Effects of Glucose, Glucose Metabolites and Calcium Ions on Adenylate Cyclase Activity in Homogenates of Mouse Pancreatic Islets

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The effects of glucose, a series of glucose metabolites, nicotinamide nucleotides, Ca^{2+} and *p*-chloromercuribenzenesulphonate on adenylate cyclase activity in homogenates of mouse pancreatic islets were studied. The basal activity of the adenylate cyclase was approx. 6 pmol of cyclic AMP formed/30 min per μg of DNA at 30°C . The enzyme activity was stimulated by some 150% by fluoride. Starvation of the animals for 48 h had no effect on either the basal or the fluoride-stimulated activity. The adenylate cyclase activity was increased by 40–50% when 17 mM-glucose, 10 μM -phosphoenolpyruvate or 10 μM -pyruvate was added to the assay medium. The effect of glucose was unchanged in the presence of 17 mM-mannoheptulose, and mannoheptulose alone had no effect. The other glycolytic intermediates, and the coenzymes NAD^+ , NADH and NADPH, at concentrations up to 1 mM were without any detectable effect on the rate of formation of cyclic AMP. The insulin secretagogue *p*-chloromercuribenzenesulphonate inhibited the adenylate cyclase markedly even at a concentration of 10 μM . Calculated concentrations of free Ca^{2+} of 10 μM and 0.1 mM inhibited adenylate cyclase by 29 and 71% respectively. It is concluded that both glucose itself and phosphoenolpyruvate and/or pyruvate are true activating ligands for islet adenylate cyclase and that inhibition of the cyclase by Ca^{2+} may be of physiological significance.

It is well documented that a high extracellular concentration of glucose can raise the content of cyclic AMP in pancreatic islets (Charles *et al.*, 1973; Grill & Cerasi, 1973; Capito & Hedekso, 1974; Zawulich *et al.*, 1975). It has been shown that this effect of glucose is dependent on the presence of extracellular Ca^{3+} (Charles *et al.*, 1975; Zawulich *et al.*, 1975; Hellman, 1976) and is impaired if the glycolysis in the β -cells is inhibited by mannoheptulose (Capito & Hedekso, 1974; Zawulich *et al.*, 1975), or by starvation (Capito & Hedekso, 1974; Hellman, 1976).

Other evidence also suggests that the observed stimulating effect of glucose on the formation of cyclic AMP is mediated by a glycolytic intermediate, as the effect can be simulated by glyceraldehyde (Hellman *et al.*, 1974a) and inosine (Capito & Hedekso, 1976), both of which enter the glycolytic pathway during their metabolism. This postulate has, however, been challenged by the demonstration that the α -anomer of glucose is more effective than the β -anomer in eliciting the cyclic AMP response (Grill & Cerasi, 1975), although no anomeric specificity can be shown in the phosphorylation and further oxidation of glucose (Idahl *et al.*, 1975).

Howell *et al.* (1973) have shown an increased activity of adenylate cyclase in homogenates from

isolated rat islets that have been preincubated with glucose or pyruvate for more than 2 h. However, all attempts to demonstrate a direct stimulatory effect of glucose or its metabolites on adenylate cyclase activity in islet homogenates or membrane fractions have hitherto been in vain (Atkins & Matty, 1971; Davis & Lazarus, 1972; Howell & Montague, 1973; Kuo *et al.*, 1973).

Our present data show that adenylate cyclase activity in homogenates of mouse pancreatic islets can be stimulated by glucose itself as well as by phosphoenolpyruvate and/or pyruvate. It is further demonstrated that Ca^{2+} and *p*-chloromercuribenzenesulphonate inhibit the enzyme activity in a concentration-dependent manner.

