock in dimethylsulphoxide) was 10 μ M. After incubation, the tubes containing the islets were centrifuged at 100g for 30 s and 80 μ l of medium was removed for radioimmunoassay of insulin (Ashcroft & Crossley, 1975). Metabolism was arrested and cyclic AMP extracted by the addition of 100 μ l of boiling 50 mM-sodium acetate buffer, pH 6.2, followed by boiling for 5 min. The boiled extract was sonicated for 5 s at 50 W with a Soniprobe (Dawe Instruments) and stored at -16°C until assayed. The cyclic AMP content of the sonicated material was assayed with a commercial radioimmunoassay kit according to the instructions of the manufacturers (New England Nuclear) for the procedure with acetylation, except that half the recommended volumes were used.

Incorporation of [32 P] P_i into islet ATP

Batches of 20 islets were incubated for 1 h at 37°C in 100 μ l of medium containing glucose (10 mM) and [32 P] P_i (50–100 μ Ci/ml; carrier-free) in the absence or presence of forskolin (10 μ M). In some experiments unlabelled P_i (1.2 mM) was added. The specific radioactivity of islet ATP was measured as previously described (Christie & Ashcroft, 1984).

Phosphorylation of peptides in intact islets

Batches of 25 islets were preincubated for 1 h at 37°C in 100 μ l of medium containing glucose (10 mM) and [32 P] P_i (0.5–2.5 mCi/ml; carrier-free). Forskolin (20 μ M, diluted from the 25 mM-stock) was added in 100 μ l of the above medium free of [32 P] P_i and incubation continued for the times indicated in the text. Controls were included in the absence of forskolin. Islets were rapidly washed at 37°C with 1 ml of medium containing glucose (10 mM) with or without forskolin (10 μ M) as appropriate. Islets (150 per condition) were pooled and homogenized in 50 μ l of ice-cold 0.3 M-sucrose/50 mM-sodium phosphate (pH 7)/50 mM-NaF/2 mM-EDTA/0.2 mM-EGTA/1 mM-phenylmethanesulphonyl fluoride with 20 strokes of a motor-driven Teflon homogenizer. Islets were fractionated by differential centrifugation as described below. SDS and Bromophenol Blue were

added to concentrations of 1% and 0.01% (w/v) respectively, and the samples were boiled for 5 min. 2-Mercaptoethanol was added (final concn. 10%, v/v) and phosphopeptides were analysed by SDS/polyacrylamide-gel electrophoresis.

Phosphorylation of islet peptides by catalytic subunit of cyclic AMP-dependent protein kinase

Islets (400) were collected into 50 μ l of 0.3 M-sucrose/50 mM-sodium phosphate (pH 7)/1 mM-phenylmethanesulphonyl fluoride and homogenized with 20 strokes of a motor-driven Teflon homogenizer. Fractionation by differential centrifugation or Percoll-density-gradient centrifugation was performed as described below. Islet fractions (10 μ l) were incubated at 30°C for 2 min with 230 μ units of catalytic subunit of cyclic AMP-dependent protein kinase (1 unit catalyses the transfer of 1 μ mol of phosphate/min from ATP to histone at 30°C) in 25 μ l of 50 mM-sodium phosphate (pH 7)/5 mM-MgCl₂/25 μ M-[γ -³²P]ATP (3 Ci/mmol)/0.5 mM-EGTA/1 mM-dithiothreitol. Control experiments were performed in the absence of catalytic subunit. Incubation was terminated by the addition of 25 μ l of 50 mM-sodium phosphate (pH 7)/SDS (2%, w/v)/Bromophenol Blue (0.02%, w/v)/sucrose (50 mg/ml) and boiling for 5 min. Phosphorylated peptides were analysed by SDS/polyacrylamide-gel electrophoresis.

Fractionation by differential centrifugation

Homogenized islets were centrifuged at 600 g for 5 min at 4°C. The supernatant was removed and the pellet fraction rehomogenized in 50 μ l of sucrose solution of the same composition as that used for the initial homogenization (see above) with 20 strokes of a motor-driven Teflon homogenizer and centrifuged for a further 5 min at 600 g. The combined supernatants were centrifuged at 24000 g for 10 min at 4°C. The supernatant was removed and centrifuged at 190000 g for 30 min at 4°C. Pellet fractions were resuspended in 100 μ l of the same sucrose solution and sonicated for 5 s at 50 W with a Dawe Soniprobe.

