Regulation of Insulin Release from Isolated Islets of Langerhans of the Rat in Pregnancy

THE ROLE OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE

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1. The concentrations of cyclic AMP were compared in islets of Langerhans isolated from the pancreases of normal female and pregnant rats and were higher in islets in pregnancy. 2. There was also a significant increase in adenylate cyclase activity in homogenates of islets from pregnant rats compared with those from normal rats. 3. Increased cyclic AMP concentration in islets from pregnant rats was reflected in increased protein kinase activity. When the cyclic AMP-dependent protein kinase activity was increased by 3-isobutyl-1-methylxanthine this stimulated activity was significantly greater in pregnancy. 4. Insulin-secretion studies with islets from normal and pregnant rats showed that theophylline or 3-isobutyl-1-methylxanthine, which raise intracellular cyclic AMP concentrations, caused a significantly greater insulin secretion in pregnancy. 5. It was also found that in the presence of a glucose concentration too low to stimulate insulin secretion, the latter could be induced if the cyclic AMP concentrations were raised sufficiently with 3-isobutyl-1-methylxanthine. 6. It is suggested that the higher cyclic AMP concentrations observed in islets in pregnancy mediate the greater insulin-secretory capacity, as well as the greater sensitivity of these islets to low glucose concentrations.

It has been reported previously that stimulation by glucose of isolated islets of Langerhans from pregnant rats gives rise to hypersecretion of insulin (Malaisse *et al.*, 1969; Costrini & Kalkhoff, 1971; Green & Taylor, 1972). Green & Taylor (1972) found that islets from pregnant rats show an increased response to substances other than glucose, and in particular to theophylline, an agent that is thought to stimulate insulin secretion by increasing the intracellular concentration of cyclic AMP in β -cells (Turtile & Kipnis, 1967). Other workers have drawn attention to the importance of cyclic AMP concentration in determining insulin-secretory response in a variety of systems (Sussman & Vaughan, 1967; Malaisse *et al.*, 1967; Cerasi *et al.*, 1969; Burr *et al.*, 1970; Lambert *et al.*, 1969; Chez *et al.*, 1971). In the present paper we have examined the adenylate cyclase activity, cyclic AMP concentrations and protein kinase activity in islets from pregnant and non-pregnant rats, and related our findings to the insulin-secretory response obtained from these islets.

Materials and Methods

Materials

Reagents. The Radiochemical Centre, Amersham, Bucks., U.K., supplied [α - 32 P]ATP of specific radioactivity in the range 1.8–0.5 Ci/mmol, [γ - 32 P]ATP of

specific radioactivity 2.35 Ci/mmol and cyclic [8- 3 H]AMP of specific radioactivity 20.7 Ci/mmol. Calf thymus histone (type IIA), glucagon ('beef-pork'), cyclic AMP, imidazole (grade III), disodium β -glycerophosphate, EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] and Trizma base were from Sigma (London) Chemical Co. Ltd., Kingston, Surrey, U.K. Crystallized bovine serum albumin and theophylline hydrate were from BDH Ltd., Poole, Dorset, U.K. Dowex-50 resin (AG 50W; H⁺ form; X8; 100–200 mesh) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Insulin-binding reagent was from Wellcome Research Laboratories, Beckenham, Kent, U.K., and 3-isobutyl-1-methylxanthine was from Searle Co., Chicago, Ill., U.S.A.

Animals. Female Sprague–Dawley rats weighing 250–300 g were used. Pregnancy was achieved by natural timed matings and was checked by measuring weight gain; 19-day-pregnant rats were used throughout the experiments. The rats received a diet of 53.4% carbohydrate, 20% protein and 3.8% fat, and drinking water *ad libitum*.

Isolation of islets of Langerhans

Preparation. Islets of Langerhans were prepared by the collagenase digestion of pancreas tissue as described by Howell & Taylor (1968). In all experiments islets were isolated in a bicarbonate buffer (Gey

& Gey, 1936) supplemented with 2mM-glucose; in experiments where insulin secretion was being measured bovine plasma albumin (2mg/ml) was also added to the incubation media.

