Biochem. J. (1972) **129**, 551–560
Printed in Great Britain

The Mode of Action of Adenosine 3′:5′-Cyclic Monophosphate in Mammalian Islets of Langerhans

PREPARATION AND PROPERTIES OF ISLET-CELL PROTEIN PHOSPHOKINASE

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(*Received* 14 *April* 1972)

1. A protein was demonstrated in mammalian islets of Langerhans that after purification appeared as a single component possessing both cyclic-AMP (adenosine 3′:5′-cyclic monophosphate)-binding and cyclic-AMP-dependent protein phosphokinase activities. 2. The protein had an intrinsic association constant for cyclic AMP of 1.15×10^{-8} M, which was similar to the K_m for cyclic AMP (1.11×10^{-8} M) of the protein phosphokinase activity. 3. Incubation of the protein in the presence of cyclic AMP resulted in its dissociation into cyclic-AMP-independent protein phosphokinase (catalytic) and cyclic-AMP-binding (receptor) subunits, which could be separated on Sephadex G-200. 4. The cyclic-AMP-dependent protein phosphokinase was capable of phosphorylating a variety of proteins, the most readily phosphorylated being histone, casein and protein components of subcellular fractions prepared from islets of Langerhans. 5. The cyclic-AMP-dependent phosphorylation of histone had a K_m for ATP of 1.1×10^{-5} M. 6. The endogenous protein phosphokinase activity in rat islets incubated with agents that are known to alter the intracellular concentration of cyclic AMP was investigated. Theophylline and 3-isobutyl-1-methylxanthine, agents that raise cyclic AMP concentrations in islets, increased the activity of the protein phosphokinase, whereas adrenaline, which lowers islet cyclic AMP concentrations, decreased its activity. 7. It is suggested that cyclic AMP may exert its effects on insulin release by increasing the activity of a protein phosphokinase and may thereby promote the phosphorylation and activity of a rate-determining component of the secretory mechanism.

The secretion of insulin from the mammalian endocrine pancreas is stimulated under conditions likely to be associated with raised intracellular concentrations of cyclic AMP (adenosine 3′:5′-cyclic monophosphate) (Malaisse *et al.*, 1967). Moreover, it has been shown directly that the concentration of cyclic AMP in islets of Langerhans parallels rates of insulin secretion in response to a variety of agents (Turtle & Kipnis, 1967; Montague & Cook, 1971). These observations suggest that cyclic AMP may play an important role in determining the rate of insulin secretion from this tissue and therefore it is of considerable interest to determine the mechanism of its action on insulin release.

The effects of cyclic AMP in a variety of tissues appear to be related to the binding of the nucleotide to a specific receptor protein, which may lead directly to the activation of a protein phosphokinase (Miyamoto *et al.*, 1969; Gill & Garren, 1970; Reimann *et al.*, 1971*b*). We report here an investigation of the occurrence and properties of a cyclic-AMP-binding protein in islets of Langerhans and its relationship to cyclic-AMP-dependent protein phosphokinase activity in the same tissue. A preliminary account of some of these findings has been published (Montague & Howell, 1972).

Experimental

Sources of reagents

The following reagents were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.: adrenaline, bovine serum albumin, casein, cytochrome *c*, DEAE-cellulose (medium mesh, 0.9m-equiv./g), ethanedioxybis(ethylamine)tetra-acetic acid (EGTA), calf thymus histone (type IIA), 2-mercaptoethanol, ovalbumin, protamine, disodium β-glycerophosphate, Trizma base and theophylline. Cellulose acetate membranes (HAWP) were from Millipore Co. Ltd., Wembley, Middx., U.K. Sephadex G-200 and Blue Dextran were from Pharmacia (G.B.) Ltd., London W.5, U.K. 3-Isobutyl-1-methylxanthine was from Searle Co.,

Chicago, Ill., U.S.A. ATP and cyclic AMP were from Boehringer Corp. (London) Ltd., London W.5, U.K. Cyclic [^{3}H]AMP (specific radioactivity 21 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and was purified by ion-exchange chromatography before use (Butcher *et al.*, 1965). [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). Acrylamide and bisacrylamide were from Kodak Ltd., Kirkby, Liverpool, U.K.

Table 1. *Subcellular distribution of cyclic-AMP-binding and protein phosphokinase activities in islets of Langerhans*

Subcellular fractions of a homogenate of 500 rat islets of Langerhans were prepared as described in the text and assayed for cyclic-AMP-binding activity, protein phosphokinase activity and protein content (see the text). Each result is the mean of three observations performed in triplicate. The S.D. did not exceed the mean value by more than 15% for any observation.

