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

EFFECTS OF INSULIN SECRETAGOGUES ON ISLET-CELL PROTEIN KINASE ACTIVITY

By W. MONTAGUE and S. L. HOWELL
*School of Biological Sciences, University of Sussex, Falmer,
Brighton BN1 9QG, Sussex, U.K.*

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1. Protein kinase activity was measured in islets of Langerhans that had been incubated in the presence of agents known to affect insulin release. 2. Glucagon, theophylline, caffeine and 3-isobutyl-1-methylxanthine, agents that raise cyclic AMP concentrations in islet cells and stimulate insulin release, increased protein kinase activity. Adrenaline and diazoxide, agents that decrease cyclic AMP concentrations and inhibit insulin secretion, decreased the activity. 3. The increase in protein kinase activity produced by different concentrations of 3-isobutyl-1-methylxanthine was apparently related to the increase in intracellular concentrations of cyclic AMP. 4. The sulphonylureas, tolbutamide and glibenclamide, agents that increase insulin release, also increased the protein kinase activity; however, leucine, arginine and xylitol, which also stimulate insulin release, were without effect on the kinase activity. 5. Increasing the glucose concentration of the incubation medium from 2 to 20 mM had no effect on protein kinase activity. Further, the ability of 3-isobutyl-1-methylxanthine to increase the protein kinase activity was not affected by the glucose concentration of the incubation medium. 6. These results suggest that agents which affect insulin secretion by altering cyclic AMP concentrations may exert their effects on hormone release by altering the activity of a cyclic AMP-dependent protein kinase in islet cells.

Montague & Howell (1972) suggested that cyclic AMP may exert its effect on insulin release by increasing the activity of a cyclic AMP-dependent protein kinase in islets of Langerhans. It was therefore decided to establish whether there was a direct relationship between changes in the concentration of cyclic AMP in islet cells and the activity of this enzyme. This was attempted by measuring the protein kinase activity in extracts prepared rapidly from islets of Langerhans which had been incubated in medium containing agents known to alter rates of insulin release. Two types of agents were used, those which appear to affect the release process by altering the concentration of cyclic AMP in islet cells, and those whose effects do not appear to be mediated by cyclic AMP.

Experimental

Reagents

The following reagents were purchased from Sigma (London) Chemical Co. Ltd., Kingston, Surrey, KT2 7BH, U.K.: adrenaline, ethanedioxybis(ethylamine)tetra-acetic acid (EGTA), calf thymus histone (type IIA), disodium β -glycerophosphate, theophyl-

line, caffeine, collagenase (type I), imidazole (grade III), xylitol, L-leucine and L-arginine (free base). Tolbutamide and glibenclamide were from Hoechst Pharmaceuticals, Brentford, U.K.; diazoxide was from Schering Inc., Bloomfield, N.J., U.S.A.; 3-isobutyl-1-methylxanthine was from Searle Co., Chicago, Ill., U.S.A. [γ - 32 P]ATP was prepared by the method of Glynn & Chappell (1964).

Source of tissue

Islets of Langerhans were prepared by the collagenase digestion of pancreatic tissue taken from male Sprague-Dawley rats as described in detail by Howell & Taylor (1968).

Methods

Incubation of islets. After isolation the islets were incubated for 30 min at 37°C in groups of 20 in tubes (0.6 cm $\times$ 4 cm) containing 1 ml of incubation medium. The incubation medium was a bicarbonate-buffered salt solution (Gey & Gey, 1936) containing (per litre): 7.0 g of NaCl, 0.37 g of KCl, 2.27 g of NaHCO₃, 0.17 g of CaCl₂, 0.21 g of MgCl₂·6H₂O, 0.15 g of

Na_2HPO_4 , 0.07 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.03 g of KH_2PO_4 . It was gassed for 15 min with $\text{O}_2 + \text{CO}_2$ (95:5) before use. Glucose and agents under study were added to this medium as required. At the end of the incubation, the medium was rapidly removed from the islets by using a Pasteur pipette. After the addition of 0.3 ml of protein kinase assay buffer (see 'Assay of protein kinase activity') the islets were immediately treated ultrasonically for 15 s by using an ultrasonic disintegrator fitted with a microtip attachment (Soniprobe, type 7530A; Dawe Instruments Ltd., London W.3, U.K.). The Soniprobe was set at position 3 and tuned to give a maximum meter reading of 3 A corresponding to a power rating of approx. 72 W. A sample (0.1 ml) of the resulting tissue extract was removed for protein determination and the protein kinase activity in the remaining extract was determined as described below. The time-interval between removal of the incubation medium and commencement of the assay was standardized at 1 min.