Experimental

Materials

Human serum albumin was from Behringwerke A.G., Marburg/Lahn, W. Germany. 3-Isobutyl-1-methylxanthine was from EGA-Chemie, Steinheim/Albuch, W. Germany. Cyclic [8- ^3H]AMP (27.5 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Collagenase, creatine kinase, coenzymes and glucose metabolites were from Boehringer, Mannheim, W. Germany. Glyceraldehyde 3-

phosphate and dihydroxyacetone phosphate were supplied as the acetal and ketal forms respectively, and were hydrolysed by the manufacturers' instructions before use. *p*-Chloromercuribenzenesulphonic acid and calf thymus DNA were from BDH Chemicals, Poole, Dorset, U.K. Instagel was from Packard Instrument Co., La Grange, IL, U.S.A. Cyclic AMP-binding protein from rabbit skeletal muscle was prepared as described by Cooper *et al.* (1972). Other reagents used were of the purest grade obtainable.

Preparation of islets

Pancreases from male albino mice (26 g) were used. The animals were either fed on a standard laboratory diet or starved for 48 h (with free access to drinking water) before being killed by decapitation. Islets were separated by a collagenase method and harvested with a loop as described by Coll-Garcia & Gill (1969). The amount of collagenase that was used for preparation of the islets was minimized for each batch of collagenase, as too intense treatment with collagenase diminished the observed activations of the adenylate cyclase. About 4–12 mg of collagenase from different batches was used per 2 ml of medium for preparation of cells from five pancreases.

Adenylate cyclase activity

Some 100–200 islets were collected in 150 μ l of 25 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-MgCl₂ and 1 mM-EDTA. A homogenate was prepared by sonication (10 s, 40 W). We checked by phase-contrast microscopy that all cells were disrupted by this procedure. A portion of the homogenate (containing approx. 5 μ g of protein) was added to the reaction mixture, which consisted of 25 mM-Tris/HCl buffer, pH 7.6, 5 mM-MgCl₂, 1 mM-EDTA, 0.15% human serum albumin, 1.5 mM-3-isobutyl-1-methylxanthine, 3.0 mM-ATP, an ATP-regenerating system (0.5 mg of creatine kinase/ml and 5.5 mg of phosphocreatine/ml), and further additions as stated in the text and Table. The total incubation volume was 40 μ l. A substrate concentration of 3.0 mM-ATP was chosen because preliminary results revealed that the substrate (ATP) activation curve had a maximum at 2–3 mM-ATP. After incubation for 30–60 min at 30°C the reaction was stopped by the addition of 8 μ l of 2.5 M-H₂SO₄. The non-cyclic nucleotides were precipitated by addition of 16 μ l of 1 M-barium acetate (Cooper *et al.*, 1973) and the precipitate was removed by centrifugation at 5000g for 5 min. The pH of the supernatant was adjusted to 5.5 by the addition of 0.4 M-Na₃PO₄, and the samples were stored at –20°C until the content of cyclic AMP was determined in duplicate as described below. The activity was proportional to the amount of homo-

genate added and was linear with time for at least 60 min.

Cyclic AMP assay

Cyclic AMP was measured by a protein-binding radioassay (Gilman, 1970); portions (50 μ l) of the samples and standards (made up in buffer similar to the samples) were placed in polypropylene tubes (10 mm $\times$ 30 mm). Then 50 μ l of 0.25 M-potassium phosphate buffer, containing 1 mM-3-isobutyl-1-methylxanthine, 6 mM-mercaptoethanol and 0.35 pmol of cyclic [³H]AMP (27.5 Ci/mmol) were added, followed by the addition of 100 μ l of the same buffer without cyclic AMP but including 0.04% human serum albumin and 0.02 mg of binding protein. The tubes were incubated for 1.5 h at 4°C, after which the protein-bound cyclic AMP was precipitated with (NH₄)₂SO₄ and CaSO₄ as described by Geisler *et al.* (1977). Then 0.1 ml of distilled water and 1.2 ml of Instagel were added to the precipitate and the radioactivity was counted in a Packard Tri-Carb liquid-scintillation spectrometer.

Protein assay

The protein content of samples of the islet homogenate was assayed spectrophotometrically as the difference between the absorbance at 224 nm and 236.5 nm (Groves *et al.*, 1968). Bovine serum albumin was used as standard.