		Cyclic-AMP-binding activity (pmol of cyclic [^{3}H]AMP bound)		Protein phosphokinase activity (pmol of ^{32}P incorporated/min per mg of histone)			
				−Cyclic AMP		+Cyclic AMP	
Fraction	Protein content (μg)	Total activity	Activity/mg of protein	Total activity	Activity/mg of protein	Total activity	Activity/mg of protein
Homogenate	464	0.284	0.613	152	327	240	519
Nuclear pellet (600*g* for 5 min)	178	0.083	0.466	61	340	91	510
Granule pellet (20000*g* of 10 min)	95	0.026	0.274	42	432	42	443
Microsomal pellet (105000*g* for 60 min)	68	0.025	0.360	21	309	24	354
Postmicrosomal supernatant	140	0.241	1.720	71	508	201	1430

Table 2. *Purification of islet-cell cyclic-AMP-binding protein and cyclic-AMP-dependent protein phosphokinase*

The activities in an homogenate prepared from 2000 guinea-pig islets were purified by the procedures shown (see the text for details). Cyclic-AMP-binding activity, protein phosphokinase activity and protein content were determined at each stage of the purification as described in the text. The results presented are from a single extraction procedure but are representative of those obtained as a routine.

		Cyclic-AMP-binding activity			Cyclic-AMP-dependent protein phosphokinase activity		
		(pmol of cyclic [^{3}H]AMP bound)			(pmol of ^{32}P incorporated/min per mg of histone)		
Stage	Concn. of total protein (mg)	Total activity	Activity/mg of protein	Degree of purification	Total activity	Activity/mg of protein	Degree of purification
1. Homogenate	3.56	2.3	0.65	1.0	2400	670	1.0
2. 105000*g* supernatant	1.20	1.8	1.50	2.3	2000	1670	2.5
3. $(NH_4)_2SO_4$ precipitate	0.76	2.1	2.76	4.3	2300	3040	4.5
4. DEAE-cellulose eluate	0.15	3.0	20.0	30.7	3500	23400	34.9

Source of tissue

Islets of Langerhans were prepared by the collagenase digestion of pancreatic tissue taken from male Sprague–Dawley rats or male guinea pigs of the Duncan–Hartley strain as described in detail by Howell & Taylor (1968).

Methods

Preparation of subcellular fractions. After isolation 500 rat islets of Langerhans were homogenized in 1ml of 10mM-sodium phosphate buffer, pH6.5, containing 0.3M-sucrose. This and all subsequent steps were performed at 4°C. The homogenate was separated by differential centrifugation into nuclear and debris (600*g* for 5min), mitochondrial and secretory-granule (20000*g* for 10min) and microsomal pellets (105000*g* for 60min), and a postmicrosomal supernatant fraction, as described in detail by Howell *et al.* (1969). Particulate material was resuspended in the homogenization buffer and all the fractions were assayed for cyclic-AMP-binding and cyclic-AMP-dependent protein phosphokinase activities and protein as described below.

Purification of islet-cell protein phosphokinase. Some 2000 islets of Langerhans isolated from guinea-pig pancreas were homogenized in 2ml of 10mM-sodium phosphate buffer, pH6.5, containing 0.3M-sucrose (stage 1). This and all subsequent steps were carried out at 4°C. The homogenate was spun at 105000*g* for 60min in the SW39L rotor of a Spinco L2 centrifuge (stage 2). The pellet was discarded and solid $(NH_4)_2SO_4$ (325mg/ml) was added to the supernatant. The mixture was left for 15min before centrifugation to remove the resultant precipitate. This precipitate was dissolved in 1ml of buffer containing 5mM-potassium phosphate, pH7.0, and 6mM-2-mercaptoethanol, and the solution was dialysed for

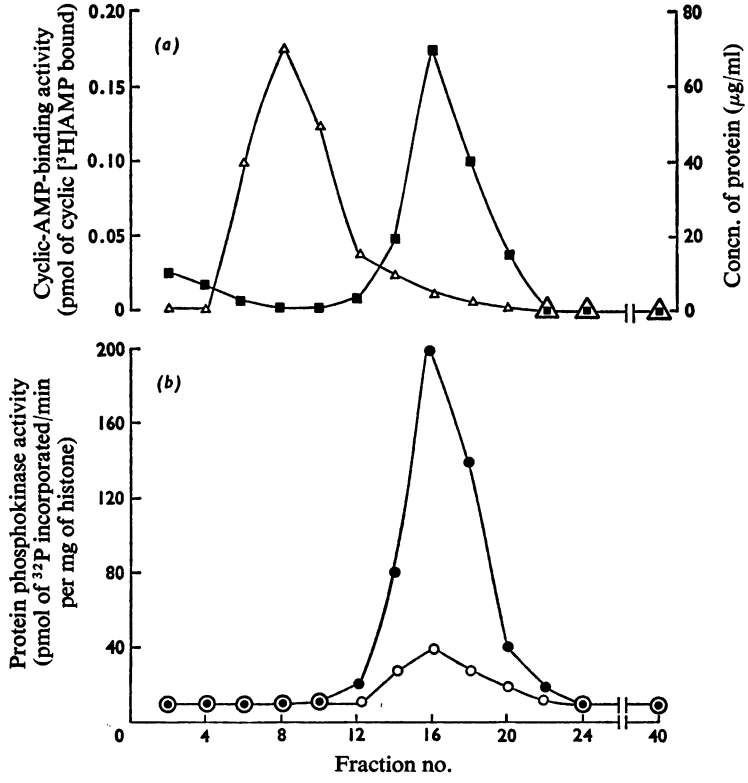

Fig. 1. *Ion-exchange chromatography of islet-cell protein phosphokinase and cyclic-AMP-binding activities*

