Biochem. J. (1971) **122**, 115–120
Printed in Great Britain

The Role of Adenosine 3′:5′-Cyclic Monophosphate in the Regulation of Insulin Release by Isolated Rat Islets of Langerhans

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(*Received* 30 *October* 1970)

1. Concentrations of cyclic AMP (adenosine 3′:5′-cyclic monophosphate) and rates of insulin release were measured in islets of Langerhans isolated from rat pancreas and incubated for various times in the presence of glucose, 3-isobutyl-1-methylxanthine, caffeine, theophylline, adrenaline and diazoxide. 2. Caffeine and theophylline produced small but significant increases in both cyclic AMP and release of insulin when they were incubated in the presence of 10mM-glucose. 3. 3-Isobutyl-1-methylxanthine produced a marked increase in the intracellular concentration of cyclic AMP in the presence of 5mM- and 10mM-glucose. However, insulin release was stimulated only in the presence of 10mM-glucose. 4. In response to rising concentrations of extracellular glucose (5–20mM) there was no detectable increase in the intracellular concentration of cyclic AMP even though there was a marked increase in the rate of insulin release. 5. In response to 10mM-glucose insulin release occurred in two phases and 3-isobutyl-1-methylxanthine potentiated the effect of glucose on both phases. The intracellular concentration of cyclic AMP remained constant with glucose and rose within 10min to its maximum value with 3-isobutyl-1-methylxanthine. 6. Adrenaline and diazoxide inhibited insulin release and lowered the intracellular concentration of cyclic AMP when islets were incubated with glucose or 3-isobutyl-1-methylxanthine. 7. It is suggested that glucose does not stimulate insulin release by increasing the concentration of cyclic AMP in islet cells. However, the concentration of cyclic AMP in islet cells may modulate the effect of glucose on the release process.

Evidence has accumulated suggesting that cyclic AMP may play a role in the regulation of insulin secretion from the mammalian pancreas. Thus agents such as glucagon, adrenocorticotrophic hormone, theophylline and caffeine, which increase the concentration of cyclic AMP in a variety of tissues, potentiate the effect of glucose on insulin release (Malaisse, Malaisse-Lagae & Mayhew, 1967).

Turtle & Kipnis (1967) were the first to attempt to measure the concentration of cyclic AMP in islets of Langerhans. They found that theophylline and glucagon raised the cyclic AMP concentration and stimulated insulin release, whereas adrenaline lowered the cyclic AMP concentration and inhibited insulin release. However, no attempt was made to determine the effect of glucose, the major physiological stimulus to insulin release, on cyclic AMP concentrations. It was therefore decided to determine whether the effect of glucose on insulin release was paralleled by changes in the concentration of cyclic AMP in islets of Langerhans incubated *in vitro*. Because of the possibility that small changes in the concentration of cyclic AMP in islet cells might be important in regulating insulin release, the amount of cyclic AMP released into the medium was also measured, since it has been shown that the measurement of cyclic AMP released from the perfused rat liver may provide a sensitive index of changes in the intracellular concentration of cyclic AMP (Lewis, Exton, Ho & Park, 1970).

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EXPERIMENTAL

Reagents. Collagenase (CLS) was obtained from Worthington Biochemical Co., Freehold, N.J., U.S.A.; 3-isobutyl-1-methylxanthine (SC-2964) was from Searle Co., Chicago, Ill., U.S.A.; adrenaline was from Parke, Davis and Co., Detroit, Mich., U.S.A.; diazoxide (7-chloro-3-methyl-1,2,4-benzothiadiazine 1,1-dioxide) was from Schering Inc., N.J., U.S.A.; caffeine and theophylline were from Merck and Co., Rahway, N.J., U.S.A.; tritiated cyclic AMP was from Schwartz BioResearch Inc.,

Orangeburg, N.J., U.S.A. [The cyclic AMP was purified by ion-exchange chromatography (Butcher, Ho, Meng & Sutherland, 1965) before use.] Dowex 50 (AG 50W; X8; 100–200 mesh; H^+ form) was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

Incubation medium. In all incubations with isolated rat islets of Langerhans the basal medium was Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) containing bovine serum albumin fraction V (1mg/ml) and 5mM-glucose. The albumin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Isolation of islets of Langerhans. 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) and Montague & Taylor (1968). In a typical experiment with eight rats weighing 350–400g it was possible to obtain 1200 islets free of acinar tissue in 15min. The isolated islets were incubated together in basal medium for 30min at 37°C under O_2+CO_2 (95:5) before use in experiments. Collection of the islets was performed at room temperature with finely drawn out Pasteur pipettes.

