Protein Kinase Activities in Rat Pancreatic Islets of Langerhans

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1. Protein kinase activities in homogenates of rat islets of Langerhans were studied. 2. On incubation of homogenates with [γ - ^{32}P]ATP, incorporation of ^{32}P into protein occurred: this phosphorylation was neither increased by cyclic AMP nor decreased by the cyclic AMP-dependent protein kinase inhibitor described by Ashby & Walsh [(1972) *J. Biol. Chem.* **247**, 6637–6642]. 3. On incubation of homogenates with [γ - ^{32}P]ATP and histone as exogenous substrate for phosphorylation, incorporation of ^{32}P into protein was stimulated by cyclic AMP (approx. 2.5-fold) and was inhibited by the cyclic AMP-dependent protein kinase inhibitor. In contrast, when casein was used as exogenous substrate, incorporation of ^{32}P into protein was not stimulated by cyclic AMP, nor was it inhibited by the cyclic AMP-dependent protein kinase inhibitor. 4. DEAE-cellulose ion-exchange chromatography resolved four peaks of protein kinase activity. One species was the free catalytic subunit of cyclic AMP-dependent protein kinase, two species corresponded to 'Type I' and 'Type II' cyclic AMP-dependent protein kinase holoenzymes [see Corbin, Keely & Park (1975) *J. Biol. Chem.* **250**, 218–225], and the fourth species was a cyclic AMP-independent protein kinase. 5. Determination of physical and kinetic properties of the protein kinases showed that the properties of the cyclic AMP-dependent activities were similar to those described in other tissues and were clearly distinct from those of the cyclic AMP-independent protein kinase. 6. The cyclic AMP-independent protein kinase had an $s_{20,w}$ of 5.2S, phosphorylated a serine residue(s) in casein and was not inhibited by the cyclic AMP-dependent protein kinase inhibitor. 7. These studies demonstrate the existence in rat islets of Langerhans of multiple forms of cyclic AMP-dependent protein kinase and also the presence of a cyclic AMP-independent protein kinase distinct from the free catalytic subunit of cyclic AMP-dependent protein kinase. The presence of the cyclic AMP-independent protein kinase may account for the observed characteristics of ^{32}P incorporation into endogenous protein in homogenates of rat islets.

It has been proposed that changes in the concentration of cyclic AMP in islets of Langerhans may modulate the rate of insulin release in response to primary initiators of insulin release, e.g. glucose (Cooper *et al.*, 1973; Hellman *et al.*, 1974). How cyclic AMP affects insulin release is not known. In mammalian tissues, the only well-defined action of cyclic AMP is to stimulate protein phosphorylation via activation of cyclic AMP-dependent protein kinase. It is thus likely that such a mechanism may underlie the effects of cyclic AMP on insulin release.

Activation of cyclic AMP-dependent protein kinase by cyclic AMP (for reviews see Krebs, 1972; Rubin & Rosen, 1975) involves dissociation of the inactive holoenzyme into its constituent regulatory (R) and catalytic (C) subunits, possibly according to eqn. (1):

This dissociation releases the catalytic subunit from inhibition. There are two major isoenzymes of cyclic

AMP-dependent protein kinase in mammalian tissues (Corbin *et al.*, 1975, 1976). These have been referred to as 'Type I' and 'Type II' according to their order of elution from DEAE-cellulose columns with increasing salt concentrations (Corbin *et al.*, 1975, 1976). The isoenzymes differ in several properties, which may affect the equilibrium position of the reaction shown in eqn. (1). Although the existence of cyclic AMP-dependent protein kinase in pancreatic islets has been demonstrated (Montague & Howell, 1972; Dods & Burdowski, 1973; Schubart *et al.*, 1973; Howell *et al.*, 1974), no attempts have been made to show the presence of different molecular forms and little information is available on the molecular properties of the enzyme. In this study we report the existence of isoenzymes of cyclic AMP-dependent protein kinase in rat islets of Langerhans and describe some of their physical and kinetic properties.

There are also protein kinases whose activity is independent of the presence of cyclic AMP. These kinases include those with specificity towards

glycogen synthase (for a review, see Nimmo & Cohen, 1977) and pyruvate dehydrogenase (Linn *et al.*, 1969*a,b*), and a less-well-defined group of which the physiological substrate(s) is not known, but whose activity can be demonstrated by the phosphorylation of model substrates such as casein or phosphitin (see, e.g., Schlender & Reimann, 1975; Nimmo & Sloane, 1976). We have demonstrated the presence of a cyclic AMP-independent protein kinase in rat islets and describe some properties of the enzyme.