Material obtained at stage 3 of the purification procedure was subjected to ion-exchange chromatography as described in detail in the text. Fractions were collected and assayed for protein (△), cyclic-AMP-binding activity (■) and protein phosphokinase activity in the presence (●) and absence (○) of cyclic AMP. The results presented are from a single experiment, each observation being made in triplicate, and are representative of the results obtained by this procedure.

12h against 1 litre of the same buffer, with four further changes each with 1 litre of buffer; this procedure was termed purification stage 3. The protein was further purified by ion-exchange chromatography on DEAE-cellulose (stage 4) or by electrophoresis on polyacrylamide gels.

Ion-exchange chromatography. Ion-exchange chromatography was performed on a column (1cm × 5cm) of DEAE-cellulose equilibrated with 5mM-potassium phosphate buffer, pH7.0. All buffers used contained 1mM-EDTA. The enzyme solution was applied to the column and the activity was eluted with 40ml of a linear gradient (0.005–0.3M) of potassium phosphate buffer (pH7.0). Fractions (1ml) were collected and assayed for cyclic-AMP-binding and cyclic-AMP-dependent protein phosphokinase activity as described below. The active fractions were pooled and dialysed at 4°C for 12h against 1 litre of 10mM-tris–HCl buffer, pH7.0, containing 6mM-2-mercaptoethanol, with four changes of the buffer. The dialysed fractions were concentrated to dryness by freeze-drying and stored at −76°C until required. Activity to this stage (stage 4) was termed purified protein phosphokinase and was used to investigate the properties of islet-cell protein phosphokinase.

Gel filtration. Gel filtration was performed on columns (60cm × 1.2cm) of Sephadex G-200 equilibrated with 50mM-tris–HCl buffer, pH7.4, containing 5mM-$MgCl_2$ and 6mM-2-mercaptoethanol. Samples in a volume of 0.6ml were applied to the columns and eluted with the equilibration buffer, 1ml fractions being collected. The fractions were assayed for cyclic-AMP-binding and cyclic-AMP-dependent protein phosphokinase activities as described below. For molecular-weight determinations the columns were calibrated by chromatography of Blue Dextran, rabbit γ-globulin, bovine serum albumin, cytochrome *c* and insulin. Elution positions were determined by measurement of the E_{280} of the column effluent in each case.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed at pH8.9

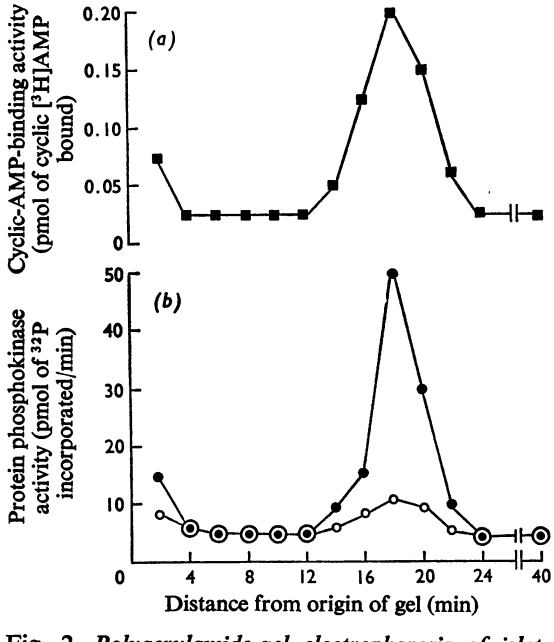

Fig. 2. *Polyacrylamide-gel electrophoresis of islet-cell protein phosphokinase and cyclic-AMP-binding activities*

Material obtained at stage 3 of the purification procedure was subjected to gel electrophoresis as described in detail in the text. The gels were either assayed for cyclic-AMP-binding activity (■) or were cut into 4mm segments for determination of protein phosphokinase activity in the presence (●) and absence (○) of cyclic AMP by the methods described in the text. The results presented are representative of those obtained by this procedure.