Insulin secretion from isolated rat islets. Groups of 50 islets selected at random were incubated in 15ml polythene tubes containing 5ml of medium. Selection took 10min. The incubation medium contained either glucose alone or glucose plus the agent under study and the gas phase was O_2+CO_2 (95:5). The islets were incubated at 37°C for 30min with gentle shaking. At the end of this period a sample of the medium was removed for the determination of its insulin content by the immunoassay method of Hales & Randle (1963) by using the assay kits available from Schwartz BioResearch Inc.

Determination of cyclic AMP concentrations in medium and tissue. Islets were incubated in groups of 50 as described above for the study of insulin secretion. At the end of the incubation period the incubation medium was rapidly removed (1–2s) from the islets after centrifugation as described by Montague & Taylor (1968), and the islets were rapidly frozen in liquid N_2. Medium trapped by the islets during the separation was negligible. The medium was immediately acidified by the addition of 0.5ml of 1M-HCl and the islets were treated ultrasonically after the addition of 2ml of 0.1M-HCl. To provide cyclic AMP concentrations high enough for reliable determinations the media and islet extracts from three tubes were pooled (150 islets). Purified tritiated cyclic AMP (approx. 10000c.p.m.; 1pmol) was added to both the medium and islet extract. Recovery of this radioactivity (usually 70–80%) provided a correction factor for losses of cyclic AMP in the purification step that followed (Butcher *et al.* 1965). A sample of the islet extract was removed for the determination of its protein by the method of Lowry, Rosebrough, Farr & Randall (1951), with purified bovine serum albumin as standard. The cyclic AMP in the islet extract and the incubation medium was purified by ion-exchange chromatography. The medium and extract were separately applied to columns (30cm×0.7cm) of Dowex 50, previously equilibrated with 0.1M-HCl and followed with 0.1M-HCl (procedure 1 of Ishikawa, Ishikawa, Davis & Sutherland, 1969). The fraction containing cyclic AMP was collected in 100ml serum bottles and freeze-dried to dryness. The contents of the serum bottles were redissolved in 1ml of 10mM-tris–HCl buffer, pH7.4, and 0.1ml was removed for measurement of its radioactivity so that the recovery of cyclic AMP could be determined. Radioactivity was measured in a Packard liquid-scintillation spectrometer by using an internal standard. Counting efficiency was 20%. The remainder of the final extract was then used directly for the determination of cyclic AMP by a method involving the activation of inactive liver phosphorylase (Butcher *et al.* 1965).

RESULTS

The results in Table 1 show that the cyclic nucleotide phosphodiesterase inhibitors caffeine, theophylline and 3-isobutyl-1-methylxanthine stimulated insulin release from rat islets of Langerhans

Table 1. *Effects of 3-isobutyl-1-methylxanthine, caffeine and theophylline on insulin release, the intracellular concentration of cyclic AMP and the amount of cyclic AMP released into the medium from islets of Langerhans incubated with glucose* in vitro

Isolated rat islets of Langerhans were incubated for 30min at 37°C in Krebs–Henseleit bicarbonate buffer containing albumin (1mg/ml). The medium contained either glucose (10mM) alone or glucose (10mM) with caffeine (10mM), theophylline (10mM) or 3-isobutyl-1-methylxanthine (1mM). Insulin released into the medium was measured immunologically (see the text). At the end of the incubation the islets were separated from the medium and rapidly frozen. The medium was acidified and the islets were extracted in HCl. The islet extracts and the incubation media, after purification by ion-exchange chromatography, were assayed for cyclic AMP as described in the Experimental section. Each value is the mean±S.D. of ten observations. Levels of statistical significance were obtained by the use of the Student's *t* test.