Experimental

Materials

Collagenase (type I), histone (type II-A), casein, haemoglobin (type II), bovine serum albumin (crystalline), ovalbumin, soya-bean trypsin inhibitor, rabbit muscle phosphorylase *b*, bovine pancreatic ribonuclease and dithiothreitol were from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. Bovine liver catalase, cyclic AMP and ATP were from Boehringer Corp. (London) Ltd., Lewes, East Sussex BN7 1LG, U.K. 3-Isobutyl-1-methyl-xanthine and benzamidine hydrochloride were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Whatman DE-11 and DE-52 DEAE-cellulose and Whatman 3MM paper were from Whatman Ltd., H. Reeve Angel Scientific Ltd., London SE1 6BD, U.K. Bio-Gel HTP hydroxyapatite was from Bio-Rad Laboratories, Watford, Herts., U.K. Gel-filtration materials were from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Norit GSX Charcoal was from Norit-Clydesdale Co., Glasgow G31 1TG, Scotland, U.K. Millipore filters (pore size 0.45 μ m) were from Millipore House, London NW10 7SP, U.K. Other reagents were from BDH Chemicals, Poole, Dorset BH12 4NN, U.K., and were of the purest grade available. All radiochemicals were from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. X-ray film was from Kodak, Hemel Hempstead, Herts., U.K.

Preparation of islets

Islets were prepared by a collagenase method (Coll Garcia & Gill, 1969) from the pancreases of 200–300g male albino Wistar rats fed *ad libitum*. Islets were placed into suitable buffers and disrupted by sonication with three intervals of 5s at position 2 on a Soniprobe (Dawe Instruments Ltd., London W.3, U.K.).

Assay of protein kinase activity

Protein kinase activity was measured by the incorporation of 32 P from [γ - 32 P]ATP into trichloroacetic acid-precipitable material. For the assay of protein kinase in homogenates, 300–600 islets were sonicated in 200 μ l of incubation medium [50mm-

Mes (4-morpholine-ethanesulphonic acid)/10mm-MgCl₂/0.25mm-EGTA/10mm-benzamidine, pH 6.9]. Suitably diluted samples [40–135 μ g of islet protein, assuming a mean islet protein content of 0.8 μ g of protein/islet (Sener & Malaisse, 1978)] were added to incubation medium containing the additions described in the legends to the Figures and Tables. The reaction was initiated by the addition of [γ - 32 P]ATP (100–200 c.p.m./pmol) to a final concentration of 0.5mm after a preincubation time of 1 min. The final volume was 300 μ l. At each specified time, a sample was pipetted on to a filter-paper square (Whatman 3MM), which was immersed in ice-cold 10% (w/v) trichloroacetic acid. The papers were processed as described by Corbin & Reimann (1974). Suitable blanks were included.

Protein kinase activity in fractions from sucrose gradients and ion-exchange or gel-filtration columns was measured as follows. A sample (30–50 μ l) of enzyme solution was added to 50 μ l of a mixture containing 40mm-Mes/8mm-MgCl₂/8mm-benzamidine/0.2mm-EGTA/0.33mm-[γ - 32 P]ATP (100–200 c.p.m./pmol), pH 6.9, and either 0.5mg of histone or 0.5mg of casein. Cyclic AMP (3 μ M) or cyclic AMP-dependent protein kinase inhibitor (over 1200 units/ml) was added as indicated in the text and Tables. The reaction mixture was incubated at 30°C for 90 min, after which the reaction was terminated and the filter papers were processed as described above. One unit of protein kinase activity is that activity which catalyses the incorporation of 1 pmol of 32 P from [γ - 32 P]ATP into protein per min at 30°C. In some cases, cyclic AMP-dependent protein kinase activity is expressed as an activity ratio, i.e. the ratio of the activity in the absence of cyclic AMP to that in the presence of cyclic AMP (3 μ M).

Preparation of catalytic subunit of cyclic AMP-dependent protein kinase from islets of Langerhans

For the preparation of the catalytic subunit from rat islets of Langerhans, a modification of the method of Reimann & Corbin (1976) and Sugden *et al.* (1976) was used: 700 islets from four rats were collected in 300 μ l of 10mm-potassium phosphate (pH 6.8)/1mm-dithiothreitol and homogenized by sonication (see above). The homogenate was applied to a column (0.5cm $\times$ 3.0cm) of DEAE-cellulose (Whatman DE-11) equilibrated with homogenization buffer. The column was washed with 10ml of 10mm-potassium phosphate (pH 6.8)/0.1mm-dithiothreitol. Catalytic subunit was eluted with 20ml of 10mm-potassium phosphate (pH 6.8)/0.1mm-dithiothreitol/100 μ M-cyclic AMP. This wash, containing catalytic-subunit activity, was applied to a column (0.5cm $\times$ 0.5cm) of hydroxyapatite (Bio-Rad Bio-Gel HTP) equilibrated with 50mm-potassium phosphate (pH 6.8)/0.1mm-dithiothreitol. The column was washed with 10ml of 50mm-potassium phosphate (pH 6.8)/0.1mm-dithio-

threitol. Catalytic subunit was eluted with 350 mM-potassium phosphate (pH 6.8)/0.1 mM-dithiothreitol. Fractions (0.5 ml) were collected and assayed for catalytic-subunit activity.