Fractionation of islets by Percoll-density-gradient centrifugation

Islets (400) were homogenized in 50 μ l of 0.3 M-sucrose/50 mM-sodium phosphate (pH 7)/1 mM-phenylmethanesulphonyl fluoride and fractionated by differential centrifugation as described above. The 24000 g pellet fraction was resuspended by homogenization in 100 μ l of the above sucrose solution and layered on to Percoll (36%, v/v)/0.3 M-sucrose/50 mM-sodium phosphate (pH 7)/1 mM-phenylmethanesulphonyl fluoride of density 1.084 g/ml. The density gradient was generated by centrifuging at 24000 g for 30 min, and fractions

(100 μ l) were collected from the top of the gradient. The gradient profile was assessed by measuring, with a refractometer, the density of fractions obtained from a similar balance tube in the absence of islet material.

SDS/polyacrylamide-gel electrophoresis

Samples prepared for SDS/polyacrylamide-gel electrophoresis were run on 12.5% polyacrylamide gels (Laemmli, 1970). In some experiments the olefinic agarose derivative AcrylAide replaced *NN'*-methylenebisacrylamide as the cross-linker (ratio of acrylamide/AcrylAide, 32:1, w/w) and the final concentration of acrylamide was increased to 16%; this gave a separation of peptides equivalent to that with 12.5%-acrylamide/bisacrylamide gels. These polyacrylamide concentrations were selected after preliminary experiments using 10–25%-polyacrylamide gradient gels to determine the *M_r* range of peptides for which significant changes in phosphorylation were observed. Dried gels were autoradiographed by using Kodak X-OMAT AR or S film. The extent of phosphorylation of individual peptides was assessed by densitometric scanning of autoradiograms on a Joyce-Loebl Chromoscan 3 densitometer, by using peak height as a measure of phosphate incorporation. Densitometric traces shown are representative of at least three similar experiments.

Miscellaneous methods

Fractions obtained from differential centrifugation or Percoll-density-gradient centrifugation of islet homogenates were analysed for protein, RNA, insulin and marker enzymes of subcellular organelles. Protein content was assayed by the Coomassie Blue-binding method of Bradford (1976). RNA was measured by the u.v. absorbance method of Fleck & Munro (1962). Lactate dehydrogenase was measured as previously described (Christie & Ashcroft, 1984). Insulin was measured by radioimmunoassay as described by Ashcroft & Crossley (1975). NADH-cytochrome *c* reductase was measured by the method of Dallner *et al.* (1966) and cytochrome oxidase by the method of Cooperstein & Lazarow (1951). 5'-Nucleotidase was assayed by incubating islet fractions at 30°C for 1 h in 100 μ l of 50 mM-Tris (pH 8)/3 mM-MgCl₂/bovine serum albumin (0.1 g/litre)/2 mM-[2-³H]AMP (500 μ Ci/mmol). Reaction was terminated by boiling. Adenosine (0.4 ml; 0.1 mM) in 20 mM-ammonium formate was added and [³H]adenosine was separated from unchanged [³H]AMP by passage through 0.75 ml columns of QAE-Sephadex A-25 (formate form). Columns were washed with 1.5 ml of 20 mM-ammonium formate and eluates counted for radioactivity by liquid-scintil-

lation spectrometry in methoxyethanol scintillation fluid (Severson *et al.*, 1974).

Results

Effects of forskolin on insulin release and islet cyclic AMP content

The data are given in Table 1. When islets were incubated in the absence of phosphate, insulin release was stimulated 11-fold by raising the glucose concentration from 2 to 10 mM. Insulin release stimulated by 10 mM-glucose was potentiated more than 3-fold by 10 μ M-forskolin. The islet content of cyclic AMP was almost doubled by 10 mM-glucose and increased a further 5-fold by 10 μ M-forskolin. Similar results were obtained when the medium was supplemented with 1.2 mM- P_i . Islet cyclic AMP content was also elevated by forskolin when islets were incubated for only 5 min with the drug, from 5.32 ± 0.92 fmol/islet in the presence of 10 mM-glucose to 37 ± 3.05 fmol/islet ($n=10$) in the presence of 10 mM-glucose plus 10 μ M-forskolin. Glucose itself did not affect islet cyclic AMP content after a 5 min incubation.