Addition to medium	Insulin release (munits/30min per mg of protein)	Concn. of cyclic AMP (pmol/mg of protein)	
		Tissue	Medium
Control	1.8±0.2	40± 4	30±3
Caffeine	2.6±0.2*	62± 5*	42±3*
Theophylline	3.0±0.3*	73± 6*	51±4*
3-Isobutyl-1-methylxanthine	4.0±0.5*	130±10*	80±7*

* Values significantly different from the control value ($P<0.01$).

Table 2. *Effects of 3-isobutyl-1-methylxanthine and various concentrations of glucose on insulin release, the intracellular concentration of cyclic AMP and the amount of cyclic AMP released into the medium from islets of Langerhans incubated* in vitro

The experimental conditions were as described in Table 1. The results are given as means ± S.D. of ten observations.

Addition to medium	Insulin release (munits/30 min per mg of protein)	Concn. of cyclic AMP (pmol/mg of protein)	
		Tissue	Medium
Glucose (5 mM)	0.8 ± 0.2	39 ± 4	30 ± 3
Glucose (5 mM) + 3-isobutyl-1-methylxanthine (1 mM)	0.8 ± 0.2	129 ± 9*	78 ± 7*
Glucose (10 mM)	1.8 ± 0.3*	40 ± 5	29 ± 4
Glucose (10 mM) + 3-isobutyl-1-methylxanthine (1 mM)	4.0 ± 0.5*	130 ± 9*	80 ± 7*
Glucose (20 mM)	3.2 ± 0.4*	39 ± 4	29 ± 3

* Values significantly different from the 5 mM-glucose control value ($P<0.01$).

Table 3. *Effects of diazoxide and adrenaline on glucose and 3-isobutyl-1-methylxanthine induced insulin release and cyclic AMP concentrations in islets of Langerhans incubated* in vitro

The experimental conditions were described in Table 1 and the incubation medium contained glucose (10 mM) alone or glucose (10 mM) with the following additions: adrenaline (1 μM), diazoxide (0.5 mM) and 3-isobutyl-1-methylxanthine (1 mM). The results are given as means ± S.D. of ten observations.

Addition to medium	Insulin release (munits/30 min per mg of protein)	Intracellular concn. of cyclic AMP (pmol/mg of protein)
Glucose	1.8 ± 0.3	40 ± 4
Glucose + adrenaline	0.8 ± 0.2*	20 ± 3*
Glucose + diazoxide	0.9 ± 0.2*	25 ± 4*
Glucose + 3-isobutyl-1-methylxanthine	4.0 ± 0.5	130 ± 9
Glucose + 3-isobutyl-1-methyl-xanthine + adrenaline	1.6 ± 0.2†	60 ± 5†
Glucose + 3-isobutyl-1-methyl-xanthine + diazoxide	1.8 ± 0.2†	70 ± 6†

* Values significantly different from the 10 mM-glucose control value ($P<0.01$).
† Values significantly different from the 10 mM-glucose + 1 mM-3-isobutyl-1-methylxanthine control value ($P<0.01$).

incubated in the presence of 10 mM-glucose and increased cyclic AMP concentration in both medium and tissue. The quantitative effects of these agents on insulin release and cyclic AMP concentrations parallel their potency as inhibitors of the phosphodiesterase activity in fat-cells (Beavo *et al.* 1970).

Since β-cells constitute 70–80% of the total cells in rat islets of Langerhans (Carpenter, 1966) it has been assumed that the concentration of cyclic AMP in islet cells reflects mainly that of β-cells. The mean protein content of the islets used in the present study was 300 μg/100 islets (S.D. ± 20 for 100 observations).

Table 2 shows that, although 3-isobutyl-1-methylxanthine had a dramatic effect on cyclic AMP concentrations in tissue and medium in the presence of 5 mM- or 10 mM-glucose, it stimulated insulin release only in the presence of 10 mM-glucose. The effects of raising the glucose concentration of the incubation medium from 5 to 10 or 20 mM are also shown in Table 2. There was no measurable effect on cyclic AMP concentrations in either medium or tissue even though there was a marked increase in insulin release under these conditions.

The results in Table 3 show that adrenaline (1 μM) and diazoxide (0.5 mM) significantly inhibited the release of insulin in response to glucose and 3-isobutyl-1-methylxanthine and lowered the intracellular concentration of cyclic AMP in islets.