Preparation of bovine liver catalytic subunit of cyclic AMP-dependent protein kinase

Bovine liver catalytic subunit of cyclic AMP-dependent protein kinase was prepared by the method of Reimann & Corbin (1976) as described by Sugden *et al.* (1976) up to and including the second hydroxyapatite column. Its activity was inhibited by the heat-stable cyclic AMP-dependent protein kinase inhibitor (see below) to 7 or 12% of the activity observed in the absence of inhibitor with histone or casein as substrates, respectively.

Determination of cyclic AMP binding

(a) *In islet of Langerhans homogenates.* For this 300 islets were sonicated in 300 μ l of 10 mM-potassium phosphate (pH 6.8)/1 mM-EDTA, and a sample (5–25 μ l suitably diluted in homogenization buffer) was added to 110 μ l of 50 mM-potassium phosphate (pH 6.8)/1 mM-EDTA/2 M-NaCl/1 μ M-cyclic [3 H]AMP (10000 c.p.m./pmol)/0.5 mg of histone/ml (binding mixture). The total volume was 135 μ l. The mixture was incubated for 90–120 min at 30°C and then 110 μ l of the mixture was filtered through a Millipore filter (HA 0.45 μ m) previously moistened with 10 mM-potassium phosphate (pH 6.8)/1 mM-EDTA. The filter was rinsed with 8 ml of the same buffer (ice-cold) and dried in an oven. Radioactivity retained by the filter was measured by counting in 10 ml of methoxyethanol scintillation fluid (Severson *et al.*, 1974).

(b) *In fractions from Sepharose 6B.* The method used was essentially that above except that 400 μ l of each fraction was added to 150 μ l of the binding mixture and a 500 μ l portion was filtered.

Determination of ATPase activity

Islet homogenates were incubated with [γ - 32 P]-ATP as described for the assay of protein kinase activity above and ATPase activity was determined by the method of Cooper *et al.* (1974). At various times, 5 μ l samples were removed and added to 1 ml of 1 M-HCl containing Norit GSX Charcoal (5 mg/ml), which selectively adsorbs [γ - 32 P]ATP and ADP, but not 32 P_i. The tubes were centrifuged (800g for 5 min). Samples (50 μ l) of supernatants were counted for radioactivity in methoxyethanol scintillation fluid (see above).

DEAE-cellulose chromatography

For this 600 islets were sonicated in 250 μ l of 5 mM-potassium phosphate (pH 6.8)/1 mM-EDTA. The homogenate was applied to a column (0.5 cm $\times$ 3.0 cm) of DEAE-cellulose (Whatman DE-11) in a Pasteur

pipette. The column was washed with homogenization buffer (8 ml). A linear NaCl gradient (60 ml total volume, 0–0.65 M) in the same buffer was started and fractions (1 ml) were collected and assayed for protein kinase activity. Na⁺ concentrations in fractions were measured with a Corning 455 flame photometer.

Sucrose-density-gradient centrifugation

Sedimentation coefficients were determined by the method of Martin & Ames (1961). Linear sucrose gradients (13 ml; 5–20%, w/v) were formed in 5 mM-Tris/HCl/1 mM-EDTA, pH 7.5. Bovine haemoglobin (50 μ l of 15 mg/ml), bovine liver catalase (50 μ l of 15 mg/ml, previously dialysed for 24 h against 5 mM-Tris/HCl/1 mM-EDTA at pH 7.5) and rabbit muscle phosphorylase *b* (50 μ l of 5 mg/ml) were used as standards. For this experiment 600–800 islets were sonicated in 300 μ l of 5 mM-Tris/HCl/1 mM-EDTA/10 mM-benzamidine, pH 7.5, and 100–150 μ l of the homogenate was applied to each tube. Centrifugation was carried out in a Beckman L5-65 ultracentrifuge in a Beckman SW 40 rotor at 200000g (at r_{av} , 11.1 cm) at 4°C for 18 h. Fractions (0.5 ml) were collected and assayed for phosphorylase *b* (Krebs *et al.*, 1964), catalase (Beers & Sizer, 1952) and protein kinase activity. Cyclic AMP-dependent protein kinase activity was assayed in the presence of cyclic AMP with histone as phosphate acceptor. Cyclic AMP-independent protein kinase activity was assayed in the absence of cyclic AMP and in the presence of cyclic AMP-dependent protein kinase inhibitor (1200 units/ml) with casein as substrate. The position of haemoglobin was determined from its A_{411} . Sedimentation coefficients of 8.2 S for phosphorylase (Keller & Cori, 1953), 4.6 S for haemoglobin (Schachman & Edelstein, 1966) and 11.2 S for catalase (Sumner & Gralen, 1938) were used to calculate unknown sedimentation coefficients. In each gradient, the plot of migration of standard proteins versus sedimentation coefficient was linear.