Assay of protein kinase activity. Protein kinase activity was measured at pH 6.0 in a final volume of 0.3 ml of an assay buffer containing: 50 mM-sodium β -glycerophosphate, 0.3 mM-EGTA, 20 mM-KF, 5 mM-theophylline and 10 mM-magnesium acetate. The reaction was started by the addition of 0.1 ml of assay buffer, containing 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2×10^6 c.p.m.) and 0.5 mg of histone, to 0.2 ml of islet extract remaining in the incubation tube. The reaction was terminated after 4 min at 37°C by the addition of 2 ml of 10% (w/v) trichloroacetic acid. The precipitated proteins were washed three times with 2 ml of 10% trichloroacetic acid and the radioactivity in the final precipitate was determined as described below. Blank values for the assay were obtained by the addition of trichloroacetic acid to islet extracts immediately after the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and histone but before incubation at 37°C for 4 min.

Determination of cyclic AMP in islet tissue. Islets were incubated in groups of 50 as described above. At the end of the incubation the medium was removed from the islets, which were then treated ultrasonically (as described above) after the addition of 1 ml of 6% (w/v) HClO_4 to precipitate the islet proteins. After centrifugation the supernatant was removed and the protein content of the precipitate was determined after dissolving it in 1 ml of 0.2 M-NaOH. The cyclic AMP in the supernatant was purified by ion-exchange chromatography as described in detail by Montague & Cook (1971) and then assayed by the method of Brown *et al.* (1971).

Determination of radioactivity. 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 0.1 M-NaOH, the counting efficiency being approx. 30%.

Determination of protein. The protein content of tissue extracts and HClO_4 precipitates was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (Sigma) as standard.

Results

The rate of ^{32}P incorporation into histone by the protein kinase activity of control islets and islets incubated with 1 mM-3-isobutyl-1-methylxanthine was linear with time of assay for only 4 min (Fig. 1). The non-linearity of response during prolonged assay incubations did not appear to be due to depletion of

Fig. 1. Effects of various assay incubation periods on the protein kinase activity in extracts prepared from islets of Langerhans

Isolated rat islets were incubated in groups of 100 in 2 ml of incubation medium containing 6 mM-glucose alone (○) or 6 mM-glucose plus 1 mM-3-isobutyl-1-methylxanthine (●). After 30 min incubation at 37°C the medium was removed from the islets, which were then treated ultrasonically as described in the text after the addition of 4 ml of the protein kinase assay buffer. Portions (12×0.3 ml) of the resulting extract were incubated in duplicate at 37°C for 2, 4, 6 or 8 min after the addition of 0.1 ml of buffer containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and histone (see the text). The reaction was terminated by the addition of 10% trichloroacetic acid and the radioactivity incorporated into protein was determined as described in the text. Each point is the mean of six observations with the s.d. represented by the vertical bars.

an essential component of the assay system, since the total ^{32}P incorporated after 4 and 8 min of incubation was linearly related to the concentration of islet-tissue protein over the range 20–200 $\mu\text{g}/\text{ml}$ of extract (results not shown). All subsequent experiments were performed with extracts containing 30–40 μg of protein/ml and with an assay incubation time of 4 min.

