Concentration of Adenosine 3':5'-Cyclic Monophosphate in Mouse Pancreatic Islets Measured by a Protein-Binding Radioassay

By R. H. COOPER, S. J. H. ASHCROFT and P. J. RANDLE
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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A protein-binding radioassay for cyclic AMP was modified to detect less than 0.025 pmol of the nucleotide. The method was applied to the measurement of cyclic AMP in small numbers of mouse pancreatic islets (as little as 25 μ g of tissue) by use of barium acetate–H₂SO₄ for deproteinization. The concentration of cyclic AMP in mouse islets incubated in media containing 3.3 or 20 mM-glucose was 0.016 pmol/10 islets (approx. 1 μ M in intracellular water). Glucose concentration (3.3 or 20 mM) had no detectable effect on islet concentrations of cyclic AMP with periods of incubation or perfusion ranging from 0.5 to 60 min, although insulin release rate was rapidly increased by 20 mM-glucose. Caffeine (5 mM) or 3-isobutyl-1-methylxanthine (1 mM), which are known inhibitors of islet cyclic AMP phosphodiesterase, produced marked and rapid increases in islet cyclic AMP concentration at 3.3 or 20 mM-glucose, but only enhanced the insulin release rate at the higher glucose concentration. The role of cyclic AMP in insulin release induced by glucose is discussed.

Insulin release by pancreatic islets is stimulated by hormones that in other tissues activate adenylate cyclase (e.g. glucagon, adrenocorticotrophin, β -effects of adrenaline) and by agents that inhibit cyclic AMP phosphodiesterase (e.g. caffeine or theophylline) provided that initiators of release such as glucose are present at an appropriate concentration (Malaisse *et al.*, 1967; Ashcroft *et al.*, 1972*a,b*). These observations have suggested that increases in cyclic AMP concentration in β -cells may facilitate release of insulin and it has been speculated that glucose may stimulate insulin release by this mechanism (Cerasi & Luft, 1970). It was therefore decided to investigate effects of glucose concentration and of inhibitors of cyclic AMP phosphodiesterase on concentrations of cyclic AMP and rates of insulin release in islets.

Measurement of the concentration of cyclic AMP in pancreatic islets is difficult. Islet tissue can only be prepared in relatively small quantities and the concentration of the cyclic nucleotide is low. By using relatively large quantities of rat islets, Montague & Cook (1971) measured cyclic AMP by a bioassay utilizing the phosphorylase cascade after extraction and partial purification of cyclic AMP. In the present study the protein-binding radioassay of Gilman (1970) as modified by Cooper *et al.* (1972) was further adapted to detect and assay less than 0.025 pmol of cyclic AMP. These adaptations enabled cyclic AMP to be assayed in small numbers of islets and permitted an investigation of effects of glucose concentration and of caffeine and 3-isobutyl-1-methylxanthine with various periods of incubation and perfusion.

Experimental

Reagents

Nucleotides, collagenase and cyclic AMP phosphodiesterase were from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Bovine plasma albumin (fraction V) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. The 3-isobutyl-1-methylxanthine was a gift from Dr. W. Montague, School of Biological Sciences, University of Sussex, Sussex, U.K.; caffeine and all other chemicals were from British Drug Houses Ltd., Poole, Dorset, U.K. Millipore filters (1EA HAWP 00010) were obtained from Millipore Corp., Bedford, Mass., U.S.A., and were cut to discs (2.4 cm diam.) before use. Cyclic [³H]AMP was from The Radiochemical Centre, Amersham, Bucks., U.K. (sp. radioactivity 20.7 Ci/mmol), or from NEN Chemicals G.m.b.H., Dreieichenhain, Germany (sp. radioactivity 24.1 Ci/mmol). The ¹²⁵I-labelled insulin (sp. radioactivity >50 μ Ci/ μ g) was from The Radiochemical Centre.

Cyclic AMP-binding protein from rabbit skeletal muscle was prepared as described by Cooper *et al.* (1972), and was further purified on DEAE-cellulose (Whatman DE-52; 8 cm $\times$ 5 cm column) as described by Gilman (1970). Fractions containing the first peak of cyclic AMP-binding activity were pooled and stored in small portions at a concentration of 0.55 mg/ml at –15°C.