Gel filtration

Stokes radii were determined on a column (1.5 cm $\times$ 90 cm) of Sepharose 6B equilibrated with 50 mM-Tris/HCl/1 mM-EDTA/0.1 mM-dithiothreitol, pH 7.5. For this study 800–1000 islets were collected in 300 μ l of 10 mM-Tris/HCl/1 mM-EDTA/10 mM-benzamidine, pH 7.5, and sonicated. Sucrose in 10 mM-Tris/HCl/1 mM-EDTA (700 μ l of 15%, w/v) was added, and the 1 ml sample placed on the column. Fractions (2.5 ml) were collected (flow rate 5 ml/h). The column was standardized, in the absence of dithiothreitol, with the following proteins for which Stokes radii had been calculated (Siegel & Monty, 1966) from values of physical parameters given in the literature, namely bovine serum albumin (Stokes radius 3.5 nm; Creeth, 1952), bovine serum albumin dimer (4.75 nm; Hughes, 1950; Creeth, 1952), soya-bean trypsin

inhibitor (2.45 nm; Rackis *et al.*, 1962), ovalbumin (2.95 nm; Kegeles & Gutter, 1951; Castellino & Barker, 1968) and bovine liver catalase (5.2 nm; Sumner & Gralen, 1938). Inclusion (122.5 ml) and exclusion (45 ml) volumes were determined with $^3\text{H}_2\text{O}$ and Blue Dextran respectively. All protein standards were applied to the column separately at a concentration of 10 mg/ml and were determined spectrophotometrically at 280 nm. $^3\text{H}_2\text{O}$ was determined by liquid-scintillation counting. Blue Dextran was determined at 580 nm. Data were plotted by the method of Laurent & Killander (1964); see also Siegel & Monty (1966).

Identification of phosphorylated amino acids

Cyclic AMP-independent protein kinase from 500 rat islets was partially purified by DEAE-cellulose chromatography (see above). The peak fractions (1 ml) were added to 2.5 ml of 40 mM-Mes/8 mM-MgCl₂/8 mM-benzamide/0.2 mM-EGTA/0.5 mM-[γ - ^{32}P]ATP (200 c.p.m./pmol), pH 6.9, containing 25 mg of casein and over 5000 units of cyclic AMP-dependent protein kinase inhibitor, and incubated at 30°C for 4 h. Trichloroacetic acid was added to a final concentration of 10% (w/v). After centrifugation (600 g for 5 min) and removal of the supernatant, the pellet was taken up in 1 ml of 8 M-urea/1% (w/v) NH₄HCO₃ and dialysed extensively at 4°C against 2 M-urea/1% (w/v) NH₄HCO₃ until all trichloroacetic acid-soluble radioactivity was removed. It was then dialysed for 24 h at 4°C against 1% (w/v) NH₄HCO₃ (four changes of 2 litres). An equal volume of 10 M-HCl was added to the non-diffusible material and hydrolysis was carried out for 3 h at 110°C. The hydrolysate was dried *in vacuo* over solid KOH/P₂O₅ and taken up in 50 μ l of water. Phosphoserine (100 nmol), phosphothreonine (100 nmol) and $^{32}\text{P}_i$ (10000 c.p.m.) were added to the samples, which were electrophoresed on Whatman 3MM paper in 8% (v/v) acetic acid/2% (v/v) formic acid at 4 kV for 2 h. Phospho-amino acids were detected by ninhydrin staining and ^{32}P -containing material was detected by radioautography by exposure of the electrophoretogram on Kodak Blue Brand BB5 X-ray film for 24 h.

Miscellaneous

Casein was treated for use in the protein kinase assay as described by Reimann *et al.* (1971). The cyclic AMP-dependent protein kinase inhibitor protein was prepared from rat hearts and brains as described by Ashby & Walsh (1972), except that the inhibitor was eluted from the final DEAE-cellulose column with 0.25 M-sodium acetate/1 mM-EDTA, pH 5.0. One unit of inhibitor activity is that amount which inhibited the transfer of 1 pmol of ^{32}P from [γ - ^{32}P]ATP to histone per min at 30°C.

All potassium or sodium phosphate buffers were prepared by mixing equimolar solutions of K₂HPO₄ and KH₂PO₄ or Na₂HPO₄ and NaH₂PO₄ to give the desired pH. EGTA and EDTA were neutralized to pH 6.8 with KOH before use. The pH of Mes was adjusted with KOH.

All statistics are given as the mean $\pm$ S.E.M. with the numbers of observations (*n*).