The results in Fig. 2 show that the protein kinase activity in control islets and islets incubated with 3-isobutyl-1-methylxanthine decreases with time when the islet extract was prepared and then incubated at 4°C for various periods of time before assay. The activation of the protein kinase in an extract of islets by the addition of cyclic AMP at a final concentration of

Fig. 2. Effect of incubation at 4°C before assay on the protein kinase activity in extracts of islets of Langerhans

Isolated rat islets were incubated as described in Fig. 1, in groups of 100 in medium containing glucose alone (○) or glucose plus 1 mM-3-isobutyl-1-methylxanthine (●). At the end of the incubation the medium was removed and the islets were treated ultrasonically (see the text) after the addition of 5 ml of protein kinase assay buffer. Samples ($12 \times 0.3 \text{ ml}$) of the resulting extracts were incubated at 4°C for various periods of time before the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and histone and subsequent incubation at 37°C for 4 min. The reaction was then terminated by the addition of 10% trichloroacetic acid and the ^{32}P incorporated into protein was determined as described in the text. The results are given as mean values and the bars represent $\pm \text{s.d.}$ of six observations.

0.1 μM followed a similar time-course at 4°C (Fig. 3). The maximal activity produced by the addition of cyclic AMP to an islet extract was similar to the activity found in extracts prepared from islets previously incubated in the presence of 0.5 mM-3-isobutyl-1-methylxanthine.

The relationship between intracellular concentrations of cyclic AMP and protein kinase activity produced by incubation of islets in the presence of different concentrations of 3-isobutyl-1-methylxanthine is shown in Fig. 4. The protein kinase activity appeared to parallel the increase in cyclic AMP concentration produced by various concentrations of 3-isobutyl-1-methylxanthine. The effect of addition of different concentrations of cyclic AMP on the

Fig. 3. Time-course of the activation by cyclic AMP of protein kinase activity in extracts of islets of Langerhans incubated at 4°C

Extracts were prepared from groups of 100 islets that had been incubated in 6 mM-glucose as described in Fig. 1. Each extract was divided into two parts and both fractions were incubated at 4°C . After 15 min cyclic AMP in assay buffer was added to one fraction (●) to give a final concentration of 0.1 μM ; the other fraction received buffer alone (○). The incubation of both fractions at 4°C was continued. Protein phosphokinase activity in the fractions was determined at intervals by removing samples (0.3 ml) and incubating at 37°C for 4 min after the addition of 0.1 ml of assay buffer containing histone and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the reaction being terminated by the addition of 10% trichloroacetic acid. Radioactivity incorporated into protein was determined as described in the text. The results are given as mean values and the bars represent $\pm \text{s.d.}$ of six observations.

Fig. 4. Protein kinase activities and cyclic AMP concentrations in islets of Langerhans incubated with various concentrations of 3-isobutyl-1-methylxanthine

Islets of Langerhans were incubated in groups of 50 (for cyclic AMP determination) and 20 (for protein kinase determination) in medium containing different concentrations of 3-isobutyl-1-methylxanthine. After incubation for 30 min at 37°C the cyclic AMP concentrations (●) and protein kinase activities (○) in the islets were determined as described in the Experimental section. Each point is the mean of four observations, with the s.e.m. represented by the vertical bars.

Fig. 5. Activation of the protein kinase activity in an extract of islets of Langerhans by various concentrations of cyclic AMP

Extracts were prepared from groups of 100 islets of Langerhans which had been incubated with 6mM-glucose as described in Fig. 1. Samples (0.2ml) of the extract were incubated for 30 min at 4°C with various concentrations of cyclic AMP in 0.1 ml of protein phosphokinase assay buffer. At the end of this incubation 0.1 ml of assay buffer containing histone and [γ -³²P]ATP was added and the reaction continued for 4 min at 37°C. The reaction was terminated by the addition of 10% trichloroacetic acid and the radioactivity incorporated into protein was determined as described in the text. The results shown are each the mean of six observations with the s.e.m. represented by the vertical bars.

protein kinase activity of an extract of control islets is shown in Fig. 5. The cyclic AMP concentration that produced half the maximum activation of the protein kinase activity was 10 nM.

Protein kinase activities in islets incubated with agents previously shown to affect insulin release are shown in Tables 1 and 2. Glucagon, theophylline, caffeine, 3-isobutyl-1-methylxanthine, glibenclamide and tolbutamide all increased the activity; adrenaline, diazoxide and imidazole decreased it, and arginine, leucine and xylitol were without effect. There was no change in the protein kinase activity with variations in the glucose concentration of the incubation medium. In addition, the ability of 3-isobutyl-1-methylxanthine to increase the activity of the enzyme was not affected by the glucose concentration of the incubation medium.