Measurement of insulin secretion. Isolated islets were preincubated for 30min at 37°C in bicarbonate buffer with 2mM-glucose. The incubation medium for insulin-secretion studies contained 6mM- or 20mM-glucose alone or with one of the following additions: 5mM-theophylline, 1mM-3-isobutyl-1-methylxanthine or 13mM-imidazole. Groups of three islets were placed in 0.6ml of incubation buffer in tubes (50mm×11mm), which were capped after gassing with O₂+CO₂ (95:5). Usually five replicates of eight treatments were tested simultaneously. The tubes were incubated for 60min at 37°C with shaking; diluted samples of incubation medium were then assayed for insulin by using a double-antibody technique based on that of Hales & Randle (1963).

Determination of protein. Protein was measured by the method of Lowry *et al.* (1951), with crystallized bovine albumin as standard.

Assay of adenosine 3':5'-cyclic monophosphate

A saturation assay method as described by Brown *et al.* (1971) was used to measure cyclic AMP concentrations in isolated rat islets. The detection limit of this assay was 0.03 pmol of cyclic AMP.

Preparation of islet tissue for assay. Groups of 30–100 islets were incubated in 5ml of bicarbonate buffer (Gey & Gey, 1936), which contained glucose alone or was supplemented with 5mM-theophylline or 1mM-3-isobutyl-1-methylxanthine. After incubation for 1h the tubes were centrifuged before removal of the incubation medium from the islet pellet, which was immediately frozen in the tube in a solid CO₂-ethanol mixture. The islet tissue was disrupted ultrasonically for 15s [the microtip attachment of a Soni-probe (type 7530A; Dawe Instruments Ltd., London, W.3, U.K.) was used, the Soni-probe being tuned to give a maximum meter reading of 3A, corresponding to approx. 72W] in 1ml of 6% (v/v) HClO₄ and then centrifuged. The cyclic AMP in the supernatant was purified (see below) on a Dowex-50 column, and the precipitated protein was dissolved in 0.1M-NaOH and assayed for protein by the method of Lowry *et al.* (1951).

Purification of samples of islet cyclic AMP. Samples of islet cyclic AMP in 1ml of HClO₄ were applied to Dowex-50 columns (0.5cm×8cm) and were eluted in 0.05M-HCl. The cyclic AMP peak was collected, its position having been determined previously by using cyclic [³H]AMP in a similar column. Samples (1ml) of HClO₄ alone run through Dowex columns yielded 'blank' samples in the assay; both blanks and islet cyclic AMP samples were freeze-dried and then reconstituted in 0.5ml of assay buffer as described

by Brown *et al.* (1971). The percentage recovery of cyclic AMP samples from Dowex columns was determined by comparison with percentage recovery of cyclic [³H]AMP from similar Dowex columns.

Enzyme assays

Adenylate cyclase. Adenylate cyclase was measured by a method based on that described by Pohl *et al.* (1971) as modified by S. L. Howell and W. Montague (unpublished work). Batches of 100 islets were homogenized, with a glass homogenizer, in 0.7ml of buffer containing Tris-HCl (25mM; pH7.6), EDTA (1mM) and MgCl₂ (5mM). The adenylate cyclase assay medium contained in addition 3-isobutyl-1-methylxanthine (1mM), [α -³²P]ATP (3–30 μ M), an ATP-regenerating system consisting of creatine phosphokinase (1mg/ml) and phosphocreatine (20mM), and 50 μ l of islet homogenate (5–10 μ g of protein), to give a final volume of 110 μ l/assay tube. Adenylate cyclase activity was stimulated by including glucagon (20 μ g/ml) or NaF (10mM) in the assay tubes. The reaction was initiated by adding 50 μ l of islet homogenate to each assay tube and continued during incubation for 30min at 30°C. The reaction was then terminated by boiling the tubes in a water bath for 3min. A blank containing all assay constituents except islet homogenate was included in all assays. The labelled cyclic AMP formed in the reaction was separated on alumina columns (4cm×0.5cm) as described by Ramachandran (1971), and was counted for radioactivity in a Beckman liquid-scintillation spectrometer. Protein was measured in 50 μ l samples of islet homogenate.