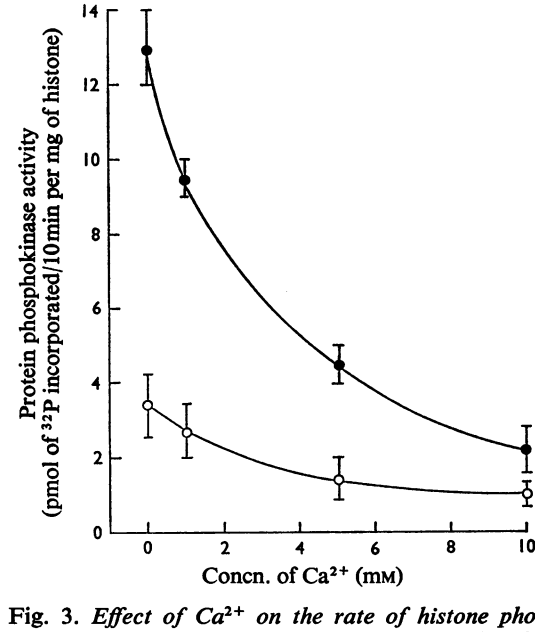

Fig. 3. *Effect of Ca^{2+} on the rate of histone phosphorylation by purified islet-cell protein phosphokinase*

The assay conditions were as described in the text with the addition of various concentrations of Ca^{2+} to the assay buffer. The effects of Ca^{2+} were investigated in the presence (●) and absence (○) of cyclic AMP. The results are given as mean values and the bars represent ±S.D. of eight observations.

by using the system of Davis (1964). Gels were prepared in tubes 6cm in length and 0.5cm in diameter and contained 7% polyacrylamide together with 0.3% bisacrylamide. Extracts to be analysed were adjusted to a sucrose concentration of 10% and one drop of an aqueous solution of Bromophenol Blue was added to serve as a tracker dye. The sample was layered directly on to the separation gel and the electrophoresis buffer was layered over the sample. Electrophoresis was performed at 4mA per tube until the tracker dye had migrated a standard distance of 4cm. Gels were then removed from the tubes, washed in water for 10min and used intact or sectioned into 20 segments for one of the procedures described below.

Assay of protein phosphokinase activity. Protein phosphokinase activity in tissue extracts and column fractions was measured at pH6.0 in a final volume of 0.2ml of an assay buffer containing 50mM-sodium β-glycerophosphate, 20mM-KF, 0.3mM-EGTA, 5mM-theophylline, 10mM-magnesium acetate and 0.1mM-[γ-^{32}P]ATP (3×10^6c.p.m.). Cyclic AMP (1μM) and histone (2mg/ml) were added to the buffer when necessary. The reaction was started by the addition of enzyme and after 30min incubation at 37°C it was stopped by the addition of 10% (w/v) trichloroacetic acid. The precipitated proteins were collected by centrifugation, washed once with trichloroacetic acid and dissolved in 0.2ml of 2M-NaOH. The proteins were reprecipitated with trichloroacetic acid and washed once more with trichloroacetic acid. The radioactivity in the final precipitate was determined as described below. Protein phosphokinase activity in segments of polyacrylamide gel was determined by adding gel slices directly to the assay mixture. The slices were removed after the addition of 10% trichloroacetic acid.

Determination of cyclic AMP binding. The binding of cyclic AMP to proteins in subcellular fractions prepared from islet cells, and in ion-exchange and gel-filtration chromatography, was determined by a method based on that of Walton & Garren (1970). Portions (0.1ml) of the fractions were incubated at 4°C for 30min in 50mM-tris–HCl buffer, pH7.4, containing 5mM-theophylline, 5mM-$MgCl_2$ and 0.1μM-cyclic [^{3}H]AMP in a final volume of 0.4ml. Separation of protein-bound from free nucleotide was then achieved by filtration of the mixture through cellulose acetate membranes, protein-bound

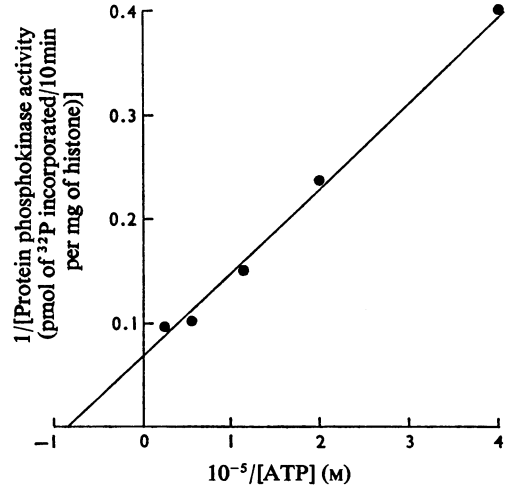

Fig. 4. *Effect of ATP concentration on the rate of histone phosphorylation by purified protein phosphokinase in the presence of cyclic AMP*

The assay conditions were as described in the text except that various concentrations of ATP in the assay buffer were used. The total radioactivity in the assay system was kept constant, and the total ATP concentration was varied by addition of unlabelled ATP. Each point represents the mean of six observations and the results are shown as a double-reciprocal plot.