Incorporation of [^{32}P]P_i into islet ATP

The specific radioactivity of islet ATP after incubation for 1 h in medium containing carrier-free [^{32}P]P_i in the presence of 10 mM-glucose or 10 mM-glucose plus 10 μ M-forskolin was 62.3 ± 3.8 and 62.0 ± 1.6 μ Ci/ μ mol ($n=5$) respectively. The corresponding values for incubation in 1.2 mM- P_i were 5.00 ± 0.64 and 4.74 ± 0.32 μ Ci/ μ mol ($n=5$). Thus forskolin failed to affect the rate of incorporation of [^{32}P]P_i into islet ATP under either condition.

Effect of forskolin on protein phosphorylation

Fig. 1 shows densitometric scans of autoradiographs obtained after incubating intact islets with

[^{32}P]P_i to label ATP and stimulating with forskolin for 5 min. Islets were subsequently fractionated by differential centrifugation and the subcellular fractions obtained were subjected to electrophoresis on SDS/polyacrylamide gels. Comparison of the upper traces (forskolin-treated) with the controls show that forskolin caused increased labelling of particulate peptides of M_r 23000 and 32000 and cytosolic peptides of M_r 25000 and 30000. Effects of forskolin were quantified by measuring heights of the appropriate peaks; results are expressed as percentages of controls. As shown in Table 2, these increases were maintained for periods of up to 1 h. Forskolin also caused a decrease in labelling of cytosolic peptides of M_r 18000 and 34000; the change in the former was the more marked. This effect was lost after 1 h incubation. Table 2 also shows a somewhat slower increase in labelling of a peptide of M_r 15000 appearing in the 600 g pellet fraction. The magnitude of response to forskolin was considerably greater for this peptide than for the other peptides affected.

Effect of catalytic subunit on protein phosphorylation in islet fractions

Incubation of subcellular fractions of islets with [γ - ^{32}P]ATP and catalytic subunit of cyclic AMP-dependent protein kinase resulted in the phosphorylation of a number of proteins. Fig. 2 illustrates typical densitometric traces of autoradiographs of such peptides separated by SDS/polyacrylamide-gel electrophoresis. Catalytic subunit stimulated the phosphorylation of particulate peptides of M_r 17000, 23000, 32000, 45000 and 53000, and cytosolic peptides of M_r 20000, 25000, 30000, 57000 and 60000. Peptides of M_r 23000, 25000, 30000 and 32000 comigrated on SDS/polyacrylamide gels with those of the same M_r phosphorylated in intact islets in response to forskolin.

The distribution of phosphopeptides in the frac-

Table 1. *Effects of forskolin on insulin release and islet cyclic AMP content*

Batches of five islets were preincubated at 37°C for 10 min in Hepes-buffered bicarbonate medium containing albumin (2 g/l) and glucose (2 mM), and either zero or 1.2 mM- P_i . Glucose and forskolin were then added to give the indicated final concentrations and incubation was continued for 60 min. Insulin released into the medium and the islet contents of cyclic AMP were measured by radioimmunoassays. Data are given as means $\pm$ S.E.M. for the numbers of observations in parentheses and the statistical significance of differences was assessed by the Mann-Whitney U-test for 10 mM-glucose versus 2 mM-glucose and for 10 μ M-forskolin plus 10 mM-glucose versus 10 mM-glucose alone: * $P<0.05$; ** $P<0.002$.