The alterations in protein kinase activity observed when islets were incubated in the presence of adrenaline or 3-isobutyl-1-methylxanthine and then assayed for protein kinase activity could not be obtained by the addition of the agents to islet extracts during the assay incubation.

Discussion

The total protein kinase activity in islet cells represents the sum of the cyclic AMP-dependent and -independent activities, changes in the cyclic AMP-dependent activity being regulated directly by the prevailing cyclic AMP concentration (Montague & Howell, 1972). The preparation of islet extracts for

Table 1. *Protein kinase activity in islets of Langerhans incubated with agents known to affect insulin secretion*

Isolated rat islets of Langerhans were incubated in groups of 20 in medium containing 6 mM-glucose alone or 6 mM-glucose plus the agent under study. After incubation for 30 min at 37°C the medium was removed and the protein kinase activity in the islets was determined as described in the Experimental section. Each value is the mean $\pm$ S.D. of eight observations.

Addition to medium	Protein phosphokinase activity (nmol of ^{32}P incorporated/min per g of islet protein)
Glucose	29 $\pm$ 4
Glucose + 3-isobutyl-1-methylxanthine (0.25 mM)	78 $\pm$ 9*
Glucose + glucagon (1 μM)	50 $\pm$ 5*
Glucose + caffeine (5 mM)	43 $\pm$ 5*
Glucose + theophylline (5 mM)	48 $\pm$ 6*
Glucose + glibenclamide (10 μM)	43 $\pm$ 4*
Glucose + tolbutamide (2.5 mM)	45 $\pm$ 5*
Glucose + arginine (5 mM)	30 $\pm$ 4
Glucose + leucine (5 mM)	28 $\pm$ 5
Glucose + xylitol (20 mM)	29 $\pm$ 4

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

Table 2. *Protein kinase activity in islets of Langerhans incubated in vitro*

The experimental conditions were as described in Table 1. Each value is the mean $\pm$ S.D. of eight observations.

Addition to medium	Protein phosphokinase activity (nmol of ^{32}P incorporated/min per g of islet protein)
Glucose (2 mM)	32 $\pm$ 4
Glucose (2 mM) + 3-isobutyl-1-methylxanthine (0.5 mM)	78 $\pm$ 9*
Glucose (6 mM)	30 $\pm$ 5
Glucose (6 mM) + 3-isobutyl-1-methylxanthine (0.5 mM)	75 $\pm$ 9*
Glucose (20 mM)	34 $\pm$ 5
Glucose (20 mM) + adrenaline (1 μM)	23 $\pm$ 3*
Glucose (20 mM) + diazoxide (0.5 mM)	24 $\pm$ 3*
Glucose (20 mM) + imidazole (27.8 mM)	21 $\pm$ 3*
Glucose (20 mM) + 3-isobutyl-1-methylxanthine (0.5 mM)	80 $\pm$ 9*

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

protein kinase determination that was used in the present study resulted in a decrease of cyclic AMP concentration by a factor of at least 3000 from that found in the intact islets, and such a decrease would be expected to decrease the cyclic AMP-dependent kinase activity. This decrease in activity was not instantaneous, since it was possible to detect differences in activity resulting from variations of intracellular cyclic AMP concentrations at the time of extraction (Fig. 4). However, such an inactivation probably occurred during a prolonged assay incubation, since the rate of reaction was linear only for a short period of time (Fig. 1). The inactivation could be followed in detail at 4°C, and exhibited a similar time-

course to the activation by cyclic AMP of the protein kinase in islet extracts (Figs. 2 and 3). It seems likely that the decline in activity of the protein kinase during prolonged incubation may be accounted for by the progressive dissociation of cyclic AMP from its receptor protein, consequent on the decrease in cyclic AMP concentration produced by the preparation of the islet extracts. Further, the constant activity reached after 45 min (Fig. 2) may represent the basal cyclic AMP-independent activity.