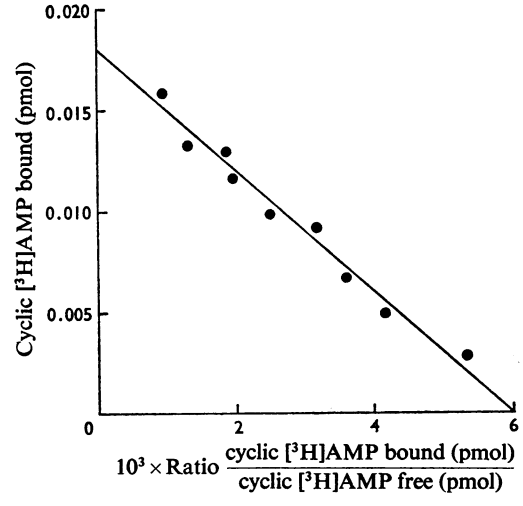

Fig. 5. *Effect of cyclic AMP concentration on the binding of cyclic AMP to purified protein phosphokinase*

Cyclic-AMP-binding activity of the purified protein phosphokinase (stage 4) was determined as described in the text with various concentrations of cyclic [^{3}H]-AMP. The results are the mean of four observations and have been plotted according to Scatchard (1949) to allow an estimate of the intrinsic association constant of the protein with cyclic AMP.

cyclic AMP being retained by the membrane. The membranes were dried at 60°C and their radioactivity was determined after the addition of scintillator as described below. The distribution of cyclic-AMP-binding protein in polyacrylamide gels was determined by the method of Gill & Garren (1971), in which the intact gels were soaked for 3h at 4°C in 50mM-tris-HCl buffer, pH7.4, containing 8mM-theophylline, 5mM-$MgCl_2$ and 0.1μM-cyclic [^{3}H]-AMP. The gels were then rinsed free of unbound cyclic AMP in running tap water for 12h before being sliced. The radioactivity in gel slices was determined as described below.

Radioactivity determination. Radioactivity was determined by using a Beckman LS 233 liquid-scintillation spectrometer. The ^{32}P content of protein precipitates was determined by the use of Čerenkov radiation, after the precipitate had been dissolved in 0.1M-NaOH, the counting efficiency being approx. 30%. The ^{3}H content of gel slices was determined after the gel had been dissolved in 30% (v/v) H_2O_2 overnight at 60°C, followed by the addition of scintillator (toluene–Triton X-100–2,5-diphenyloxazole (140:60:1, v/v/w), 10ml of scintillator being required for 1ml of solution. The ^{3}H content of cellulose membranes was determined directly after the addition of scintillator. The counting efficiency for ^{3}H under these conditions was approx. 20%.

Protein determinations. The protein content of tissue extracts and column fractions was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Results

Subcellular distribution of cyclic-AMP-binding and protein phosphokinase activities

The results in Table 1 show that 85% of the cyclic-AMP-binding and cyclic-AMP-dependent protein phosphokinase activities present in the original homogenate were recovered in the 105000*g* supernatant, whereas only 47% of the protein phosphokinase activity obtained in the absence of cyclic AMP was recovered in this fraction. The protein phosphokinase activity in the particulate fractions was relatively insensitive to cyclic AMP, and these fractions demonstrated much lower cyclic-AMP-binding activities.

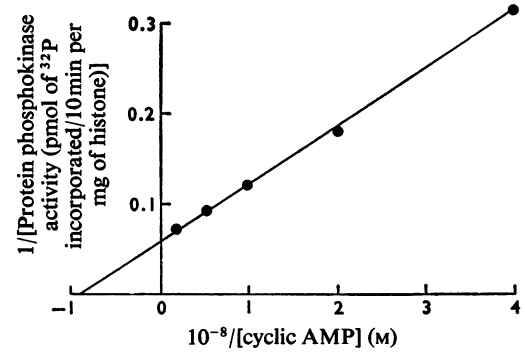

Fig. 6. *Effect of cyclic AMP concentration on the rate of histone phosphorylation by purified protein phosphokinase*

Purified protein phosphokinase (stage 4) was incubated at 4°C for 30min with various concentrations of cyclic AMP in 0.1ml of the protein phosphokinase assay buffer. At the end of this incubation, 0.1ml of the protein phosphokinase assay buffer containing histone (2mg/ml) was added and the reaction continued for 10min at 37°C. The reaction was terminated by the addition of 10% trichloroacetic acid and the ^{32}P incorporated into protein was determined as described in the text. The results shown are each the mean of six observations and are shown as a double-reciprocal plot.

Table 3. *Relative effectiveness of various proteins as substrates for purified islet-cell protein phosphokinase*

The assay conditions were as described in the text and each protein was used at a final concentration of 1mg/ml. The results are given as the means±S.D. of six observations.