Methods and Procedure

Preparation of islets. Islets were prepared from 3–4-week-old male white mice by a collagenase method described by Coll-Garcia & Gill (1969). All incubations were carried out at 37°C in bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) containing the additions given in the text, figures or tables.

Incubation of islets for assay of insulin release. Batches of six islets were incubated in 0.6 ml of medium containing albumin (2 mg/ml) and other additions as stated. After incubation (2 h) the medium was separated by gentle centrifugation and aspiration, diluted with phosphate–albumin buffer and stored at –15°C until assay.

Incubation of islets for assay of cyclic AMP content in the medium plus islets. Batches of islets (numbers given in the text) were collected in 10 µl of medium containing 3.3 mM-glucose in small tubes (Gallenkamp TF 123; size 60 mm × 7 mm, cut to approx. 30 mm × 7 mm) and placed inside stoppered scintillation vials. After gassing for 2 min with O₂ + CO₂ (95:5), islets were incubated for the times shown in the text or tables and then deproteinized.

Perfusion of islets. A diagram of the perfusion system is shown in Fig. 1. Disposable plastic tips for an Oxford sampler [no. 810, Oxford Laboratories,

obtained through Boehringer Corp. (London) Ltd.; 4.5 cm × 0.8 cm cut off to give a tip 2.5 cm × 0.4 cm] were used as perfusion chambers. A length of cotton button thread was passed through the chamber, projecting at both ends by about 3 mm, and with a knot partially occluding the aperture in the lower end of the chamber; this served to hold the islets in the chamber. The upper part of the chamber was connected through a short piece of plastic tubing and a three-way tap to two water-jacketed reservoirs. The perfusate from the chamber passed through a short length of tubing to collecting vessels. The perfusion chamber was immersed in a small water jacket at 37°C. Medium in the stoppered reservoirs was kept gassed by an inlet for O₂ + CO₂ (95:5). The chamber with the outflow tube attached was filled with medium and islets were transferred to the chamber. After connexion to the system, the chamber was perfused for at least 10 min with medium containing 3.3 mM-glucose before transferring to the other reservoir as described in the text, figures or tables. The flow rate was approx. 0.1 ml/min and for insulin-release studies 1- or 2-min samples of perfusate were collected in disposable polystyrene tubes.

For measurement of cyclic AMP content of perfused islets the chamber was disconnected from the system after termination of the flow and the islets were frozen *in situ* by immersion in liquid N₂. After the outflow tube had been disconnected from the chamber, the frozen plug of islets and medium was pulled from the silicone-treated conical chamber by the button thread passing through it. The frozen plug was trimmed and immediately deproteinized as described below.

Assay of insulin released into the perfusate or incubation medium. The radioimmunoassay (Hales & Randle, 1963) was used with mouse insulin as standard (Coll-Garcia & Gill, 1969).

Extraction of cyclic AMP from incubated islets. To 15 µl of medium and islets was added 7.5 µl of 2.5 M-H₂SO₄ followed by 22.5 µl of water and sonication (5–10 s at position 2 on a Dawe Soniprobe). Then 15 µl of 1 M-barium acetate was added to each extract and the BaSO₄ and coprecipitated protein were removed by centrifugation. After centrifugation, supernatants were removed into polystyrene tubes and adjusted to pH 6.5 (against indicator paper, Whatman, pH 6–8) by addition of 400 mM-trisodium phosphate (40 µl) and 200 mM-potassium phosphate, pH 6.5 (15 µl). Deproteinized samples were stored at –15°C until assayed (in duplicate).

Extraction of cyclic AMP from perfused islets. The frozen plug of medium and islets was added to 7.5 µl of 2.5 M-H₂SO₄ in a small tube (30 mm × 7 mm). After sonication, the thread was removed and 15 µl of 1 M-barium acetate was added. Thereafter the procedure was as for incubated islets.