Additions to medium			Insulin release (μ units/h per islet)	Cyclic AMP (fmol/islet)
Glucose (mM)	P_i (mM)	Forskolin (μ M)		
2	0	0	7.3 ± 1.4 (10)	4.57 ± 0.59 (10)
10	0	0	77.2 ± 13.0 (10)**	8.71 ± 1.58 (10)*
10	0	10	265.4 ± 45.3 (10)**	46.13 ± 8.18 (10)**
2	1.2	0	18.4 ± 7.5 (9)	6.85 ± 0.75 (10)
10	1.2	0	88.3 ± 13.8 (9)**	9.71 ± 1.02 (10)*
10	1.2	10	196.8 ± 34.5 (9)*	51.15 ± 6.42 (10)**

Fig. 1. Effects of forskolin on protein phosphorylation in intact islets of Langerhans

Islets were preincubated for 1 h in Hepes-buffered bicarbonate medium containing [32 P]P $_i$ (carrier free), albumin (2g/l) and 10mM-glucose. Incubation was continued for a further 5 min either in the absence of forskolin (lower traces in each panel) or after addition of 10 μ M-forskolin (upper traces). After incubation, islets were washed in Hepes-buffered bicarbonate medium and homogenized in 0.3M-sucrose/50mM-sodium phosphate (pH 7)/50mM-NaF/2mM-EDTA/0.2mM-EGTA/phenylmethanesulphonyl fluoride. Differential centrifugation was carried out as described in the Materials and methods section to yield 600g, 24000g and 190000g particulate fractions and a supernatant fraction. Fractions were subjected to electrophoresis on SDS/16% acrylamide/0.5% AcrylAide gels. Dried gels were autoradiographed, and the Figure shows densitometric traces of the autoradiographs. The arrows indicate the M_r values ($\times 10^{-3}$) of the major bands whose phosphorylation was changed by forskolin.

Table 2. Time course of effects of forskolin on protein phosphorylation in intact islets

The effect of forskolin on phosphorylation of islet proteins was studied as described in the legend to Fig. 1, except that exposure to forskolin was for the incubation periods given in the Table. Phosphorylation of individual peptides was quantified by measurement of peak heights on the densitometric traces of the autoradiographs. Data are expressed as percentages of values in control incubations of the same duration in the absence of forskolin and are given as means $\pm$ S.E.M. for three experiments. The significance of the effects of forskolin was assessed by the paired Student's t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fraction	Phosphopeptides: M_r	Effect of forskolin (% of control) at time (min):			
		5	10	30	60
600g pellet	15000	110 $\pm$ 5	114 $\pm$ 10	204 $\pm$ 7**	580 $\pm$ 13***
24000g pellet	23000	188 $\pm$ 19*	218 $\pm$ 19*	156 $\pm$ 14	156 $\pm$ 4**
190000g pellet	32000	145 $\pm$ 9*	173 $\pm$ 5**	192 $\pm$ 3***	189 $\pm$ 18*
Supernatant	18000	57 $\pm$ 6*	50 $\pm$ 3**	52 $\pm$ 3**	98 $\pm$ 23
	25000	174 $\pm$ 16*	166 $\pm$ 16*	136 $\pm$ 7*	175 $\pm$ 15*
	30000	138 $\pm$ 3**	135 $\pm$ 1***	148 $\pm$ 4**	149 $\pm$ 9*
	34000	71 $\pm$ 11	73 $\pm$ 3*	88 $\pm$ 2**	112 $\pm$ 48

Fig. 2. Phosphorylation of proteins in islet subcellular fractions catalysed by exogenous catalytic subunit of cyclic AMP-dependent protein kinase

Homogenates of rat islets were fractionated by differential centrifugation into 600g, 24000g and 190000g particulate fractions and a supernatant fraction. Fractions were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence (lower traces in each panel) or presence (upper traces) of exogenous catalytic subunit of cyclic AMP-dependent protein kinase (230 μunits) and then subjected to electrophoresis on SDS/16% acrylamide/0.5% AcrylAide gels. Dried gels were autoradiographed, and the Figure shows densitometric traces of the autoradiographs. The arrows mark the positions of the major bands whose phosphorylation was enhanced by catalytic subunit and their M_r values ($\times 10^{-3}$) are indicated above the arrows.

tions was quantified by measuring heights of the appropriate peaks on the densitometric traces. The fractions were also assayed for markers of subcellular organelles. Results expressed as percentages recovered in each fraction are shown in Fig. 3. The distribution of the M_r -25000 and -30000 peptides correlated with the cytosolic marker lactate dehydrogenase. The greatest proportion of the M_r -32000 peptide appeared in the 190000g pellet fraction, which was low in activity of all subcellular markers except RNA. The M_r -23000 peptide appeared mainly in the 24000g pellet, which was rich in 5'-nucleotidase, cytochrome oxidase, NADH-cytochrome *c* reductase, insulin and RNA.