	Protein phosphokinase activity (pmol of ^{32}P incorporated/min per mg of protein)		Ratio $\frac{+\text{cyclic AMP}}{-\text{cyclic AMP}}$
Protein substrate	−Cyclic AMP	+Cyclic AMP	
Histone	2.4±0.5	13.0±2.0	5.4
Casein	2.1±0.4	8.7±1.8	4.2
Protamine	2.1±0.4	6.5±1.5	3.1
Bovine serum albumin	1.6±0.3	2.9±0.7	1.8
Ovalbumin	1.3±0.2	2.1±0.5	1.6

Table 4. *Subcellular distribution of substrates for protein phosphokinase in islets of Langerhans*

Subcellular fractions of an homogenate of 2000 guinea-pig islets of Langerhans were prepared as described in the text and they were incubated with purified islet-cell protein phosphokinase (stage 4) in the assay buffer in the presence and absence of cyclic AMP. After 10min incubation at 37°C the reaction was terminated by the addition of 10% trichloroacetic acid, and carrier protein (0.1 ml of a 10mg/ml solution of bovine serum albumin in water) was added. The precipitated proteins were washed repeatedly with trichloroacetic acid and their radioactivity was determined (see the text). The results presented have been corrected for values obtained without the addition of purified protein phosphokinase, since some endogenous protein phosphokinase activity is present in each of the fractions utilized (Table 1). Each value is the mean±S.D. of eight observations.

		Protein phosphokinase activity (pmol of ^{32}P incorporated/10min)			
		Total activity		Activity/mg of protein	
Fraction	Protein content (mg)	−Cyclic AMP	+Cyclic AMP	−Cyclic AMP	+Cyclic AMP
Homogenate	3.05±0.5	400±80	510±130	130	170
Nuclear pellet	1.05±0.2	150±40	180±50	140	170
Granule pellet	0.55±0.1	40±10	50±10	70	90
Microsomal pellet	0.36±0.2	30±8	30±7	90	90
Postmicrosomal supernatant	0.96±0.3	80±20	240±70	80	250

Purification of protein phosphokinase and cyclic-AMP-binding protein activities

Typical results of the procedure used for the purification of cyclic-AMP-dependent protein phosphokinase activity from islets of Langerhans are shown in Table 2, from which it is clear that the protein phosphokinase and cyclic-AMP-binding activities are enriched in parallel throughout the purification procedure. Further, both activities appeared in the same fraction when the extracts were further purified by ion-exchange chromatography or polyacrylamide-gel electrophoresis (Figs. 1 and 2).

Properties of purified protein phosphokinase

The rate of histone phosphorylation by purified protein phosphokinase under the assay conditions used was linear over a 30-min incubation period in the presence and absence of cyclic AMP and was proportional to the enzyme concentration under these conditions (results not shown). Histone phosphorylation was dependent on the presence of Mg^{2+}, and the optimum concentration was in the range 10–20mM. The effect of Ca^{2+} on the reaction is shown in Fig. 3. It inhibited protein phosphokinase activity in the presence and absence of cyclic AMP, at all Ca^{2+} concentrations studied. The cyclic-AMP-dependent protein phosphokinase activity showed a pH optimum of 6.2 when histone was used as the substrate. The binding of cyclic AMP to the protein phosphokinase was independent of pH over the range 5.5–7.5.

The effect of ATP concentration on the rate of histone phosphorylation by cyclic-AMP-dependent protein phosphokinase is shown in Fig. 4. The K_m for ATP under the conditions of the assay was 1.1×10^{-5}M. The interaction of cyclic AMP with protein phosphokinase, as a function of the total concentration of the nucleotide, was determined, and a Scatchard plot (Scatchard, 1949) of these results was drawn to determine the intrinsic association constant of the protein–cyclic AMP complex (Fig. 5). The value of 1.15×10^{-8}M agrees closely with the K_m value for cyclic AMP of the cyclic-AMP-dependent protein phosphokinase activity, which was 1.10×10^{-8}M (Fig. 6).

The relative effectiveness of various proteins as substrates for the protein phosphokinase is shown in Table 3, which indicates that histone was the most effective substrate on a weight basis. Protein components of subcellular fractions prepared from an islet-cell homogenate were also readily phosphorylated (Table 4).

Gel filtration of protein phospkinase

The protein phosphokinase and cyclic-AMP-binding activities from any stage of purification emerged as a single peak, corresponding to a molecular weight of 180000, when subjected to gel filtration on Sephadex G-200 (Fig. 7). Treatment of the protein with cyclic AMP before gel filtration resulted in the dissociation of the cyclic-AMP-binding activity from the protein phosphokinase activity, and in these conditions the protein phosphokinase activity was no longer stimulated by cyclic AMP (Fig. 7). The

