The Role of Adenosine 3':5'-Cyclic Monophosphate in the Regulation of Insulin Release

PROPERTIES OF ISLET-CELL ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE PHOSPHODIESTERASE

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1. An assay has been developed with sufficient sensitivity for determination of the adenosine 3':5'-cyclic monophosphate diesterase activity in islets of Langerhans, and has been used to investigate the response of the enzyme to various agents which are known to affect insulin release. 2. The subcellular distribution of the enzyme in islets of Langerhans prepared from guinea-pig pancreas was investigated and over 70% of the activity present in the original homogenate was recovered in the supernatant fraction. 3. Gel filtration of the activity present in the supernatant fraction on Sephadex G-200 gave a single peak of activity with an apparent molecular weight of 200000. The phosphodiesterase activity in the peak fraction showed two apparent K_m values for adenosine 3':5'-cyclic monophosphate (cyclic AMP) of $3\mu\text{M}$ and $30\mu\text{M}$, suggesting the presence of two activities. The pH optimum of the activity with the low K_m value was 8.7. 4. Theophylline, caffeine, 3-isobutyl-1-methylxanthine (SC-2964), glibenclamide, tolbutamide, xylitol and leucine were inhibitors of the activity with the low K_m value; imidazole and arginine stimulated the activity, and glucose and diazoxide were without significant effect. 5. It is suggested that the agents theophylline, caffeine, SC-2964, glibenclamide, tolbutamide, leucine and imidazole may alter the intracellular concentration of cyclic AMP in islets of Langerhans by affecting the cyclic AMP phosphodiesterase activity in islet cells and in this way may affect insulin release.

The release of insulin from the mammalian pancreas appears to be stimulated under conditions likely to be associated with an increased intracellular concentration of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in islet cells, since agents such as glucagon, theophylline and caffeine, which increase cyclic AMP concentrations in a variety of tissues, stimulate insulin release (Malaisse *et al.*, 1967; Sussman & Vaughan, 1967). Further, the intracellular concentration of cyclic AMP in islets of Langerhans parallels the effects of several agents on insulin release (Turtle & Kipnis, 1967; Montague & Cook, 1971). These observations suggest that rates of insulin release may be determined directly by the concentration of cyclic AMP in islet cells. The concentration of cyclic AMP in cells is controlled largely by the relative rates of its formation by adenylate cyclase and destruction by cyclic AMP phosphodiesterase (Robison *et al.*, 1968). Agents which alter rates of insulin release by an effect on the concentration of cyclic AMP in islet cells may therefore affect one or both of these activities. Since it is of considerable importance to determine the mode of action of agents that control insulin release, we have investigated the effects of several agents on islet-cell cyclic AMP phospho-

diesterase and adenylate cyclase activities. In the present paper we report kinetic analyses of the effects on islet-cell phosphodiesterase activity of several agents that are known to alter rates of insulin release. Studies on the phosphodiesterase activity of mouse islets of Langerhans have been reported (Atkins & Matty, 1971; Ashcroft *et al.*, 1972; Bowen & Lazarus, 1972).

Experimental

Reagents

Collagenase (CLS) was purchased from Worthington Biochemical Co., Freehold, N.J., U.S.A.; L-arginine, imidazole, caffeine, Trizma base and 5'-nucleotidase were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; 3-isobutyl-1-methylxanthine (SC-2964) was from Searle Co., Chicago, Ill., U.S.A.; diazoxide (7-chloro-3-methyl-1,2,4-benzathiadiazine 1,1-dioxide) was from Schering Inc., Bloomfield, N.J., U.S.A.; Dowex 2 (AG2; X8; 100-200 mesh) was supplied by Bio-Rad Laboratories, Porterswood, St. Albans, Herts., U.K.; 3':5'-[^3H]adenosine monophosphate (sp. radioactivity $20.7\text{Ci}/\text{mmol}$) was obtained from The Radio-

chemical Centre, Amersham, Bucks., U.K. Other chemicals of the purest grade available were from British Drug Houses Ltd., Poole, Dorset, U.K.

Methods

Isolation of islets of Langerhans. Islets of Langerhans were prepared by the collagenase digestion of pancreatic tissue (Howell & Taylor, 1968) from guinea pigs weighing 800–1000g.