Fig. 1. Diagram of islet perfusion apparatus

A, Inlet for O₂ + CO₂ (95:5); B, water-jacketed reservoirs containing perfusion media; C, water-manometer to adjust flow rate of perfusate; D, three-way tap; E, perfusion chamber containing islets and the cotton button thread with a knot to hold the islets in the chamber; F, outflow tube for collection of perfusate; G, water bath maintained at 37°C. For further details see the text.

Assay of cyclic AMP. The method was that of Cooper *et al.* (1972) modified by the use of a more purified preparation of cyclic AMP-binding protein as described under 'Reagents', and by performing the assay at pH 6.5 instead of 5.5 as previously used and filtering on Millipore filters.

Results

Sensitivity and validity of cyclic AMP assay

Fig. 2 shows a typical standard curve for the binding protein radioassay of cyclic AMP. A sensitivity better than 0.025 pmol of cyclic AMP has been achieved (displacement of $20.3 \pm 2.9\%$ of bound cyclic [^3H]AMP by 0.025 pmol; $P < 0.001$). This was achieved by a 2h incubation of unlabelled cyclic AMP and binding protein before the addition of cyclic [^3H]AMP as described previously (Cooper *et al.*, 1972), and by the use of less binding protein and of cyclic [^3H]AMP of higher specific radioactivity. It has been shown (Cooper *et al.*, 1972) that

Fig. 2. Typical standard curve for binding protein radioassay of cyclic AMP

Cyclic AMP-binding protein (5 μg) and unlabelled cyclic AMP were incubated for 120 min and then cyclic [^3H]AMP (1 pmol, 22 nCi) was added and incubation continued for a further 90 min. Bound cyclic AMP was then separated by filtration and assayed for radioactivity (see the Experimental section).

the only cyclic nucleotide to affect binding of cyclic [^3H]AMP in the assay was inosine 3':5'-cyclic monophosphate (cyclic IMP), which was about 15 times less effective than cyclic AMP. Addition of ATP, ADP or AMP at concentrations greater than those present in islet extracts (i.e. 5×10^4 times greater than cyclic AMP concentration) had no effect on binding of cyclic [^3H]AMP in the assay. [In an earlier paper by Cooper *et al.* (1972) the amounts of ATP, ADP and AMP added were incorrectly given as pmol; they should have read nmol.]

Medium, as used for islet incubations, containing caffeine (5 mM) or 3-isobutyl-1-methylxanthine (1 mM) and subjected to the deproteinization procedure, had no effect on binding of cyclic [^3H]AMP in the assay.

Measurement of cyclic AMP in islet extracts

For the more sensitive assay used in these experiments deproteinization with HClO_4 or trichloroacetic acid resulted in interference in the assay. Deproteinization with H_2SO_4 -barium acetate as described in the Experimental section gave rise to no interference and recoveries of cyclic AMP added before deprotein-

Fig. 3. Relationship between concentration of islet extract and apparent cyclic AMP concentration in the binding protein assay for cyclic AMP

Islets (400) were incubated in batches of 50 at 37°C for 15 min in 3.3 mM-glucose and cyclic AMP was extracted by deproteinization with barium acetate- H_2SO_4 and sonication (see the Experimental section). Cyclic AMP was assayed in quadruplicate in volumes of extract corresponding to 10, 20 and 40 islets. Vertical lines indicate $\pm$ S.E.M.

ization were 80–111% of added unlabelled cyclic AMP.

The absence of interference by islet extracts deproteinized by this method was confirmed by treatment of such extracts with cyclic AMP phosphodiesterase. Mouse islets (250) were incubated in two batches in 15 μ l of medium containing 3.3 mM-glucose for 15 min and then deproteinized as described in the Experimental section, except that the extract was adjusted to pH 7.5 by addition of 45 μ l of 400 mM-trisodium phosphate. The extracts were pooled and to 100 μ l (in duplicate) was added 5 μ l of 100 mM-MgCl₂ followed by incubation at 37°C for 18 h with or without cyclic AMP phosphodiesterase (20 units). The incubation was terminated with 15 μ l of 2.5 M-H₂SO₄, followed by 30 μ l of 1 M-barium acetate. After centrifugation, the supernatant was adjusted to pH 6.5 by addition of 40 μ l of 400 mM-trisodium phosphate.