DNA assay

The DNA content of samples of the islet homogenate was determined by the method of Kissane & Robins (1958), with calf thymus DNA as standard.

Calculations

Some of the additions to the incubation medium, e.g. Ca²⁺ and fructose 1,6-bisphosphate, caused a change in the concentration of free Mg²⁺ in the assay mixture, which made it hard to evaluate if these additions had any direct effects on the adenylate cyclase activity. Therefore a computer program (Flodgaard & Fleron, 1974) was used to calculate the total amounts of MgCl₂ that should be added in each experiment in order to maintain the same concentration of free Mg²⁺. This program takes into account all known values of pH, ionic strength, relevant acid dissociation constants and stability constants of possible metal-ion complexes (Sillen & Martell, 1971). In all experiments the concentration of free Mg²⁺ was calculated to be 1.22 mM. The same program was used to determine the free concentration of Ca²⁺ in the assay when this ion was added.

Results

A prerequisite for the described method to be used for determination of the activity of adenylate cyclase is that no cyclic AMP is degraded during the incuba-

tions. Preliminary experiments were therefore performed to determine the ability of 3-isobutyl-1-methylxanthine to inhibit the activity of the islet phosphodiesterases. It was found that the low- K_m phosphodiesterase ($K_m = 5 \times 10^{-6} M$) was completely inhibited by 1 mM-3-isobutyl-1-methylxanthine in homogenates of islets from both fed and starved mice, whereas it was not possible to inhibit the high- K_m phosphodiesterase ($K_m = 2.7 \times 10^{-4} M$) by more than about 60% by 3-isobutyl-1-methylxanthine. It was also found that the K_i (3-isobutyl-1-methylxanthine) was unaffected by starvation. As the low- K_m phosphodiesterase must be assumed to be the one that is responsible for the possible degradation of cyclic AMP under the present conditions, where the maximum concentration of cyclic AMP formed in the assay medium would be 0.1–0.2 μM , it was concluded that the method used is fully valid.

The activity of the adenylate cyclase in islet homogenates was 5.1 ± 1.1 pmol of cyclic AMP formed/30 min per μg of DNA in the fed state and 7.3 ± 0.7 pmol of cyclic AMP formed/30 min per μg of DNA in the starved state ($n = 8$). NaF (25 mM) stimulated the activity some 150% irrespective of the nutritional state of the animals [12.9 ± 2.5 pmol of cyclic AMP formed/30 min per μg of DNA in the fed

state ($n = 10$) and 18.7 ± 2.7 pmol of cyclic AMP formed/30 min per μg of DNA in the starved state ($n = 6$]. The difference in the activities in the fed and starved state was not statistically significant ($P > 0.05$).

In homogenates prepared from islets of fed mice the adenylate cyclase activity was increased by 40–50% when 17 mM-glucose, 10 μM -phosphoenolpyruvate or 10 μM -pyruvate was added to the incubation medium (Table 1). In three experiments it was shown that the effect of glucose was still marked and unchanged when 17 mM-mannoheptulose was added together with glucose and that 17 mM-mannoheptulose alone had no effect. None of the other glycolytic intermediates had any statistically significant effect on the adenylate cyclase activity at the concentration tested. The coenzymes NAD⁺, NADH and NADPH, at concentrations up to 1 mM, were also without any effect on the rate of formation of cyclic AMP (Table 1).

The insulin secretagogue *p*-chloromercuribenzenesulphonate (Bloom *et al.*, 1972) inhibited the adenylate cyclase activity markedly, even at a concentration of 10 μM (Table 1).

Calculated free concentrations of Ca²⁺ ranging from 10 nM to 0.1 mM (total calcium concentrations

Table 1. Adenylate cyclase activity in homogenates of mouse pancreatic islets

Adenylate cyclase activity in homogenates prepared from fed islets was measured at 30°C as the amount of cyclic AMP formed during a 30 min incubation in the reaction mixture described in the Experimental section. Results are given as percentages $\pm$ S.E.M. of the control value (320 ± 44 pmol/30 min per mg of protein; mean $\pm$ S.E.M., $n = 30$). Statistical evaluation of the data was made by a paired *t* test. NS, not significant, $P > 0.05$.