Protein kinase. Protein kinase activity in islets was measured as the amount of ³²P incorporated into histone/5min per g of islet tissue (Montague & Howell, 1972). Isolated islets of Langerhans, in groups of 20, were incubated at 37°C in 2ml of buffer (Gey & Gey, 1936) containing 6mM-glucose alone or supplemented with 1mM-3-isobutyl-1-methylxanthine. After incubation for 30min this buffer was removed from the islets, which were then disrupted ultrasonically as before in 0.4ml of buffer containing 50mM-sodium β -glycerophosphate, 20mM-KF, 0.3mM-EGTA, 5mM-theophylline and 10mM-magnesium acetate, pH6.0. A sample (100 μ l) of this sonicated mixture was withdrawn for protein determination, and to the remaining 300 μ l was added 100 μ l of the buffer used for protein kinase, containing calf thymus histone (8mg/ml) and [γ -³²P]ATP (1.2mM) to give final concentrations of 2mg/ml and 0.3mM respectively in the reaction mixture. Histone was phosphorylated by islet-tissue protein kinase during a 5min reaction at 37°C; the reaction was stopped by the addition of 1ml of trichloroacetic acid (10%, w/v). The precipitated protein was washed three times in trichloroacetic acid and was then dissolved in 0.3ml

of 0.1M-NaOH, before measurement of its radioactivity.

Determination of radioactivity

Radioactivity was determined by using a Beckman LS233 liquid-scintillation spectrometer. The ^{32}P content of cyclic [^{32}P]AMP eluted in Tris buffer from alumina columns, and of protein precipitates containing [^{32}P]phosphorylated histone dissolved in 0.1M-NaOH, was determined by the use of Čerenkov radiation (Braunsberg & Guyver, 1965). The counting efficiency was approx. 30%. The radioactivity of ^{125}I -labelled insulin in equilibrium with insulin-binding protein, as used in the insulin assay, and cyclic [^3H]AMP bound to cyclic AMP-binding protein, was also determined in the Beckman liquid-scintillation spectrometer under conditions that gave counting efficiencies of 50% and 20% respectively. The scintillator solution used was toluene-Triton X-100-2,5-diphenyloxazole (140:60:1, v/v/w).

Statistical treatment of results

Results were analysed statistically by using analysis of variance (Bailey, 1959). Probability was determined by referring the variance ratio obtained to tables of the F distribution; standard errors of the means were calculated from the residual sum of squares as described by Bailey (1959).

Results

Cyclic AMP concentrations and enzyme activities

Cyclic AMP. Direct measurement of cyclic AMP concentrations in islets isolated from normal and pregnant rats and incubated in 6mM-glucose indicated that these were higher in pregnancy (Table 1). However, some of the increases seen when islets were

incubated with theophylline and 3-isobutyl-1-methylxanthine were not statistically significant, owing to wide individual variation in measurements.

Adenylate cyclase activity. A direct comparison was made of the activity of adenylate cyclase in homogenates of islets from normal and pregnant rats. The basal adenylate cyclase activity was significantly higher in pregnancy (Table 2). Adenylate cyclase activity stimulated by glucagon or fluoride was also significantly higher in islets from pregnant rats compared with normal rats, although there was no difference in the degree of stimulation of cyclase activity produced by these agents. The glucagon content of homogenates of rat islets used in the adenylate cyclase assay was estimated to be 0.36 and 0.46 $\mu\text{g}/\text{ml}$ in homogenates of normal and pregnant rat islets respectively; this is many times lower than the concentration (20 $\mu\text{g}/\text{ml}$) of glucagon used to obtain a two fold stimulation of adenylate cyclase activity *in vitro*.

Protein kinase activity. Total protein kinase activity in the islets, which consists of cyclic AMP-dependent and cyclic AMP-independent fractions (Montague & Howell, 1972), was significantly higher in islets from pregnant rats when compared with those of normal females (Table 3), and this increase can be accounted for solely by the increase in cyclic AMP in these islets. Selective stimulation of the cyclic AMP-dependent activity by 3-isobutyl-1-methylxanthine raised the total protein kinase activities almost two-fold. The increase in histone phosphorylation (pmol of ^{32}P incorporated/5 min per mg of islet protein) brought about by the increase of cyclic AMP concentration in the presence of the drug was 64.5 in normal islets and 122.7 in islets from pregnant rats.