Preparation of the cyclic AMP phosphodiesterase. About 250 isolated islets were treated ultrasonically at 0°C in 1 ml of 100mM-tris-HCl buffer, pH 8.5, for 10s with a Soniprobe (Dawe Instruments Ltd., London W.3, U.K.) set at position 3. The homogenate was centrifuged for 25 min at 12000 rev./min in an MSE 18 centrifuge. The supernatant fraction was applied to a column (40cm × 0.8cm) of Sephadex G-50 and eluted with 50mM-tris-HCl buffer, pH 8.5, to remove insulin from the preparation because it affects cyclic AMP phosphodiesterase activity (Loten & Sneyd, 1970). Fractions (1 ml) were collected. The phosphodiesterase activity eluted from the column in fractions 13–16, whereas insulin (as determined with an ^{125}I -labelled ox insulin marker) eluted in fractions 21–27. Active fractions from several batches of islets were collected, pooled and stored in 1 ml portions at –70°C.

Assay of cyclic AMP phosphodiesterase. Cyclic AMP phosphodiesterase activity was determined by measuring the rate of breakdown of cyclic [^3H]AMP to 5'-[^3H]AMP and then to [^3H]adenosine by an assay method based on that of Beavo *et al.* (1970). The assay system of total volume 50 μl consisted of cyclic [^3H]AMP (40000 c.p.m.), cyclic AMP (various concentrations), 100mM-tris-HCl buffer, pH 8.5, 10mM-MgCl₂ and 2mM-EDTA. The agents under investigation were added to this buffer when necessary. Incubations were started by the addition of 50 μl of phosphodiesterase preparation and were done in bacteriological tubes (5cm × 0.7cm) at 37°C. The duration of the incubation (10–20 min) was determined by the activity of the batch of phosphodiesterase in use, and was such that approx. 20% conversion of cyclic AMP into 5'-AMP was obtained. The reaction was stopped by placing the tubes in boiling water for 3 min. The tubes were then returned to 37°C and 50 μl of 5'-nucleotidase (0.1 mg/ml) was added to the reaction mixture and the incubation continued for a further 10 min. The contents of the tubes were transferred to columns (1.5cm × 0.4cm) of Dowex AG2 (X8) with 600 μl of 50mM-tris-HCl buffer, pH 7.4. [^3H]Adenosine was eluted from the column with 5.25 ml of 50mM-tris-HCl buffer, pH 7.4, giving a total eluted volume of 6 ml. Further elution with 6 ml of 0.1 M-HCl removed the unchanged cyclic [^3H]AMP from the column. Samples (1 ml) of all eluted fractions were placed in scintillation vials to which a further

10 ml of toluene-Triton X-100–2,5-diphenyloxazole (700:300:3, v/v/w) scintillator was added. The radioactivity was assayed in a Beckmann LS 233 liquid-scintillation counter with a counting efficiency for ^3H of 30%. Blanks were run with each experiment and contained 50 μl of assay buffer and 50 μl of 50mM-tris-HCl buffer, pH 8.5. Under the conditions of the assay the amount of product formed was proportional to the enzyme concentration and to the time of incubation.

Determination of protein. The protein contents of the phosphodiesterase preparations were determined by the method of Lowry *et al.* (1951).

Preparation of the subcellular fraction. After isolation, about 250 guinea-pig islets of Langerhans were homogenized at 4°C in 1 ml of 10mM-sodium phosphate buffer, pH 6.5, containing 0.3M-sucrose. The homogenate was separated by differential centrifugation into fractions as follows: nuclei and cell debris (600g for 5 min); secretory granules (20000g for 10 min); microsomal (105000g for 60 min); post-microsomal supernatant, as described by Howell *et al.* (1969). Particulate material was resuspended in the homogenization buffer and all the fractions were assayed for cyclic AMP phosphodiesterase activity at a final cyclic AMP concentration of 10 μM as described above.