Results and Discussion

Phosphorylation of endogenous substrate by islet homogenate

Incubation of [γ - ^{32}P]ATP with a sonicated preparation of islets of Langerhans resulted in an incorporation of phosphate into islet protein. A typical time course is shown in Fig. 1. Because of the small amounts of experimental material available, each point represents only a single observation. However, the experiment was performed on three separate islet preparations. A similar approach was used for data shown in Figs. 2 and 3. Initially, the progress curve was linear. Control experiments showed that the subsequent non-linearity was due to depletion of [γ - ^{32}P]ATP primarily as a result of breakdown of ATP by ATPase activity in the preparation. At the ATP concentration used, the ATPase activity was 0.345 ± 0.023 nmol of P_i liberated/min per islet (*n*=6). Neither the initial rate nor the plateau value of incorporation of ^{32}P into islet protein was stimulated statistically significantly by cyclic AMP or inhibited

Fig. 1. Time course of protein kinase activity in an islet homogenate

The time course of ^{32}P incorporation from [γ - ^{32}P]ATP into islet proteins was measured in the presence (●) or absence (■) of cyclic AMP (5 μ M) and 3-isobutyl-1-methylxanthine (0.5 mM) or the presence of protein kinase inhibitor plus cyclic AMP (5 μ M) and 3-isobutyl-1-methylxanthine (0.5 mM) (▲). Methodology was as described in the Experimental section.

statistically significantly by addition of the cyclic AMP-dependent protein kinase inhibitor (Ashby & Walsh, 1972). These findings extend the observation by Muller *et al.* (1976) that cyclic AMP had no effect on the phosphorylation of endogenous proteins in

homogenates of rat islets, and indicate that, under the conditions used, phosphorylation was catalysed by a protein kinase other than cyclic AMP-dependent protein kinase.

The phosphorylation of endogenous islet protein was also studied by using purified bovine catalytic subunit of cyclic AMP-dependent protein kinase. As shown in Fig. 2, bovine catalytic subunit catalysed the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into endogenous islet protein. Thus the islet homogenate does contain substrates for cyclic AMP-dependent protein kinase. The magnitude of the incorporation observed depended on the amount of catalytic subunit added. Suitable controls where islet proteins were not present were subtracted. This suggests the possibility that, in sonicated islets, a high cyclic AMP-independent phosphorylation of endogenous substrate might mask a much lower cyclic AMP-stimulated phosphorylation.

Fig. 2. Phosphorylation of islet proteins by purified catalytic subunit

The time course of ^{32}P incorporation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into islet proteins was measured as described in the Experimental section in the absence (●) or presence of bovine catalytic subunit of cyclic AMP-dependent protein kinase at a final concentration of 6000 (■), 10000 (▼) or 20000 (▲) units/ml.

Phosphorylation of exogenous substrate by islet homogenate: demonstration of cyclic AMP-dependent and -independent protein kinases

When sonicated islets-of-Langerhans homogenates were incubated with histone and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, a cyclic AMP-dependent phosphorylation of protein occurred (Fig. 3a). The ratio of initial rates in the absence and presence of cyclic AMP (the activity ratio) was 0.40 and the initial rates were statistically significantly different ($P < 0.01$ for three different islet preparations). Although this activity ratio is rather

Fig. 3. Time course of protein kinase activity in rat islets-of-Langerhans homogenates in the presence of exogenous substrates

The time course of protein phosphorylation was estimated as described in the Experimental section. Activity was measured in the presence of cyclic AMP (5 μM) and 3-isobutyl-1-methylxanthine (0.5 mM) (●), in the absence of cyclic AMP (■), or in the presence of cyclic AMP (5 μM) and 3-isobutyl-1-methylxanthine (0.5 mM) and protein kinase inhibitor protein (▲). In (a) the exogenous substrate was histone, and in (b) the exogenous substrate was casein.

high, it may contain a component representing cyclic AMP-independent phosphorylation of islet proteins (see above) or be caused by the prolonged incubation time used (since histone may cause dissociation of the holoenzyme; Corbin *et al.*, 1975, 1976). The former alternative is supported by the finding that the heat-stable cyclic AMP-dependent protein kinase inhibitor (Ashby & Walsh, 1972) in the presence of cyclic AMP did not decrease protein phosphorylation below that observed in the absence of cyclic AMP, and by the finding that the amount of phosphate incorporated into protein in the presence of histone and absence of cyclic AMP (Fig. 3a) was similar to that observed in the absence of histone and cyclic AMP (Fig. 1). These data demonstrate the presence of cyclic AMP-dependent protein kinase in islets.

A different pattern of phosphorylation occurred when casein was used as exogenous substrate (Fig. 3b). Under these conditions, protein phosphorylation was not stimulated by cyclic AMP, nor was it inhibited by the cyclic AMP-dependent protein kinase inhibitor. It was shown that these observations were not caused by insensitivity of islets-of-Langerhans catalytic subunit to the cyclic AMP-dependent protein kinase inhibitor, since, as expected, the activity of the isolated catalytic subunit from islets was inhibited by the cyclic AMP-dependent protein kinase inhibitor with histone or casein as substrate (Table 1).

Thus experiments with islets-of-Langerhans homogenates indicate that a cyclic AMP-independent protein kinase is present that is distinct from cyclic AMP-dependent protein kinase or its catalytic subunit. This conclusion was confirmed by separation of islet protein kinase activities by ion-exchange chromatography as described below.