Insulin secretion from isolated islets of normal and pregnant rats

Insulin secretion in response to glucose alone was significantly higher in isolated islets from pregnant

Table 1. Cyclic AMP concentrations in isolated islets from normal and pregnant rats

Groups of 30–100 islets were incubated for 1 h under the experimental conditions shown; the incubation medium was then removed and the islets were frozen immediately in solid CO_2 -ethanol mixture. The islets were sonicated in HClO_4 , and cyclic AMP was determined as described in the Materials and Methods section. The precipitated protein was dissolved in 0.1M-NaOH before measurement by the method of Lowry *et al.* (1951). Each value is the mean of approx. 15 observations, $\pm$ s.e.m. calculated from the residual sum of squares.

Addition to medium	Cyclic AMP (pmol/mg of islet protein)	
	Islets of control rats	Islets of pregnant rats
Glucose (6mM)	8.15 ± 1.27	$11.60 \pm 1.27^*$
Glucose (6mM)+theophylline (5mM)	13.60 ± 1.75	18.66 ± 1.75
Glucose (6mM)+3-isobutyl-1-methylxanthine (1mM)	78.23 ± 7.51	100.59 ± 7.51

* Values significantly different from controls ($P < 0.05$) by analysis of variance.

Table 2. *Adenylate cyclase activity in rat islets*

Cyclic [^{32}P]AMP was formed when 50 μl samples of islet homogenates were incubated with [$\alpha\text{-}^{32}\text{P}$]ATP and an ATP-regenerating system as described in the Materials and Methods section. The radioactivity of the cyclic [^{32}P]AMP formed and the concentration of protein in the homogenates were determined. The results were analysed statistically by using analysis of variance; each result is the mean of 15–18 observations $\pm$ S.E.M. calculated from the residual sum of squares.

Source of islets	Cyclic [^{32}P]AMP formed (nmol/30 min per mg of islet protein)		
	Basal activity	Glucagon-stimulated activity	Fluoride-stimulated activity
Normal rats	0.747 ± 0.074	$1.472 \pm 0.113^*$	$3.561 \pm 0.35^*$
Pregnant rats	$1.022 \pm 0.075^\dagger$	$1.974 \pm 0.113^{*\dagger}$	$4.875 \pm 0.349^{*\dagger}$

* Values significantly different from basal activity ($P < 0.01$).

† Values significantly different from those for normal islets ($P < 0.01$).

Table 3. *Protein kinase activity in islets from control and pregnant rats*

Groups of 20 islets isolated from control and pregnant rats were incubated for 30 min at 37°C in 2 ml of buffer (Gey & Gey, 1936) supplemented with glucose alone (6 mM) or glucose plus 3-isobutyl-1-methylxanthine (1 mM). After incubation this buffer was removed and the islets were disrupted ultrasonically; a portion of the sonicated mixture was used for protein determination and the remainder was used to determine the incorporation of ^{32}P into histone by islet protein kinase as described in the Materials and Methods section. The results were subjected to an analysis of variance. Each result is the mean of 15 observations $\pm$ S.E.M. calculated from the residual sum of squares.

Source of islets	Addition to medium	Protein kinase activity (pmol of ^{32}P incorporated/5 min per mg of islet protein)
Normal rats	Glucose (6 mM)	99.7 ± 7.7
	Glucose (6 mM) + 3-isobutyl-1-methylxanthine (1 mM)	$164.2 \pm 22.5^*$
Pregnant rats	Glucose (6 mM)	$138.2 \pm 7.7^\dagger$
	Glucose (6 mM) + 3-isobutyl-1-methylxanthine (1 mM)	$260.9 \pm 22.5^{*\dagger}$

* Values significantly different from glucose controls ($P < 0.01$).