The final volume of each extract was brought to 200 μ l by the addition of 200 mM-potassium phosphate, pH 6.5. Cyclic AMP concentrations were determined in quadruplicate; the addition of phosphodiesterase decreased the cyclic AMP content from 0.020 ± 0.001 pmol/10 islets to a value which was not significantly different from zero.

The amount of cyclic AMP detected in islet extracts increased linearly with the quantity of extract added to the radioassay (Fig. 3).

Effects of glucose, caffeine and 3-isobutyl-1-methylxanthine on cyclic AMP concentrations and on the rate of release of insulin in islets

The results in Table 1 show that caffeine (5 mM) and 3-isobutyl-1-methylxanthine (1 mM) raised cyclic AMP concentrations in medium plus islets by approx.

Table 1. *Effects of glucose concentration (3.3 or 20 mM) and of caffeine (5 mM) or 3-isobutyl-1-methylxanthine (1 mM) on rates of insulin release by mouse islets and on concentrations of cyclic AMP in islets plus incubation medium*

Batches of islets were collected in 10 μ l of incubation medium containing 3.3 mM-glucose. Concentrations of glucose, caffeine and 3-isobutyl-1-methylxanthine were then adjusted to values given in the table by addition of a further 5 μ l of medium of the appropriate composition. The number of islets per batch was 30 with glucose alone, 20 with glucose+caffeine and 10 with glucose+3-isobutyl-1-methylxanthine. Cyclic AMP concentrations were measured after 5 min of incubation. Insulin release was measured over 2 h of incubation. Cyclic AMP assays were performed in duplicate and insulin assays in triplicate. Results shown are means $\pm$ S.E.M. for the numbers of batches of islets given in parentheses. * $P < 0.01$ for difference from 3.3 mM-glucose alone.

Additions to medium	Cyclic AMP (pmol/10 islets)	Insulin release (ng/2 h per 10 islets)
Glucose (3.3 mM)	0.020 ± 0.002 (27)	15.4 ± 10.8 (5)
Glucose (3.3 mM)+caffeine (5 mM)	0.059 ± 0.010 (9)*	10.3 ± 3.2 (5)
Glucose (3.3 mM)+3-isobutyl-1-methylxanthine (1 mM)	0.365 ± 0.018 (54)*	16.8 ± 5.2 (5)
Glucose (20 mM)	0.027 ± 0.007 (6)	77.4 ± 9.0 (90)*
Glucose (20 mM)+caffeine (5 mM)	0.063 ± 0.003 (13)*	244 ± 43 (5)*
Glucose (20 mM)+3-isobutyl-1-methylxanthine (1 mM)	0.312 ± 0.015 (34)*	318 ± 47 (5)*

Table 2. *Effect of 3-isobutyl-1-methylxanthine (1 mM) with 3.3 or 20 mM-glucose on the concentration of cyclic AMP in mouse islets plus incubation medium after incubation for various times from 0 to 60 min*

For experimental details see Table 1. Results shown are means $\pm$ S.E.M. for the numbers of batches of islets given in parentheses. $P < 0.01$ for all differences from the appropriate zero-time control.

Time of incubation (min)	Concn. of cyclic AMP in islets plus medium (pmol/10 islets)	
	3.3 mM-Glucose	20 mM-Glucose
0	0.023 ± 0.003 (12)	0.027 ± 0.007 (6)
2	0.620 ± 0.045 (6)	0.680 ± 0.087 (4)
5	0.585 ± 0.065 (6)	0.439 ± 0.014 (4)
10	0.532 ± 0.040 (4)	0.435 ± 0.061 (2)
15	0.362 ± 0.074 (4)	0.382 ± 0.039 (4)
20	0.414 ± 0.035 (2)	—
30	0.405 ± 0.015 (2)	—
60	0.394 ± 0.026 (2)	—

three- and 15-fold respectively over the control value at both 3.3 and 20mM-glucose, whereas release of insulin was stimulated only at 20mM-glucose. The relative effectiveness of caffeine and 3-isobutyl-1-methylxanthine in increasing concentrations of cyclic AMP in islets plus medium parallels their potency

as inhibitors of islet-cell cyclic AMP phosphodiesterase (Ashcroft *et al.*, 1972b; Sams & Montague, 1972). Table 1 also shows that whereas there was a marked increase in the rate of insulin release when glucose concentration in the medium was raised from 3.3 to 20mM there was no significant difference between the