Additions	Concn.	Activity (% of control)	<i>n</i>	<i>P</i> (versus control)
Glucose	2.5 mM	87 $\pm$ 13	7	NS
	16.7 mM	148 $\pm$ 8	10	<0.02
Glucose 6-phosphate	1 mM	69 $\pm$ 8	8	NS
Fructose 6-phosphate	1 mM	98 $\pm$ 10	3	NS
Fructose 1,6-bisphosphate	1 mM	96 $\pm$ 6	3	NS
Glyceraldehyde 3-phosphate	0.1 mM	108 $\pm$ 13	4	NS
	10 μM	100 $\pm$ 7	3	NS
Dihydroxyacetone phosphate	0.1 mM	92 $\pm$ 13	4	NS
	10 μM	89 $\pm$ 8	4	NS
3-Phosphoglycerate	10 μM	92 $\pm$ 14	4	NS
2-Phosphoglycerate	10 μM	107 $\pm$ 8	5	NS
Phosphoenolpyruvate	10 μM	142 $\pm$ 9	14	<0.05
Pyruvate	10 μM	152 $\pm$ 14	8	<0.02
NADH	1 mM	117 $\pm$ 16	5	NS
NADPH	1 mM	94 $\pm$ 13	4	NS
NAD ⁺	1 mM	83 $\pm$ 9	6	NS
Free Ca ²⁺	10 nM	94 $\pm$ 10	7	NS
	0.1 μM	80 $\pm$ 11	7	NS
	1 μM	86 $\pm$ 9	8	NS
	10 μM	71 $\pm$ 10	8	<0.05
	0.1 mM	29 $\pm$ 9	8	<0.025
<i>p</i> -Chloromercuribenzenesulphonate	10 μM	34 $\pm$ 4	4	<0.01
	0.1 mM	17 $\pm$ 12	5	<0.02
	1 mM	5 $\pm$ 3	5	<0.02

1 μ M–1.2 mM) inhibited the adenylate cyclase in a concentration-dependent manner. The inhibition was, however, only statistically significant at 10 μ M- and 0.1 mM-free Ca^{2+} .

Discussion

The activity of adenylate cyclase measured in the present experiments is of the same order of magnitude as that found by Howell & Montague (1973) in homogenates of rat islets. In contrast with the situation in rat islet homogenates (Howell *et al.*, 1973), the basal activity of adenylate cyclase was not decreased by 48 h starvation of the animals. This is, however, in good agreement with our previous observation that the basal concentration of cyclic AMP and the activities of the two phosphodiesterases are identical in islets from fed and starved mice (Capito & Hedekov, 1974).

Although it is well established that a high extracellular glucose concentration stimulates the formation of cyclic AMP in isolated pancreatic islets (Charles *et al.*, 1973; Grill & Cerasi, 1973; Capito & Hedekov, 1974; Zawulich *et al.*, 1975) and although it has been shown that preincubation of rat islets with glucose or pyruvate for more than 2 h has a stimulatory effect on adenylate cyclase activity as measured in homogenates prepared from the same islets (Howell *et al.*, 1973), we have now shown for the first time a direct activating effect of glucose itself and of two glucose metabolites on adenylate cyclase activity in homogenates of pancreatic islets.

The possibility that 10 μ M-phosphoenolpyruvate could be converted into a stimulating concentration of pyruvate or, conversely, that 10 μ M-pyruvate could be metabolized to a stimulating concentration of phosphoenolpyruvate, seems remote, as no cofactors for these reactions were added and because the cell water, cofactors and enzymes were diluted 1:2000 or more in the assay medium. The possibility can, nevertheless, not be completely dismissed and the activating metabolite(s) may therefore be either phosphoenolpyruvate or pyruvate or both.