† Values significantly different from those for normal islets ($P < 0.05$).

rats (Table 4), even when the result was expressed as ng of insulin/h per mg of islet protein (Green & Taylor, 1972). When cyclic AMP concentrations in islets were raised by theophylline or 3-isobutyl-1-methylxanthine in the presence of a physiological glucose concentration (6 mM), insulin secretion was again markedly higher in islets from pregnant than from normal rats (Table 4), and was proportional to the concentration of 3-isobutyl-1-methylxanthine used. In the presence of 2 mM-glucose there was no stimulation of insulin secretion from islets of either normal or pregnant rats. However, if the cyclic AMP concentration in these islets was raised sufficiently by 3-isobutyl-1-methylxanthine, stimulation of secretion was observed even in the presence of 2 mM-glucose, again to a much greater extent in islets from pregnant rats. Imidazole, which accelerates cyclic AMP breakdown in islets (Ashcroft *et al.*, 1972; Sams &

Montague, 1972), lowered the insulin-secretory response of islets to glucose. However, it did not decrease the insulin-secretory response of islets of pregnant rats to the same extent as it did that of normal rats, the percentage inhibition of secretion being 37% in islets of the pregnant rat and 61% in the normal rat islets.

Discussion

Green & Taylor (1972) showed that insulin-secretory capacity and the degree of stimulation of insulin secretion by a given concentration of glucose are both greater in islets isolated from 19-day pregnant rats than from normal rats, and these increases cannot be accounted for solely in terms of the observed increase in islet size during pregnancy. Further, the minimum glucose concentration (3.5 mM) capable

Table 4. *Insulin secretion from isolated islets of normal and pregnant rats*

Groups of three islets were incubated in 0.6 ml of buffer (Gey & Gey, 1936) with additions to the medium as shown. The tubes were incubated at 37°C for 60 min with shaking. Diluted samples of the incubation medium were then assayed for insulin. Each value represents the mean of a minimum of 12 observations $\pm$ S.E.M. Results were analysed statistically by an analysis of variance, from which S.E.M. values were calculated.

Addition to medium	Insulin secretion (ng of insulin/h per islet)	
	Islets of normal rats	Islets of pregnant rats
Glucose (2 mM)	0.80 $\pm$ 0.08	0.90 $\pm$ 0.08
Glucose (2 mM) + 3-isobutyl-1-methylxanthine (1 mM)	1.84 $\pm$ 0.14*	6.24 $\pm$ 0.38*†
Glucose (6 mM)	1.30 $\pm$ 0.24	3.86 $\pm$ 0.70†
Glucose (6 mM) + theophylline (5 mM)	4.12 $\pm$ 0.24*	9.56 $\pm$ 0.88*†
Glucose (6 mM)	1.20 $\pm$ 0.28	4.48 $\pm$ 0.63†
Glucose (6 mM) + 3-isobutyl-1-methylxanthine (1 mM)	7.12 $\pm$ 0.28*	21.72 $\pm$ 1.70*†
Glucose (20 mM)	6.30 $\pm$ 0.30	9.00 $\pm$ 0.76†
Glucose (20 mM) + theophylline (5 mM)	9.00 $\pm$ 0.34*	19.54 $\pm$ 1.34*†
Glucose (20 mM)	6.40 $\pm$ 0.76	11.24 $\pm$ 1.56†
Glucose (20 mM) + 3-isobutyl-1-methylxanthine (1 mM)	16.50 $\pm$ 0.76*	32.74 $\pm$ 1.28*†
Glucose (20 mM)	6.26 $\pm$ 0.34	9.60 $\pm$ 0.50†
Glucose (20 mM) + imidazole (13 mM)	2.40 $\pm$ 0.34*	6.00 $\pm$ 0.50*†

* Values significantly different from glucose controls ($P < 0.01$).

† Values significantly different from those for normal islets ($P < 0.01$).

of stimulating secretion of insulin from islets of pregnant rats is significantly lower than that required by islets from normal rats (5.5 mM). Our previous studies on insulin secretion (Green & Taylor, 1972) had suggested the possible involvement of increased adenylate cyclase activity or increased cyclic AMP concentrations in β -cells in controlling this altered secretory response. This hypothesis has been tested directly and indirectly in the present study.