Separation of protein kinases by ion-exchange chromatography

A typical separation of protein kinases from islets-of-Langerhans homogenates on DEAE-cellulose columns is shown in Fig. 4. In the presence of histone

and cyclic AMP, three peaks (peaks 1, 2 and 4) of protein kinase activity were observed. Peak 1, present in the column flow-through, was the catalytic subunit of cyclic AMP-dependent protein kinase, since its activity was not stimulated by cyclic AMP (results not shown), but was inhibited by the cyclic AMP-dependent protein kinase inhibitor. Two peaks of cyclic AMP-dependent protein kinase activity were eluted by a linear NaCl gradient. These were eluted at 0.045 ± 0.004 M-NaCl ($n=6$; peak 2) and 0.228 ± 0.007 M-NaCl ($n=6$; peak 4). Both activities were dependent on the presence of cyclic AMP (see Table 2) and inhibited by the cyclic AMP-dependent protein kinase inhibitor (Fig. 4). The elution pattern of peaks 2 and 4 from DEAE-cellulose columns is consistent with their being Type I and Type II isoenzymes of cyclic AMP-dependent protein kinase (Corbin *et al.*, 1975, 1976), respectively. This conclusion is supported by the finding (Table 2) that, whereas dissociation of peak-2 (Type I) activity into its constituent regulatory and catalytic subunits was stimulated by preincubation with histone or NaCl, such treatment had a much smaller effect on peak-4 (Type II) activity (Corbin *et al.*, 1975, 1976).

However, when fractions from a DEAE-cellulose column were assayed with casein as substrate in the presence of the cyclic AMP-dependent protein kinase inhibitor, a distinct peak of enzyme activity was eluted at 0.138 ± 0.005 M-NaCl ($n=6$; peak 3). This protein kinase activity was not stimulated by cyclic AMP (Table 2) nor inhibited by the inhibitor (Fig. 4). This activity presumably accounts for the observations with homogenates of islets of Langerhans (Figs. 1 and 3) and possibly for the observations of Muller *et al.* (1976).

Kinetic properties of protein kinases from islets of Langerhans

The kinetic properties of cyclic AMP-dependent and -independent protein kinases are distinct (Table 3). In homogenates, no phosphorylation of histone was observed in the absence of cyclic AMP. The

Table 1. *Substrate specificity of rat islet-of-Langerhans catalytic subunit*

Catalytic-subunit activity was assayed by adding 50 μ l samples of islet catalytic subunit in 350 mM-potassium phosphate (pH 6.8)/0.1 mM-dithiothreitol to 50 μ l of 40 mM-Mes (pH 6.9)/8 mM-MgCl₂/0.2 mM-EGTA/8 mM-benzamidine/0.33 mM-[γ -³²P]ATP (150 c.p.m./pmol) containing histone or casein at a concentration of 10 mg/ml and incubated for 20 min. Protein kinase inhibitor protein, when present, was at a concentration of greater than 1200 units/ml. Incubations were terminated as described in the Experimental section. Results are shown as means $\pm$ S.E.M. for four observations.

Substrate	Cyclic AMP-dependent protein kinase inhibitor	³² P incorporated (pmol/ml of catalytic subunit)
Histone	—	1342.0 $\pm$ 18.0
Histone	+	43.8 $\pm$ 7.8
Casein	—	55.6 $\pm$ 5.6
Casein	+	5.3 $\pm$ 2.4

Fig. 4. DEAE-cellulose chromatography of rat islets-of-Langerhans homogenate
Methodology was as described in the Experimental section. The protein kinase activity was assayed with histone (○, ●) or casein (△, ▲) as substrates, either in the presence of cyclic AMP (○, △) or in the absence of cyclic AMP in the presence of protein kinase inhibitor (●, ▲). NaCl concn. (□) was also determined.

Table 2. Activity ratios of protein kinases under various conditions

Fractions from DEAE-cellulose columns were pooled separately, dialysed for 18 h at 4°C against 5 mM-potassium phosphate (pH 6.8)/1 mM-EDTA, and concentrated by surrounding the dialysis tubing with dry Sephadex G-200. A sample (250 µl) of Type I or Type II cyclic AMP-dependent or cyclic AMP-independent protein kinase was incubated with water (50 µl), histone (50 µl of a 5 mg/ml solution) or NaCl (50 µl of a 3 M solution) at 30°C for 5 min, after which time the activity was measured in the absence or the presence of cyclic AMP (3 µM) in a 30 min incubation by using histone (10 mg/ml) as substrate for Type I and Type II cyclic AMP-dependent protein kinases, and casein (10 mg/ml) as substrate for the cyclic AMP-independent protein kinase. Similar results were obtained with two other preparations of enzymes. The activity ratio is the ratio of the activity observed in the absence of cyclic AMP to that observed in the presence of 3 µM-cyclic AMP.