The intracellular concentrations of cyclic AMP and the rates of insulin release when islets were incubated for various times with 10 mM-glucose or

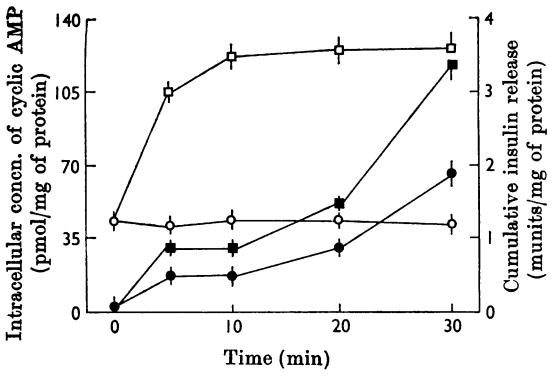

Fig. 1. Effects of various incubation periods on insulin release and the intracellular concentration of cyclic AMP in islets of Langerhans incubated with glucose or glucose plus 3-isobutyl-1-methylxanthine. Isolated rat islets of Langerhans were incubated in groups of 50 in 1ml of incubation medium containing 2mM-glucose. At zero time 4ml of medium containing 12mM-glucose or 12mM-glucose plus 1mM-3-isobutyl-1-methylxanthine was added. The islets were incubated for various times and the rates of insulin release (●, glucose; ■, glucose plus 3-isobutyl-1-methylxanthine) together with the intracellular concentration of cyclic AMP (○, glucose; □, glucose plus 3-isobutyl-1-methylxanthine) were determined as described in Table 1. The results are the means of six observations with the S.D. represented by the vertical bars.

10mM-glucose plus 1mM-3-isobutyl-1-methylxanthine are shown in Fig. 1. In response to raising the glucose concentration of the incubation medium from 2 to 10mM there was no detectable increase in the intracellular concentration of cyclic AMP at any time-interval studied, even though insulin secretion appeared to follow the biphasic pattern reported for other pancreatic preparations (Curry, Bennett & Grodsky, 1968). The addition of 3-isobutyl-1-methylxanthine at the same time as raising the glucose concentration produced a marked elevation in the intracellular concentration of cyclic AMP within 5min. The secretion of insulin in response to 3-isobutyl-1-methylxanthine followed the same biphasic pattern as for glucose but at an increased rate.

DISCUSSION

It is now often assumed that glucose, the major physiological stimulus to insulin secretion, is largely effective in regulating release as a consequence of its metabolism in the pancreatic β-cell (Montague & Taylor, 1968; Randle, Ashcroft & Gill, 1968). This metabolism may provide both the signal or signals that trigger the release mechanism and some of the factors necessary for the process to occur. In addition, the work of Turtle & Kipnis (1967) and the results in Table 1 show that the concentration of cyclic AMP in islet cells parallels the effects of various agents on insulin release, suggesting that cyclic AMP may also play a role in regulating this process.

There has been much discussion as to the site of action of cyclic AMP in the series of events that lead from stimulation to the release of insulin from the β-cell. Several authors have suggested that cyclic AMP is an essential component of the release mechanism and that the concentration of cyclic AMP in the β-cell may directly regulate the rate of insulin release (Cerasi, Effendic & Luft, 1969; Lambert, Junod, Stauffacher, Jeanrenaud & Renold, 1969). However, the results in Table 2 show that the maintenance of an increased concentration of cyclic AMP in islet cells is not necessary for the release of insulin in response to glucose from rat islets of Langerhans. The possibility existed that glucose-induced changes in the concentration of cyclic AMP may have occurred over a shorter time-period than the 30min that was used in these experiments. The results in Fig. 1 show that it was not possible to detect any effect of glucose on the concentration of cyclic AMP even at 5min, when an increased rate of secretion could be measured. These results strongly suggest that the effect of glucose on the insulin-release mechanism is not mediated by an increase in the concentration of cyclic AMP in the β-cell, and therefore an increase in the concentration of cyclic AMP cannot be regarded as an essential component of the release mechanism.

It might be argued that the method used to measure cyclic AMP concentrations was not sensitive enough to detect the small changes that may be important in regulating insulin release. However, there was no detectable increase in cyclic AMP concentration in the medium when islets were incubated with various concentrations of glucose (Table 2), and the measurement of extracellular cyclic AMP concentrations appears to provide a sensitive index of intracellular changes (Lewis *et al.* 1970). Further, since caffeine and theophylline produced significant increases in the concentration of cyclic AMP but had less effect on insulin release than did 20mM-glucose, which itself did not alter the cyclic AMP concentration, it is unlikely that small changes in the concentration of cyclic AMP are important in the regulation of insulin release.