That none of the glucose metabolites above the phosphoenolpyruvate level, even at a concentration of 1 mM, activated the adenylate cyclase activity can be taken as circumstantial evidence against the possibility that glucose exerted its stimulating effect by being metabolized to phosphoenolpyruvate or pyruvate. The fact that the activating effect of glucose was also observed and unchanged when a high concentration of mannoheptulose (which inhibits phosphorylation of glucose) was added together with a high concentration of glucose also indicates that glucose itself activates the adenylate cyclase. Thus both glucose and phosphoenolpyruvate and/or pyruvate must be regarded as true activating ligands for the adenylate cyclase molecule.

Our results therefore offer an explanation of the apparent contradiction implied in the observations that the glucose-stimulated formation of cyclic AMP on the one hand has been shown to be preferential for the α -anomer of D-glucose (Grill & Cerasi, 1975) and on the other hand has been thought to be dependent on formation of a glucose metabolite (Capito & Hedekov, 1974, 1976; Hellman *et al.*, 1974a), although the glycolytic metabolism of glucose in the islets displays no anomeric specificity (Idahl *et al.*, 1975).

One explanation of the failure of other attempts to show an effect of glucose on adenylate cyclase activity could be that the enzyme in some of the assay systems from the outset was much stimulated by the very high concentration of phosphoenolpyruvate that was added together with pyruvate kinase as an ATP-regenerating system. Other explanations would include differences in collagenase treatment of the tissue and in homogenization procedure.

In contrast with the situation in intact islets (Charles *et al.*, 1975; Zawulich *et al.*, 1975; Hellman, 1976), glucose stimulation of adenylate cyclase activity in islet homogenates did not require the addition of Ca^{2+} to the medium. This finding may indicate either that the homogenization procedure has caused a sufficient release of bound Ca^{2+} , or that Ca^{2+} is needed for maintenance of some function in the intact β -cell that is unimportant under the present incubation conditions. Another explanation would be that the formation of cyclic AMP is secondary to the secretion of insulin, and that the observed requirement for extracellular Ca^{2+} in the glucose-induced formation of cyclic AMP in fact reflects the well-known need for extracellular Ca^{2+} for glucose-induced insulin secretion. Such an assumption is in accordance with the demonstration by Leitner *et al.* (1975) that the cyclic AMP found in granules from pancreatic islets seems to be formed during the extrusion of the secretory granules.

It has been shown that Ca^{2+} inhibits the activity of adenylate cyclase in homogenates or membrane fractions prepared from mouse islets (Davis & Lazarus, 1972) and from other tissues (Birnbaumer, 1973; Steer & Levitzki, 1975). These results are confirmed here and further extended to show that the inhibition by Ca^{2+} is concentration-dependent but only achieves statistical significance when the concentration of free Ca^{2+} reaches 10 μ M. The concentration of free Ca^{2+} in the cytosol has been reported to be between 10 nM and 10 μ M in different tissues (Rasmussen, 1970) and it is therefore possible that the observed inhibition of the adenylate cyclase by Ca^{2+} may be of physiological importance.

It is known from various tissues that thiol reagents inhibit adenylate cyclase, suggesting that thiol groups are necessary for the catalytic activity (Pohl *et al.*, 1971; Storm & Dolginow, 1973). This is

confirmed in the present study, where it is shown that 10 μM -*p*-chloromercuribenzenesulphonate inhibits the adenylate cyclase by approx. 65%. In contrast with these results, Hellman *et al.* (1974b) have shown that 0.1 mM-*p*-chloromercuribenzenesulphonate increases the content of cyclic AMP in islets from obese-hyperglycaemic mice if 3-isobutyl-1-methyl-xanthin is present. This seems to suggest that in intact islets the essential thiol groups in the catalytic site of the adenylate cyclase molecule must be inaccessible for the slowly (Hellman *et al.*, 1973) permeating mercurial compound.

The demonstration here of a direct effect of glucose on islet adenylate cyclase activity should encourage the testing of Cerasi's (1975) hypothesis that pancreatic β -cells contain two or more pools of adenylate cyclase, only one of which is capable of being activated by glucose.

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