	Activity ratio		
	Control (+water)	+Histone preincubation	+NaCl preincubation
Type I (peak 2)	0.17	0.86	1.03
Cyclic AMP-independent protein kinase (peak 3)	0.91	0.92	1.14
Type II (peak 4)	0.38	0.45	0.48

Eadie (1942) plot for cyclic AMP-independent protein kinase, with casein as variable substrate, was biphasic (not shown), with both 'high'- and 'low'- K_m components. This type of behaviour may indicate negative co-operativity or heterogeneity in enzyme or substrate. The K_m of the cyclic AMP-independent protein kinase for ATP is approx. 10 times that of cyclic AMP-dependent protein kinase. The K_m for

ATP of the latter does not vary between Type I or Type II cyclic AMP-dependent protein kinase, since it is probable that the catalytic subunit is identical in the two species (Corbin *et al.*, 1976). Furthermore, the protein substrate used does not affect the K_m for ATP. (It was not possible to perform kinetic experiments for the Type-I activity with casein as substrate because of the small amounts of material available.)

Table 3. *Kinetic properties of cyclic AMP-dependent and -independent protein kinases*

The K_m values of the protein kinases for their substrates were measured by adding various amounts of histone or casein to islet homogenates. The histone kinase activity was measured in the presence of cyclic AMP ($5 \mu\text{M}$) and 3-isobutyl-1-methylxanthine (0.5 mM). The % cyclic AMP-independent protein kinase activity was measured in the presence of heat-stable cyclic AMP-dependent protein kinase inhibitor (over 1200 units/ml). The apparent K_a for cyclic AMP of the cyclic AMP-dependent protein kinase was measured with histone as substrate. The K_m values of the two protein kinases for ATP were measured by using partially purified enzymes after DEAE-cellulose chromatography and concentration (see Table 2). The substrate used is given in parentheses.

Preferred substrate in islets-of-Langerhans homogenates ...	Cyclic AMP-dependent protein kinase Histone	Cyclic AMP-independent protein kinase Casein
K_m for protein substrates in homogenates (mg/ml)	0.08 ± 0.01 (3)	Biphasic Eadie (1942) plot, 0.14 ± 0.01 (3) and 1.59 ± 0.13 (3)
K_a for cyclic AMP in homogenates (μM)	0.08 ± 0.03 (4)	Not required for activity
K_m for ATP with partially purified enzyme (μM)	Type I (histone), 16.1 ± 2.4 (3) Type II (histone), 15.4 ± 2.1 (5) Type II (casein), 13.5 ± 1.8 (3)	134.0 ± 11.5 (3) (casein)

Table 4. *Physical characteristics of cyclic AMP-dependent and -independent protein kinases*

The $s_{20,w}$ and Stokes radii were measured as described in the Experimental section. The molecular weights and frictional ratios (f/f_0) were calculated by using these data (Siegel & Monty, 1966). A partial specific volume of $0.725 \text{ cm}^3/\text{g}$ was assumed.

	Cyclic AMP-dependent protein kinase	Cyclic AMP-independent protein kinase
$s_{20,w}$ (S)	6.73 ± 0.07 (9)	5.20 ± 0.08 (6)
Stokes radius (nm)	5.20 ± 0.20 (5)	2.81 ± 0.09 (6) and 4.26 ± 0.23 (6)
Mol.wt.	144 200	60 300 and 91 400
f/f_0	1.5	1.4 and 1.1

Physical properties of protein kinases from islets of Langerhans

The physical properties of cyclic AMP-dependent and -independent protein kinases are shown in Table 4. The two kinases were observed as single peaks after sucrose-density-gradient centrifugation and their sedimentation coefficients differed. It has been reported that products of proteolysis of cyclic AMP-dependent protein kinases have sedimentation coefficients of about 5.2S (Corbin *et al.*, 1972; Sugden & Corbin, 1976). However, the cyclic AMP-independent protein kinase reported here must be distinct from these species, since (a) assays were performed in the absence of cyclic AMP, (b) the cyclic AMP-independent protein kinase activity was not inhibited by the cyclic AMP-dependent protein kinase heat-stable inhibitor, and (c) all operations were performed in the presence of the proteinase inhibitor benzamidine.

The Stokes radii of the cyclic AMP-dependent and -independent protein kinases were determined by Sepharose 6B chromatography. A typical gel-filtration pattern of a rat islets-of-Langerhans homogenate is shown in Fig. 5. A single peak of cyclic

AMP-dependent protein kinase activity, which was not observed when assayed in the presence of cyclic AMP-dependent protein kinase inhibitor, was eluted at 72.5 ml. This was co-eluted with a shoulder of cyclic AMP-binding activity and presumably represented the holoenzyme of cyclic AMP-dependent protein kinase. The major peak of cyclic AMP-binding was eluted at 80 ml. It is considered that this was probably free regulatory-subunit dimer, since its Stokes radius corresponded to reported values (Corbin *et al.*, 1972; Sugden & Corbin, 1976). Adenosine in a 100-fold excess over cyclic [^3H]AMP did not decrease binding of cyclic [^3H]AMP, thereby excluding the possibility that this protein was the adenosine-analogue-binding protein (Sugden & Corbin, 1976). There was insufficient material available to test whether this protein would inhibit catalytic-subunit activity. The reason for the presence of this large peak of binding is not understood. Cyclic AMP-independent protein kinase activity was relatively unstable on gel filtration. Two small peaks of activity were observed (Table 4). These peaks were clearly resolved from the peak of cyclic AMP-dependent protein kinase activity.