An investigation of the properties of the cyclic AMP-dependent protein kinase in islets of Langerhans indicated that the cyclic AMP concentration that produced half the maximum activation of the purified

enzyme was 11 nM (Montague & Howell, 1972); a similar value was obtained for the activity in the crude islet extracts used in the present study (Fig. 5). However, estimates of the total cyclic AMP concentration in islet cells gives values in the 0.5–1.0 μ M range (Ashcroft *et al.*, 1972; Montague & Cook, 1971), which suggests that the enzyme should be fully activated even at basal cyclic AMP concentrations. However, the results given in Fig. 4 show that there was a direct relationship between the intracellular concentration of cyclic AMP and the activity of the protein kinase in islet cells. Moreover, the maximum activity produced by the addition of cyclic AMP to islet extracts was similar to the activity found in extracts of islets previously incubated in the presence of 0.5 mM-3-isobutyl-1-methylxanthine (Figs. 2 and 3). These observations suggest that the protein kinase activity in islet cells at basal cyclic AMP concentrations is not maximal and that changes in intracellular cyclic AMP concentrations do directly affect the activity.

These results raise the problem of why the protein kinase is not fully active if the cyclic AMP concentration in the vicinity of the kinase is the same as that found in total islet extracts. Exton *et al.* (1971) suggested that cyclic AMP may exist in cells in at least two pools, a free and metabolically active pool and a second bound and inert pool. On this basis the effective cyclic AMP concentration in the metabolically active pool, which could alter the protein kinase activity, would then be less than that which is measured in total tissue extracts. An alternative and perhaps equally plausible explanation appears to be simply that there are not pathways of free diffusion of cyclic AMP between its site of production and its site of action. Thus, adenylate cyclase activity, located exclusively at the plasma membrane (Howell & Whitfield, 1972), may produce very high local concentrations of cyclic AMP which may diffuse through the cytoplasm only to a limited extent, so that at the location of protein kinase activity the cyclic AMP concentration is much lower than that observed in total islet extracts.

Glucagon, theophylline, caffeine and 3-isobutyl-1-methylxanthine stimulate rates of insulin release and increase both intracellular concentrations of cyclic AMP (Turtle & Kipnis, 1967; Montague & Cook, 1971) and protein kinase activity (Table 1) in islets of Langerhans. In contrast, adrenaline and diazoxide inhibit insulin release and decrease both intracellular concentrations of cyclic AMP (Turtle & Kipnis, 1967; Montague & Cook, 1971) and protein kinase activity (Table 2), whereas imidazole, an agent that stimulates islet-cell phosphodiesterase activity (Ashcroft *et al.*, 1972; Sams & Montague, 1972) and inhibits insulin release (Malaisse *et al.*, 1968) also decreased the protein kinase activity. Thus there is a relationship between protein kinase activity and

rates of insulin secretion when islets of Langerhans are incubated with agents that appear to affect the release process by altering the intracellular cyclic AMP concentration. Further, the alterations of protein kinase activity appear to be a direct result of changes in cyclic AMP concentrations rather than of changes in rates of secretion, since glucose, an agent that appears to stimulate insulin release without altering cyclic AMP concentrations (Montague & Cook, 1971), had no effect on protein kinase activity during periods of incubation up to 30 min (Table 2). In addition, glucose did not appear to affect the ability of the protein kinase to respond to changes in the intracellular concentration of cyclic AMP, since 3-isobutyl-1-methylxanthine produced comparable increases in activity at various glucose concentrations (Table 2).

It has been shown that tolbutamide and glibenclamide may exert inhibitory effects on cyclic nucleotide phosphodiesterase activity in extracts from islets of Langerhans (Ashcroft *et al.*, 1972; Sams & Montague, 1972). The increases in protein kinase activity during incubation of intact islets with tolbutamide or glibenclamide (Table 1) suggest that the effects on phosphodiesterase activity may be important in intact cells and may represent a possible mode of action of these agents in stimulating insulin release.

The results presented here show that changes in islet-cell protein kinase activity parallel alterations of the intracellular concentration of cyclic AMP and suggest that agents that affect insulin secretion by altering cyclic AMP concentrations may exert their effects on hormone release by altering the activity of a cyclic AMP-dependent protein kinase. The nature of the endogenous substrates of this enzyme in islet cells and their role in the regulation of insulin secretion remain to be established.

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