In order to determine the subcellular localization of this peptide, the 24000g pellet fraction was subjected to Percoll-density-gradient centrifugation, and fractions were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and catalytic subunit or assayed for marker enzymes. Results from a typical experiment are shown in Fig. 4. The plasma-membrane marker 5'-nucleotidase sedimented at a density of 1.05g/ml. The mitochondrial marker cytochrome oxidase and the endoplasmic-reticulum marker NADH-cytochrome *c* reductase sedimented at a density of 1.07g/ml. Insulin showed a triphasic distribution profile, with peak activities at the origin and at densities of 1.06 and 1.11g/ml. The M_r -23000 peptide sedimented with the third peak of insulin.

Fig. 3. Distribution of phosphopeptides and markers in islet subcellular fractions

Islet subcellular fractions were phosphorylated with [γ - 32 P]ATP and catalytic subunit of cyclic AMP-dependent protein kinase as described in the legend to Fig. 2. Phosphopeptides were quantified by densitometry of autoradiographs. Fractions were assayed for their content of protein, RNA, insulin, 5'-nucleotidase, cytochrome oxidase, NADH-cytochrome c reductase and lactate dehydrogenase by methods described in the text. Data for each fraction are expressed as a percentage of the total value for all fractions, and are given as means $\pm$ S.E.M. for three experiments.

Discussion

The modulatory effect of cyclic AMP on insulin secretion is well established (Sharp, 1979) and the presence and properties of islet cyclic AMP-dependent protein kinase have been documented (Sugden *et al.*, 1979). Very few studies, however, have been carried out to explore directly the proposition that effects of cyclic AMP on insulin secretion do involve phosphorylation of islet proteins by cyclic AMP-dependent protein kinase. Schubart *et al.* (1977) found that only one peptide, of M_r 28000, showed enhanced phosphorylation when hamster insulinoma cells were incubated under conditions that increased intracellular cyclic AMP. Since the M_r -28000 peptide was identified as a component of the 40S ribosomal subunit, it appeared unlikely to play any role in secretion. More recently, the same group, using two-dimensional electrophoresis, were able to detect increased phosphorylation of two cytosolic peptides of M_r 16000 in hamster insulinoma cells incubated with glucagon. Whether similar proteins occur in normal B-cells was not investigated; nor was any evidence given that the peptides were substrates for cyclic AMP-dependent protein kinase. Suzuki *et al.* (1983) reported that glucagon or dibutyl cyclic AMP enhanced phosphorylation of 15 peptides in rat islets. The changes were small (19–51%), no information was given on the subcellular location or

possible nature of the peptides, and it was not established that cyclic AMP was indeed increased under the conditions used; moreover, individual radioactive peaks were not clearly resolved, owing to the use of a gel-slicing technique rather than autoradiography for analysis of gels. In a previous study (Christie & Ashcroft, 1984), we have shown that potentiation of insulin release from rat islets by several agents that increase islet cyclic AMP content was accompanied by phosphorylation of islet protein. However, the major substrate detected in unfractionated islets was identified as histone H3, and thus unlikely to be involved in effects of cyclic AMP on insulin release. Centrifugation of islet homogenates before analysis of phosphopeptides revealed several cytosolic peptides phosphorylated in response to an increase in cyclic AMP; the possible occurrence of substrates in particulate fractions was not investigated.