Fig. 5. Sepharose 6B chromatography of rat islets-of-Langerhans homogenate

A volume (1 ml) of islet homogenate was applied to a column of Sepharose 6B and fractions were collected and assayed for cyclic AMP binding (△) or protein kinase activity with histone as substrate in the absence of cyclic AMP in the presence (●) or absence (○) of protein kinase inhibitor protein, or in the presence of 3 μM-cyclic AMP (▲).

From the values of sedimentation coefficients and Stokes radii, and assuming a partial specific volume ($\bar{v}$) of 0.725 cm³/g (Siegel & Monty, 1966), molecular weights for the cyclic AMP-dependent and -independent protein kinases were calculated (Table 4). Since it was not known whether one or the other or both peaks of the cyclic AMP-independent protein kinase were derived from the 5.2S species, molecular weights were calculated for both species by assuming they both had an $s_{20,w}$ of 5.2S. Frictional coefficients were calculated from the Stokes radii and molecular weights (Cohn & Edsall, 1943; Siegel & Monty, 1966). Thus, by using the model of a prolate ellipsoid, the cyclic AMP-dependent protein kinase has an axial ratio of 6, and the higher- and lower-molecular-weight species of cyclic AMP-independent protein kinases have axial ratios of 6 and 1 respectively.

Substrate specificity of cyclic AMP-independent protein kinase

The islet cyclic AMP-independent protein kinase phosphorylated casein, but not histone, whereas for both islet cyclic AMP-dependent protein kinase and bovine catalytic subunit, histone was the preferred substrate. Cyclic AMP-dependent protein kinase has been shown generally to phosphorylate serine residues (for a review, see Nimmo & Cohen, 1977) and the cyclic AMP-independent myosin light-chain

Fig. 6. Cyclic AMP binding by a sonicated islet preparation. Binding assays were as described in the Experimental section. The line includes data obtained with four different preparations of islets (●, ■, ▲, ▼). The results are given as mean values.

kinase also phosphorylates a serine residue in its substrate (Perrie *et al.*, 1973). The nature of the amino acids phosphorylated in casein by islet cyclic AMP-independent protein kinase was determined. ³²P radioactivity was found to be incorporated only into serine residue(s). Thus substrate specificity is determined by protein topographical features other than the amino acid residue phosphorylated.

Cyclic AMP binding in islets-of-Langerhans homogenates

The total cyclic AMP-binding capacity in rat islet homogenates measured at a cyclic [³H]AMP concentration of 1 μM was found to be 0.016 pmol/islet (Fig. 6). Binding was not decreased in the presence of a 100-fold excess of non-radioactive adenosine over cyclic [³H]AMP. Thus cyclic [³H]AMP binding probably represented binding by the regulatory subunit of cyclic AMP-dependent protein kinase. Published values for rat islet cyclic AMP content under basal conditions, i.e. non-stimulatory glucose concentration and absence of phosphodiesterase inhibitors, are approx. 0.013 pmol/islet (Zawalich *et al.*, 1975; Charles *et al.*, 1976). This approximate equivalence of cyclic AMP and regulatory-subunit concentrations has been noted in other tissues and has important implications in assessing the con-

centration of free catalytic subunit in tissues. The concentration of cyclic AMP giving half-maximal activation of cyclic AMP-dependent protein kinase in rat islet homogenates in the present study was found to be 80 nM (Table 3). The basal concentration of cyclic AMP in islets may be calculated to be 6.5 μ M, assuming a mean intracellular volume of 2 nl/islet (Sener & Malaise, 1978). Hence it might appear that the protein kinase would be fully activated under basal conditions, as discussed by Montague & Howell (1973). However, taking into account the equivalence in the concentration of cyclic AMP and protein kinase and assuming a dissociation constant of 0.1 μ M (Beavo *et al.*, 1974), it can be calculated from eqn. (1) that the protein kinase holoenzyme R_2C_2 is only 1% dissociated under basal conditions of cyclic AMP concentrations.

General conclusions

Homogenates of rat islets of Langerhans contain cyclic AMP-dependent protein kinases which are probably identical with those reported by many other investigators. Both the Type-I and Type-II isoenzymes are present. These enzymes presumably mediate some, if not all, of the effects of cyclic AMP in islets of Langerhans. In addition, another species of protein kinase, which was cyclic AMP-independent, was detected in islets of Langerhans. Evidence was obtained that this kinase is distinct from cyclic AMP-dependent protein kinase. The role of this kinase *in vivo* is unknown: studies of the specificity of islet cyclic AMP-independent protein kinase towards potential physiological substrates may clarify this point.

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