Results and Discussion

Previous studies on the subcellular distribution of cyclic AMP phosphodiesterase have indicated that in islet-cell tumours both soluble and particulate activities exist (Rosen *et al.*, 1971). The results in Table 1 show that over 70% of the activity present in an homogenate of guinea-pig islets was recovered in the post-microsomal supernatant, suggesting that the enzyme is largely soluble. Most of the remaining activity was associated with the nuclear and debris fraction, little being found in the granule-mitochondrial or microsomal pellets. This pattern of distribution is similar to that of the enzyme in a variety of other tissues including liver (Menahan *et al.*, 1969), rabbit brain (Drummond & Perrott-Yee, 1961) and thyroid tissue (Bastomsky *et al.*, 1971).

The cyclic AMP phosphodiesterase activity present in the post-microsomal supernatant, when subjected to gel filtration on Sephadex G-200, appeared as a single peak of activity with an apparent molecular weight of 200000 (results not shown). However, kinetic analysis of the results obtained for the effect of cyclic AMP concentration on the rate of enzyme activity (Fig. 1) revealed the presence of two activities with differing apparent K_m values for cyclic AMP. Estimates of the apparent K_m values derived from four such plots yielded mean values $\pm$ s.d. of 3 ± 0.4 and $30 \pm 4.1 \mu\text{M}$. Evidence for the presence of two cyclic AMP phosphodiesterase activities in mouse

Table 1. Subcellular distribution of cyclic AMP phosphodiesterase activity in islets of Langerhans

Subcellular fractions of an homogenate of about 250 islets of Langerhans from guinea pigs were prepared as described in the text and assayed for protein and cyclic AMP phosphodiesterase activity by using a substrate concentration of 10 μ M (see the text). The results are given as the means $\pm$ S.D. of six observations.

Fraction	Protein (μ g)	Total activity (nmol of cyclic AMP hydrolysed/15 min)	Activity/mg of protein (nmol of cyclic AMP hydrolysed/15 min per mg of protein)
Complete homogenate	464 $\pm$ 40	0.728 $\pm$ 0.10	1.57
Nuclear pellet (600g $\times$ 5 min)	178 $\pm$ 16	0.107 $\pm$ 0.09	0.60
Granule pellet (20000g $\times$ 10 min)	95 $\pm$ 8	0.048 $\pm$ 0.01	0.51
Microsomal pellet (105000g $\times$ 60 min)	68 $\pm$ 7	0.048 $\pm$ 0.01	0.71
Post-microsomal super- natant	140 $\pm$ 10	0.526 $\pm$ 0.04	3.77

Fig. 1. Effect of substrate concentration on the rate of cyclic AMP hydrolysis by islet-cell phosphodiesterase

Cyclic AMP phosphodiesterase activity was determined as described in the text, with substrate concentrations over the range 0.5 μ M–5.0 μ M, by using enzyme obtained from the supernatant fraction of an homogenate of guinea-pig islets of Langerhans (see the text for details). Each point is the mean of three observations and the results are presented in the form of a double-reciprocal plot, the lines of best fit being obtained by the method of least squares.

Fig. 2. Effect of pH on the activity with the low K_m value of islet-cell cyclic AMP phosphodiesterase

Cyclic AMP phosphodiesterase activity was determined as described in the text over the pH range 6.5–9.5 by using a substrate concentration of 0.5 μ M. Each point is the mean of three observations with the S.E.M. represented by the vertical bars.

islets of Langerhans has been reported (Ashcroft *et al.*, 1972). The presence of two activities of cyclic AMP phosphodiesterase in several mammalian tissues has been reported (Brooker *et al.*, 1968; Beavo *et al.*, 1970; Loten & Sneyd, 1970), although the significance of these observations remains obscure. Since the concentration of cyclic AMP in islet cells is approx. 1 μ M (Montague & Cook, 1971;

Ashcroft *et al.*, 1972), the effects of agents on phosphodiesterase activity from these cells was studied over the substrate range 0.25 μ M to 2 μ M. At these concentrations the phosphodiesterase activity appeared to reflect the activity with the low K_m value since a K_m value of 3 μ M was consistently found (Figs. 3–7).

These results suggest that the enzyme with the low K_m value may be an important regulator of cyclic AMP concentration in islet cells. The pH optimum of the activity with the low K_m value was 8.7 (Fig. 2).