Several authors have suggested that cyclic AMP acts indirectly on the secretory process by potentiating the effect of glucose (Malaisse *et al.* 1967; Montague & Taylor, 1969). Much of the available evidence supports this idea. Thus it was shown in Table 3 that 3-isobutyl-1-methylxanthine increased the concentration of cyclic AMP in islets incubated

with 5-mM or 10mM-glucose, but only stimulated secretion in the presence of 10mM, a concentration at which glucose itself stimulates release. These observations are in agreement with those made by Malaisse *et al.* (1967), who were unable to obtain effects of theophylline or glucagon on insulin release unless glucose at a concentration greater than 5mM was also present in the incubation medium. In addition Malaisse & Malaisse-Lagae (1968) were able to show that imidazole (a stimulant of cyclic nucleotide phosphodiesterase at a concentration that inhibited the effect of theophylline on insulin release) had no effect on glucose-mediated release.

The results in Table 3 show that adrenaline and diazoxide lowered the concentration of cyclic AMP in islet cells and inhibited the secretory response to glucose and 3-isobutyl-1-methylxanthine. These observations could be interpreted as indicating that a certain concentration of cyclic AMP in the β-cell may be necessary for the release mechanism to function. However, it is possible that the effects of adrenaline and diazoxide on cyclic AMP concentrations are secondary to other effects on β-cell metabolism and that the inhibition of insulin release may be related to alterations of β-cell metabolism rather than to changes in the concentration of cyclic AMP.

The observations in Fig. 1 show that glucose-induced insulin release from rat islets appears to follow the biphasic pattern characteristic of insulin release from other pancreatic preparations (Curry *et al.* 1968). Since the intracellular concentration of cyclic AMP remained constant under these conditions the biphasic pattern of secretion appears not to be a phenomenon mediated by cyclic AMP. The results with 3-isobutyl-1-methylxanthine (Fig. 1) also support this suggestion since this compound potentiated the effect of glucose on both phases but cyclic AMP accumulation did not show a biphasic pattern.

The release of glucagon from the α-cells of the pancreas (Chesney & Schofield, 1969), growth hormone and adrenocorticotrophin from the pituitary gland (Steiner, Peake, Utiger, Karl & Kipnis, 1970; Fleischer, Donald & Butcher, 1969) and amylase from the parotid gland (Bdolah & Schramm, 1965) appear to be stimulated under conditions that would be expected to produce an increase in the intracellular concentration of cyclic AMP. The release of these proteins involves the movement of granules containing exportable material to the cell periphery for discharge, and since the release process in all cases involves the mediation of cyclic AMP it is possible that cyclic AMP acts in some way to regulate the movement of granules.

The results in the present study show that, although cyclic AMP may be important in the release of insulin from the β-cell, overriding any effect of variations in the concentration of cyclic AMP is the control by variations in the concentration of glucose. Thus glucose will stimulate insulin release independently of any increase in the concentration of cyclic AMP, and an increase in the concentration of cyclic AMP will not produce release unless a stimulatory concentration of glucose is also present. It is possible therefore that cyclic AMP could act either to increase the efficiency with which glucose produces the activation signal or to increase the efficiency with which that signal activates granule movement. The first of these two possibilities seems unlikely in view of the results in Table 3. Thus in spite of the marked increase in the concentration of cyclic AMP in the β-cell produced by 3-isobutyl-1-methylxanthine in the presence of 5mM-glucose there was no detectable increase in the rate of insulin release, indicating that cyclic AMP was unable to increase the conversion of glucose into a 'signal' compound. We suggest therefore that cyclic AMP may modulate the release of insulin in response to glucose by an effect on the movement of granules in the β-cell. However, it remains to be demonstrated that cyclic AMP is an essential component in the mechanism responsible for the release of insulin after an increase in glucose concentration.

We thank Professor C. R. Park for advice and encouragement. W.M. is in receipt of a Wellcome Travel Fellowship and a Beit Memorial Research Fellowship.

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