Fig. 4. Rates of insulin release from perifused islets at 3.3mM-glucose, 20mM-glucose, and 20mM-glucose plus 5mM-caffeine (a), or at 20mM-glucose, 20mM-glucose plus 5mM-caffeine, followed by 20mM-glucose (b)

Batches of 50 islets (a) or 40 islets (b) were collected in incubation medium containing 3.3mM-glucose in a perfusion chamber. Islets were either perifused for 60min with 20mM-glucose and then transferred to 3.3mM-glucose, 20mM-glucose and then 20mM-glucose + 5mM-caffeine (a), or perifused for 20min with 20mM-glucose and then 20mM-glucose + 5mM-caffeine, followed by 20mM-glucose alone (b), for the times indicated. Samples of perfusate for 2 or 4min periods were collected in polystyrene tubes, diluted, and assayed for insulin as described in the Experimental section. Each point is the mean of insulin determinations in triplicate.

Fig. 5. Concentration of cyclic AMP in perifused islets at 3.3 or 20mM-glucose after addition of 1mM-3-isobutyl-1-methylxanthine (a) or 5mM-caffeine (b)

Batches of 20 islets were collected in incubation medium containing 3.3mM-glucose in perfusion chambers (capacity approx. 80 μ l). Islets were perfused for 10min with medium containing glucose (3.3mM) and then transferred to medium containing glucose (3.3 or 20mM) and either (a) 3-isobutyl-1-methylxanthine (1mM) or (b) caffeine (5mM). $\bullet$, Glucose (3.3mM); $\blacktriangle$, glucose (20mM). The contents of the perfusion chambers were frozen in liquid N_2 at the times shown and then extracted and assayed for cyclic AMP as described in the Experimental section. Each point is the mean of two batches of islets assayed in duplicate, and the vertical lines represent $\pm$ S.E.M.

cyclic AMP concentrations at these two glucose concentrations. The islet cyclic AMP concentrations in Table 1 were obtained after 5 min of incubation whereas the insulin-release rates were measured over 2h. Table 2 shows the cyclic AMP concentration in medium plus islets with 3.3 or 20mM-glucose plus 3-isobutyl-1-methylxanthine (1mM), at times of incubation ranging from 2 to 60min. There was no detectable difference between cyclic AMP concentrations at 3.3 or 20mM-glucose at any of the times of incubation studied. Addition of 3-isobutyl-1-methylxanthine produced maximal increase in cyclic AMP concentration after about 2min; after 10min cyclic AMP concentrations decreased somewhat, although

they remained higher than the initial control values even after 1h of incubation.

Islet perfusion studies

The development of a perfusion system for islets allowed changes in cyclic AMP concentration to be measured over very short time-periods (1 min or less) and to be correlated with changes in the rate of insulin secretion. Fig. 4(a) shows the rapid response of insulin release from perifused islets to a change in glucose concentration from 3.3 to 20mM. Fig. 4(b) shows the potentiating effect of caffeine (5mM) on glucose-induced insulin release from perifused islets. Removal of caffeine from the perfusate results in a lowering of the rate of insulin release to that pertaining before the caffeine was present.

Fig. 5 shows the concentration of cyclic AMP in islets perfused with 3.3 or 20mM-glucose, after addition of (a) 1mM-3-isobutyl-1-methylxanthine or (b) 5mM-caffeine over periods ranging from 30s to 8min. The results show that glucose had no detectable effect on the islet concentration of cyclic AMP over an 8min period.

Discussion

Protein-binding assay for cyclic AMP in islets

The assay of cyclic AMP presents special problems because of its relatively low concentration in tissues (approx. 2.5–25nmol/g dry wt.). Assay of this nucleotide in pancreatic islets is particularly difficult because the dry weight of a single islet is only approx. 0.5 μ g. The present study has shown that the protein-binding assay of Gilman (1970) may be modified to detect less than 0.025pmol and thus to be capable of measuring the nucleotide in small numbers of islets without concentration and purification. The major factors in this increased sensitivity were the use of high-specific-radioactivity cyclic [3H]AMP, prior exposure of the binding protein to standard or unknown solutions before addition of labelled nucleotide, a procedure first used to sensitize radioimmunoassay by Hales & Randle (1963), and use of barium acetate– H_2SO_4 for deproteinization.