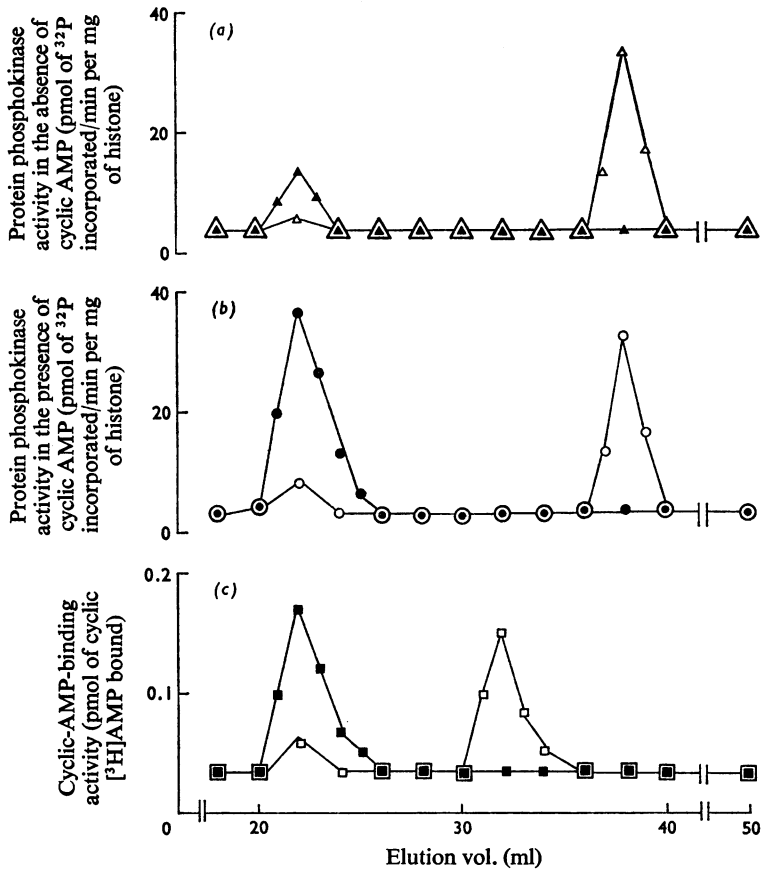

Fig. 7. *Elution of purified islet-cell protein phosphokinase from Sephadex G-200; effect of cyclic AMP*

Protein phosphokinase, purified to stage 4, from 2000 islets of Langerhans was dissolved in 1ml of buffer containing 50mM-tris–HCl, pH7.4, 5mM-$MgCl_2$ and 6mM-2-mercaptoethanol. The resulting solution was divided into two and 0.1ml of 6μM-cyclic AMP in water was added to one portion (treated enzyme) and 0.1ml of water was added to the other (untreated enzyme). The solutions were maintained at 4°C for 30min and then subjected to gel filtration on columns of Sephadex G-200 (see the text). Fractions (1ml) were collected and assayed for cyclic-AMP-binding activity (*c*) [untreated enzyme (■), treated enzyme (□)] and protein phosphokinase activity in the presence (*b*) [untreated enzyme (●), treated enzyme (○)] and absence (*a*) [untreated enzyme (▲), treated enzyme (△)] of cyclic AMP as described in the text.

protein phosphokinase was eluted in a position corresponding to a molecular weight of 75000, and the cyclic-AMP-binding activity was eluted in a position corresponding to a molecular weight of 90000.

Endogenous protein phosphokinase activity in islets incubated in vitro

The results in Table 5 show that 3-isobutyl-1-methylxanthine and theophylline, which are known to raise the intracellular concentration of cyclic AMP in rat islets of Langerhans (Montague & Cook, 1971), increased the activity of protein phosphokinase in rat islets, whereas adrenaline, which lowers cyclic AMP concentrations in islets, decreased the protein phosphokinase activity.

Discussion

The effects of cyclic AMP in a variety of mammalian tissues appear to be related to the ability of the nucleotide to activate cyclic-AMP-dependent protein phosphokinases, the activation occurring as a consequence of the nucleotide binding to a specific

Table 5. *Effects of 3-isobutyl-1-methylxanthine, theophylline and adrenaline on endogenous protein phosphokinase activity in islets of Langerhans incubated in vitro*

Isolated rat islets of Langerhans were incubated in groups of 20 in 2ml of a bicarbonate-buffered salt solution (Gey & Gey, 1936) containing 8mM-glucose alone or 8mM-glucose plus 1mM-3-isobutyl-1-methylxanthine, 5mM-theophylline or 1 μM-adrenaline. After incubation for 30min at 37°C, the medium was removed from the islets, which were disrupted ultrasonically after the addition of 0.5ml of the protein phosphokinase assay buffer (see the text) containing histone (2mg/ml). After incubation at 37°C for 10min the reaction was terminated by the addition of 10% trichloroacetic acid. The precipitated proteins were washed repeatedly with trichloroacetic acid and their radioactivity was determined as described in the Experimental section. Each result is the mean±S.D. of eight observations.