Several methylxanthines stimulate the release of insulin from the mammalian pancreas and since these compounds are inhibitors of cyclic AMP phosphodiesterase in a variety of tissues, a similar mechanism

Fig. 3. Effects of caffeine, theophylline and SC-2964 on the cyclic AMP phosphodiesterase activity with the low K_m value of islet cells

Cyclic AMP phosphodiesterase activity was determined (see the text) at substrate concentrations of 0.25, 0.5, 1.0 and 2.0 μM by using enzyme prepared from guinea-pig islets of Langerhans, as described in the Experimental section. Caffeine (■), theophylline (▲) and SC-2964 (□) were added to the assay buffer to give final concentrations of 1, 1 and 0.1 mM respectively. Control values (●) were determined in the absence of any addition. The results are presented as a double-reciprocal plot, each point being the mean of three observations. Lines of best fit were obtained by the method of least squares.

Fig. 4. Effects of leucine and arginine on the cyclic AMP phosphodiesterase activity with the low K_m value in islet cells

The assay conditions were as described in Fig. 3. The effects of leucine (▲) and arginine (■) were investigated at a final concentration of 5 mM. Control values (●) were determined in the absence of any addition. The results are presented as a double-reciprocal plot, each point being the mean of three observations. Lines of best fit were obtained by the method of least squares.

of action in islet cells has been suggested (Malaisse *et al.*, 1967; Montague & Cook, 1971). In support of this it was shown that the relative effectiveness of the methylxanthines, theophylline, caffeine and SC-2964, on insulin release parallels their effects on the con-

centration of cyclic AMP in islets of Langerhans (Montague & Cook, 1971). Moreover, the results in Fig. 3 show a similar order of potency for these compounds as inhibitors of islet-cell cyclic AMP phosphodiesterase, suggesting that the mode of action

Table 2. Comparison of the effects on islet-cell cyclic AMP phosphodiesterase activity of agents known to affect insulin release

The cyclic AMP phosphodiesterase activity was determined as described in the text, by using a substrate concentration of $1\text{ }\mu\text{M}$. Agents were added to the assay system to give the final concentrations shown in the Table. The results are given as the means $\pm$ S.E.M. of six observations.

Agents	Phosphodiesterase activity (pmol of cyclic AMP hydrolysed/15 min)	% change
Control	19.8 ± 0.66	
Theophylline (1 mM)	$8.3 \pm 0.54^*$	-57*
Caffeine (1 mM)	$11.5 \pm 0.54^*$	-41*
SC-2964 (0.1 mM)	$5.0 \pm 0.24^*$	-74*
Leucine (5 mM)	$16.5 \pm 0.31^*$	-16*
Arginine (5 mM)	$24.7 \pm 0.55^*$	+24*
Glibenclamide (0.02 mM)	$13.6 \pm 0.83^*$	-31*
Tolbutamide (2.5 mM)	$13.8 \pm 0.94^*$	-30*
Diazoxide (0.5 mM)	17.7 ± 1.05	-10
Imidazole (27.8 mM)	$24.6 \pm 1.18^*$	+24*
Glucose (20 mM)	21.2 ± 1.12	+7
Xylitol (20 mM)	$15.4 \pm 0.48^*$	-22*

* Values significantly different from control value obtained in the absence of any addition to the assay system ($P < 0.005$).

Fig. 5. Effects of glucose and xylitol on the cyclic AMP phosphodiesterase activity with the low K_m value of islet cells

The assay conditions were as described in Fig. 3. The effects of glucose (○) and xylitol (▲) were investigated at a final concentration of 20 mM. Control values (●) were obtained in the absence of any addition. The results are presented as a double-reciprocal plot, each point being the mean of three observations. Lines of best fit were obtained by the method of least squares.

on insulin release may indeed be by the inhibition of islet-cell cyclic AMP phosphodiesterase.

Leucine and arginine stimulate the release of insulin from the mammalian pancreas (Floyd *et al.*, 1966; Malaisse & Malaisse-Lagae, 1968; Milner, 1970), although the mechanism of their action has remained obscure. The results in Fig. 4 suggest that the effect of leucine may be related to an increase in the intracellular concentration of cyclic AMP in islet cells since this amino acid was shown to be an inhibitor of islet-cell cyclic AMP phosphodiesterase. Arginine, however, appeared to stimulate the phosphodiesterase activity (Fig. 4).