Several aspects of the present study were designed to overcome the limitations of previous approaches. Firstly, we considered it important to use normal rat islets and to fractionate the homogenized islets before analysis of phosphopeptides to provide information on their subcellular location. Secondly, we chose to use forskolin, which has several advantages over other agents used to study cyclic AMP effects on islet-cell function, in that the responses are rapid and sustained, in contrast with the transient effects of glucagon on cyclic

Fig. 4. Percoll-density-gradient centrifugation of a 24000g particulate fraction from islet homogenate

A 24000g particulate fraction from islet homogenate was centrifuged for 30 min at 24000g on a Percoll density gradient (original density 1.084 g/ml). Fractions 1–11 were collected from the gradient; fraction 12 consisted of pelleted material resuspended in 100 μ l of 0.3 M-sucrose. Portions of the fractions were phosphorylated with [γ - 32 P]ATP and catalytic subunit of cyclic AMP-dependent protein kinase and subjected to electrophoresis on SDS/16% acrylamide/0.5% AcrylAide gels. Phosphorylation of the M_r -23000 peptide was quantified by densitometry of autoradiographs. Fractions were also assayed for their content of 5'-nucleotidase, insulin, cytochrome oxidase and NADH-cytochrome c reductase. The density of fractions obtained from a similar balance tube in the absence of islet material was measured with a densitometer. The results are representative of three similar experiments.

AMP concentrations (Schauder *et al.*, 1977) or the relatively slow response to cholera toxin (Christie & Ashcroft, 1984); and forskolin appears to be more specific than the commonly used methylxanthines, which have been shown to have effects unrelated to cyclic AMP metabolism (Sehlin, 1970; Sugden & Ashcroft, 1978). Thirdly, we have used exogenous catalytic subunit to show which

islet peptides were substrates for cyclic AMP-dependent protein kinase. This has important advantages over reliance on endogenous kinase activity, in that the extent of incorporation of 32 P can be large, facilitating detection of phosphopeptides. Moreover it avoids the problem that endogenous cyclic AMP-dependent protein kinase is almost entirely in the supernatant fraction (Monta-

gue & Howell, 1972): hence particulate fractions which contain protein substrates may fail to show significant phosphorylation in the absence of added kinase. This may explain why Thams *et al.* (1984) did not observe effects of cyclic AMP on the phosphorylation of proteins in a particulate fraction from mouse islets. The use of exogenous catalytic subunit was validated (results not shown) by the finding that addition of cyclic AMP to whole homogenates or to 600g-supernatant fractions caused detectable labelling of all the bands seen with added catalytic subunit. It was important also to ensure that the islet proteins were not exposed to grossly supra-physiological concentrations of catalytic subunit. In our experiments the concentration of catalytic subunit was 9 munits/ml; this is clearly well below the concentration of catalytic subunit in intact islets, which we estimate from the data of Sugden *et al.* (1979), assuming a mean intracellular space of 3 nl/islet (Sener & Malaisse, 1978), to be 170 munits/ml.

Effects of forskolin on islet cyclic AMP content and insulin release have been reported previously in some detail (Wiedenkiller & Sharp, 1983; Henquin & Meissner, 1984). Therefore in the present study we restricted our measurements of these parameters to the essential demonstration that forskolin potentiated insulin release and increased islet cyclic AMP under the conditions that we used for study of protein phosphorylation. In particular, it was verified that the absence of phosphate from the incubation medium did not markedly affect these islet responses to forskolin. The forskolin-induced increase in islet cyclic AMP was rapid and sustained and was accompanied by an approx. 3-fold potentiation of insulin release stimulated by glucose. In agreement with previous observations (Christie & Ashcroft, 1984), 10 mM-glucose alone increased islet cyclic AMP significantly after 60, but not 5, min incubation.

The present study demonstrates that the effects of forskolin on insulin release and islet cyclic AMP content are accompanied by changes in phosphorylation state of a number of islet peptides. Since forskolin did not alter the rate of labelling of ATP on incubation of islets with [32 P]P_i, we could exclude the possibility that the observed changes were attributable to forskolin effects on radioactive phosphate uptake at the cell membrane or on ATP turnover rather than to true increases in protein phosphorylation. Four peptides (cytosolic peptides of M_r 25 000 and 30 000 and particulate peptides of M_r 23 000 and 32 000) showed increased phosphorylation both in intact islets incubated with forskolin and in subcellular fractions exposed to catalytic subunit. Fractions obtained by differential centrifugation of islet homogenates were assayed for organelle markers to determine the