The mean cyclic AMP concentration of mouse islets with 3.3 or 20mM-glucose was 0.016pmol/10 islets during perfusion. The intracellular water of normal mouse islets is approx. 1.75nl/islet, corresponding to an intracellular concentration of cyclic AMP of approx. 1 μ M. This concentration agrees closely with the value of 1 μ M measured in rat islets by Montague & Cook (1971), but is considerably lower than the values obtained by Turtle & Kipnis (1967).

Cyclic AMP and insulin release

The idea that cyclic AMP may be involved in the regulation of insulin secretion was first suggested by

the observations that adrenaline may inhibit and glucagon may potentiate insulin-secretory responses to glucose (Coore & Randle, 1964; Karam *et al.*, 1966). Further evidence in support of this suggestion was provided by the observation that theophylline and caffeine, potent inhibitors of cyclic AMP phosphodiesterase, may stimulate insulin release *in vivo* (Turtle *et al.*, 1967). Subsequent studies *in vitro* have shown that agents such as caffeine, theophylline or glucagon, which may increase islet cyclic AMP concentrations do not initiate insulin secretion, but that they markedly potentiate insulin release when this has been initiated by metabolic substrates such as glucose or leucine (Malaisse *et al.*, 1967; Ashcroft *et al.*, 1972a). This has been confirmed in the present study. Contrary to some speculations we have found no evidence from these measurements of cyclic AMP concentration that glucose initiates insulin release by increasing islet cyclic AMP. Moreover caffeine and 3-isobutyl-1-methylxanthine, which raise islet cyclic AMP concentrations at 3.3 mM-glucose, failed to stimulate insulin release at this concentration of the sugar. At 20 mM-glucose caffeine and 3-isobutyl-1-methylxanthine markedly potentiated insulin release, but produced no greater increase in cyclic AMP concentration than at 3.3 mM-glucose. Montague & Cook (1971) first showed clearly that caffeine and 3-isobutyl-1-methylxanthine increase islet cyclic AMP concentration and obtained no evidence for such an effect of glucose. Their observations were made with rat islets incubated for 5–30 min and we were concerned that a fast and short-lived increase in islet cyclic AMP with glucose might have been overlooked in these studies. It is known that the insulin-secretory response to glucose is biphasic and the initial fast response is complete within 4 min (Curry *et al.*, 1968). The present study has shown no effect of glucose concentration on islet cyclic AMP over various periods of exposure (0.5–60 min) to high glucose concentrations. The time-intervals selected for this study appear to be sufficiently comprehensive to cover both phases of insulin release. Therefore these findings would appear to indicate that glucose may initiate insulin release by a mechanism which does not involve increase in the concentration of islet cyclic AMP, and that increase in islet cyclic AMP concentration (i.e. by caffeine or 3-isobutyl-1-methylxanthine) does not stimulate insulin release unless this has been initiated by glucose. Our studies do not, of course, provide information about the distribution of cyclic AMP between different cell types in mouse islets, although in this species at least 80% of islet cells are β -cells, nor do they provide information about the distribution of cyclic AMP within β -cells. Nevertheless we believe that the simplest interpretation is that glucose initiates insulin release by a mechanism that does not involve increase

in cyclic AMP concentration, and that this mechanism is potentiated when cyclic AMP is increased. The mechanism of this glucose effect is not known, but it may involve an increased intracellular concentration of Ca^{2+} (Milner & Hales, 1967; Malaisse-Lagae & Malaisse, 1971; Brisson *et al.*, 1972). Evidence for such an hypothesis requires definition of mechanisms of calcium uptake and action, of the mechanism of cyclic AMP and of the role of cyclic AMP-dependent protein kinase, which has now been demonstrated in rat islets (Montague & Howell, 1972).

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