There has been much discussion on the mode of action of sugars and sugar derivatives in stimulating insulin release (Montague & Taylor, 1968; Randle *et al.*, 1968; Malaisse *et al.*, 1967). The evidence suggests that glucose does not stimulate insulin release by increasing the intracellular concentration of cyclic AMP in islet cells (Montague & Cook, 1971). Consistent with this suggestion is the observation (Table 2 and Fig. 5) that glucose does not affect islet-cell cyclic AMP phosphodiesterase activity. Similar results have been reported by Ashcroft *et al.* (1972) and Bowen & Lazarus (1972) for mouse islet cyclic AMP phosphodiesterase activity. However, the results in Table 2 also show that xylitol has a significant inhibitory effect on cyclic AMP phosphodiesterase activity and suggest that it may stimulate insulin release by increasing the intracellular concentration of cyclic AMP in islet cells.

The inhibition by the sulphonylureas, tolbutamide and glibenclamide, of islet-cell phosphodiesterase activity (Fig. 6) is of considerable interest since the mode of action of these compounds on insulin release is at present unknown. Previous work has shown a similar inhibition by tolbutamide in islet-cell tumours of the Syrian hamster (Goldfine *et al.*, 1971; Rosen *et al.*, 1971) and by glibenclamide in mouse islets of Langerhans (Ashcroft *et al.*, 1972). The order of potency of these two agents as inhibitors of islet-cell phosphodiesterase activity parallels their effects on insulin release (Fussgänger *et al.*, 1969; Löffler *et al.*, 1969). The inhibition of cyclic AMP phosphodiesterase activity and a concomitant rise in intracellular concentration in β -cells could provide the mechanism for the potentiation of insulin release by these sulphonylureas. However, a study of the cyclic AMP concentrations in islet cells subjected to sulphonylureas is necessary before any definite conclusions can be reached.

Diazoxide inhibits insulin release from the mammalian pancreas and decreases the concentration of cyclic AMP in islet cells (Montague & Cook, 1971), effects which may be related. However, the action of diazoxide on cyclic AMP concentration in islet cells does not appear to be mediated by an increase in cyclic AMP phosphodiesterase activity since the results in Fig. 7 indicate that diazoxide was without significant effect on the enzyme activity.

Imidazole inhibits the release of insulin (Malaisse *et al.*, 1968), and the results in Fig. 7 indicate that

Fig. 6. Effects of glibenclamide and tolbutamide on the cyclic AMP phosphodiesterase activity with the low K_m value of islet cells

The assay conditions were as described in Fig. 3. The effects of glibenclamide (▲) and tolbutamide (■) were investigated at final concentrations of 0.02 mM and 2.5 mM respectively. Control values (●) were obtained in the absence of sulphonylureas. The results are presented as a double-reciprocal plot, each point being the mean of three observations. Lines of best fit were obtained by the method of least squares.

Fig. 7. Effects of diazoxide and imidazole on cyclic AMP phosphodiesterase activity with the low K_m value of islet cells

The assay conditions were as described in Fig. 3. The effects of diazoxide (▲) and imidazole (■) were investigated at final concentrations of 0.5mM and 27.8mM respectively. Control values (●) were obtained in the absence of any addition. The results are presented as a double-reciprocal plot, each point being the mean of three observations. Lines of best fit were obtained by the method of least squares.

imidazole may stimulate islet-cell cyclic AMP phosphodiesterase activity, suggesting that the effect on insulin release may be related to a lowering of islet-cell cyclic AMP by this agent.

Our understanding of the regulatory mechanism for insulin release has been complicated by the wide range of compounds which alter rates of insulin release and a unifying hypothesis has yet to be derived. However, the results in the present paper suggest that several groups of compounds of widely differing chemical composition which affect insulin release may do so by a common mechanism, namely via effects on islet-cell cyclic AMP phosphodiesterase activity which may lead to alterations in the concentrations of cyclic AMP in islet cells.

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