subcellular localization of the particulate peptides. The M_r -32 000 peptide appeared in a fraction with a high RNA content and low in other markers. This may be indicative of a ribosomal location for this peptide. This is supported by the observations that the major peptide phosphorylated in hamster insulinoma tissue in response to glucagon is ribosomal protein S6 (Schubart *et al.*, 1977) and that S6 is phosphorylated in other tissues stimulated to produce cyclic AMP (Freedman & Jamieson, 1982). Differential centrifugation was not sufficient to determine the localization of the M_r -23 000 peptide, and the fraction containing this protein was analysed further by Percoll-density-gradient centrifugation. The plasma-membrane marker 5'-nucleotidase was recovered in fractions of lower density than markers of mitochondria and endoplasmic reticulum. A large proportion of insulin was recovered at the top of the gradient, which may represent insulin released from granules damaged during the fractionation procedure. Some insulin was associated with mitochondria and endoplasmic reticulum. The M_r -23 000 peptide appeared in an insulin-containing fraction of high density, which may contain the insulin-secretory granules. Hutton and coworkers have recovered insulinoma secretory granules from Percoll gradients at a similar density range (Hutton & Peshavaria, 1982).

Three further peptides showed a response to incubation with forskolin. The M_r -15 000 peptide appeared exclusively in the 600g pellet fraction. The magnitude of the response, the mobility on SDS/polyacrylamide gels and appearance in the nuclear fraction are similar to the properties of histone H3, which we have previously demonstrated to be phosphorylated in islets in response to a range of agents that increase cyclic AMP (Christie & Ashcroft, 1984). In the current study we have failed to show phosphorylation of this peptide in response to exogenous catalytic subunit of cyclic AMP-dependent protein kinase. The lack of effect, together with the observed delay in response to forskolin, may indicate secondary activation of a kinase independent of cyclic AMP. Ca²⁺-dependent phosphorylation of histone H3 has been observed in HeLa cells, where cyclic AMP was without effect (Whitlock *et al.*, 1980). However, a role for cyclic AMP-dependent protein kinase cannot be excluded. Histone H3 is a substrate for cyclic AMP-dependent protein kinase in some systems (Hashimoto *et al.*, 1976), and the lack of effect observed in the current study may be due to interaction of the protein with DNA, masking the phosphorylation site. Purified histones have been shown to be better substrates for protein phosphorylation than are those associated with the nucleosome (Walton & Gill, 1981). The

lag time in the response in intact islets could reflect a slow uptake of the activated kinase into the nucleus.

Forskolin decreased the labelling of an M_r -18000 cytosolic peptide. This may be the result of activation of a phosphoprotein phosphatase, for which several possible mechanisms could be envisaged. Alternatively a specific protein kinase may be inhibited on stimulation with forskolin. Such a situation may be analogous to the inhibition of myosin light-chain kinase after phosphorylation by cyclic AMP-dependent protein kinase in platelets and smooth muscle (Hathaway *et al.*, 1981; Conti & Adelstein, 1981). The inhibitory effect of forskolin on the M_r -18000 peptide appears to be lost after 1 h incubation. Similar, although less marked, effects of forskolin were also observed on a M_r -34000 cytosolic peptide. We have previously shown (Christie & Ashcroft, 1984) that a 3 h incubation with 3-isobutyl-1-methylxanthine caused increased phosphorylation of peptides of these M_r values; and prolonged incubation with forskolin under similar conditions also caused an increase in labelling of these peptides (M. R. Christie, unpublished work). Therefore, it appears that there are two effects of forskolin on these peptides; immediate net dephosphorylation, followed by a slow increase in the phosphorylation state.

The function of the reported phosphopeptides is unknown. The probable nuclear and ribosomal locations of the M_r -15000 and -32000 peptides respectively argue against a role for these peptides in the control of secretion, but they may be involved in DNA and protein synthesis. The appearance of the M_r -23000 substrate for catalytic subunit in an insulin-containing fraction is of interest in that a cyclic AMP-dependent protein kinase activity has been reported to be present in insulin-secretory granules (Sussman & Leitner, 1977). Cyclic AMP has been reported to inhibit both Ca^{2+} uptake (Hahn *et al.*, 1980) and Ca^{2+} -stimulated ATPase activity (Capito *et al.*, 1980) in secretory-granule-containing fractions of islets; thus cyclic AMP-dependent protein phosphorylation could conceivably modulate intracellular Ca^{2+} distribution.

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