Addition to medium	Protein phosphokinase activity (pmol of ^{32}P incorporated/min per mg of histone)
Glucose	11.4±1.2
Glucose+3-isobutyl-1-methylxanthine	20.2±1.8*
Glucose+theophylline	16.5±1.3*
Glucose+adrenaline	6.5±0.5*

* Values assessed by Student's *t* test to be significantly different from the glucose control ($P<0.01$).

receptor subunit of the enzyme (Gill & Garren, 1971; Erlichman *et al.*, 1971; Reimann *et al.*, 1971*a*). The results in Table 1 demonstrate the occurrence, in a postmicrosomal supernatant fraction prepared from an islet-cell homogenate, of cyclic-AMP-binding and cyclic-AMP-dependent protein phosphokinase activities. Further, both activities were purified in parallel (Table 2) and appeared as a single component when subjected to ion-exchange chromatography (Fig. 1), polyacrylamide-gel electrophoresis (Fig. 2) or gel filtration (Fig. 7). These results indicate that islet cells contain a cyclic-AMP-dependent protein phosphokinase with cyclic-AMP-receptor activity in a single component, and suggest that the effects of cyclic AMP in islets of Langerhans may be mediated through the activation of a cyclic-AMP-dependent protein phosphokinase. That such an activation does occur in islet cells in response to changes in the intracellular concentration of cyclic AMP is shown by the results in Table 5. Thus, incubation of isolated rat islets of Langerhans in the presence of theophylline or 3-isobutyl-1-methylxanthine, agents known to raise cyclic AMP concentrations in rat islets of Langerhans (Montague & Cook, 1971), resulted in an increased activity of the protein phosphokinase, whereas adrenaline, an agent known to decrease islet cyclic AMP concentrations, lowered the protein phosphokinase activity. These results suggest that alterations in the intracellular concentration of cyclic AMP do produce changes in the activity of the cyclic-AMP-dependent protein phosphokinase in islet cells.

The mechanism by which cyclic AMP effects the increase in catalytic activity of the islet-cell cyclic-AMP-dependent protein phosphokinase appears to be similar to that for the enzymes isolated from liver, adrenal cortex and skeletal muscle (Erlichman *et al.*, 1971; Gill & Garren, 1971; Reimann *et al.*, 1971*a*). Thus, the results in Fig. 7 suggest that the cyclic-AMP-dependent protein phosphokinase from islet cells consists of two subunits, one of molecular weight 90000, which binds cyclic AMP (receptor subunit), and the other of molecular weight 75000, which possesses protein phosphokinase activity (catalytic subunit). In the presence of cyclic AMP the receptor subunit binds the nucleotide and becomes dissociated from the catalytic subunit. The catalytic subunit is then no longer dependent on cyclic AMP for its activity and exhibits maximal phosphokinase activity in the dissociated state.

The kinetic properties of the islet-cell cyclic-AMP-dependent protein phosphokinase, with histone as substrate, are very similar to those of the enzymes extracted from other mammalian tissues. Thus its intrinsic association constant for cyclic AMP (1.15×10^{-8}M), and its K_m for cyclic AMP and ATP (1.1×10^{-8}M and 1.1×10^{-5}M respectively) are very similar to the values obtained for the enzyme from brain (Miyamoto *et al.*, 1969) and skeletal muscle (Reimann *et al.*, 1971*b*).

The demonstration of a high activity of the cyclic-AMP-dependent protein phosphokinase in the postmicrosomal supernatant fraction of an islet homogenate (Table 1) is noteworthy since this fraction also contained the highest concentration of substrates for the cyclic-AMP-dependent protein phosphokinase (Table 4).

The results presented here suggest that the mode

of action of cyclic AMP in insulin release may be to increase the activity of a cyclic-AMP-dependent protein phosphokinase, thereby promoting the phosphorylation and activity of a rate-determining component of the secretory mechanism. In view of the association between cyclic-AMP-dependent protein phosphokinase and microtubules, shown in brain (Goodman *et al.*, 1970), and the proposed role of microtubules in insulin release (Lacy *et al.*, 1968), it is possible that the phosphorylation of microtubular protein by islet-cell protein phosphokinase may be part of the mechanism of control of insulin release by cyclic AMP. Some indirect evidence for this hypothesis derives from the effects of colchicine, an agent which interferes with microtubule function, in inhibiting not only insulin secretion (Lacy *et al.*, 1968) but also cyclic-AMP-dependent phosphorylation of endogenous islet proteins (Montague & Howell, 1972). Further, over 80% of the colchicine-binding (microtubule) protein present in islet homogenates is recovered in the 105000*g* supernatant fraction (S. L. Howell, unpublished work), which provides the major substrates for the cyclic-AMP-dependent protein phosphokinase (Table 4). It will therefore be of interest to identify the endogenous substrates of the cyclic-AMP-dependent protein phosphokinase in islets and to explore in detail the role of these proteins in regulating the secretory process.

W. M. is a Beit Memorial Research Fellow. Financial assistance towards the cost of this work from the British Diabetic Association and the Medical Research Council to Dr. K. W. Taylor and from Hoechst Pharmaceuticals (to S. L. H.) is gratefully acknowledged.

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