It is clear from the results that the pancreatic islets of 19-day pregnant rats contain higher concentrations of cyclic AMP and of cyclic AMP-stimulated protein kinase activity (Tables 1 and 3), and that these increases may be mediated at least in part through elevation of the activity of β -cell adenylate cyclase (Table 2). Although adenylate cyclase is known to be associated with plasma membranes (Howell & Whitfield, 1972), these increases in activity per unit of protein did not appear to result from alteration in the available area of plasma membrane, since the DNA/protein ratio is similar in islets derived from normal and pregnant rats, suggesting that the number of cells per unit of protein is unchanged in islets in pregnancy (Green & Taylor, 1972).

Cyclic AMP as a regulator of insulin secretion in pregnancy

A number of observations are consistent with the concept that the observed increase in cyclic AMP in

islets in pregnancy may be responsible for the altered patterns of insulin secretion previously noted (Green & Taylor, 1972). Thus the inhibitors of cyclic nucleotide phosphodiesterase activity, theophylline and 3-isobutyl-1-methylxanthine, are relatively much more effective at a given glucose concentration in islets in pregnancy (Table 4). In addition, the secretory pattern of the normal islet can be altered in at least two ways as a result of increasing its cyclic AMP content by the use of phosphodiesterase inhibitors. First, the threshold for glucose stimulation of normal islets may be lowered markedly by the use of phosphodiesterase inhibitors, so that in the presence of a 10-fold increased concentration of cyclic AMP (Table 1) 2 mM-glucose can stimulate insulin secretion (Table 4). Secondly, the secretory response at a given glucose concentration can be related directly to the degree of phosphodiesterase inhibition, and hence to the intracellular cyclic AMP concentration (Fig. 1; also Montague & Howell, 1973).

Taken together these observations suggest that the alterations of insulin-secretory response observed in the islets in pregnancy may be accounted for predominantly by the observed increases in β -cell concentration of cyclic AMP.

Up to the present, the effects caused by cyclic AMP in islets have usually been considered as relatively short-term effects concerned with the minute-to-minute regulation of rates of insulin secretion. In the present experiments there is evidence of a much

Fig. 1. Insulin secretion in response to 3-isobutyl-1-methylxanthine

Groups of three islets isolated from pregnant rats were incubated for 1 h at 37°C in 0.6 ml of buffer (Gey & Gey, 1936) containing 6 mM-glucose and a range of concentrations of 3-isobutyl-1-methylxanthine. The insulin secreted into the medium was assayed as described in the Materials and Methods section. A similar response was obtained for control islets. Each value is the mean of approx. 10 observations $\pm$ S.E.M.

longer-term adaptive response in islets, mediated by cyclic AMP, to the different physiological circumstances of pregnancy. The increases in cyclic AMP reflect a greater adenylate cyclase activity of rat islets in pregnancy, and suggest that adenylate cyclase may be an important factor in the long-term regulation of insulin secretion in pregnancy.

Several hormonal changes associated with pregnancy have been shown to affect insulin secretion. There is evidence that human placental lactogen alters insulin secretion in humans (Beck & Daughaday, 1967; Kalkhoff *et al.*, 1969) and in rabbits (López-Quijada & Blázquez, 1971) and that injection of steroid hormones into rats may produce insulin-secretory responses similar in magnitude to those obtained in pregnancy (Costrini & Kalkhoff, 1971; Hager *et al.*, 1972). However, it is not known whether these changes after hormonal administration alter insulin secretion through long-term effects on the adenylate cyclase system in islets. In addition, since the nutritional state of rats has been shown to affect adenylate cyclase activity in islets (S. L. Howell, I. C. Green & W. Montague, unpublished work), dietary changes in pregnancy (Campbell *et al.*, 1953;

Scow *et al.*, 1964) may also be important in contributing to the altered pattern of insulin secretion. Further experiments are required to determine the relative importance of dietary and hormonal influences on the long-term activation of adenylate cyclase of β -cells in pregnancy.

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