Regulation of Guanylate Cyclase in Guinea-Pig Islets of Langerhans

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1. Guanylate cyclase activity was determined in homogenates of guinea-pig islets of Langerhans by measurement of the conversion of [α - 32 P]GTP into cyclic [32 P]GMP, the reaction products being separated on columns of neutral alumina. 2. The pH optimum of the enzyme was 7.3; it showed a requirement for bivalent cations, the effectiveness of the cations tested being $\text{Mn}^{2+} \gg \text{Ca}^{2+} > \text{Mg}^{2+}$. 3. About 70% of enzyme activity was sedimented by centrifugation at 105 000g for 60 min; activity was increased 2.3-fold by treatment of homogenates with 0.1% Triton X-100. 4. Guanylate cyclase activity of homogenates was increased by acetylcholine, secretin or pancreozymin, but was inhibited by adrenaline, noradrenaline or ATP. Insulin, glucagon, prostaglandins E_1 or E_2 , glucose, F^- , diazoxide or glibenclamide were ineffective. 5. Determination of cyclic GMP amounts in islets by radioimmunoassay showed a basal concentration of 2.0 pmol/mg of protein, which was increased by incubation of the islets in the presence of acetylcholine or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, but was unaffected by glucose. 6. Dibutyl cyclic GMP had significant stimulatory effects on rates of insulin biosynthesis in isolated rat islets of Langerhans. 7. These results suggest a possible role for cyclic GMP in the regulation of insulin biosynthesis and secretion.

Evidence has accumulated which suggests that adenylate cyclase and cyclic AMP may play an important role in the acute minute-by-minute regulation of insulin release in response to hormones (see review by Malaisse, 1972), and also in the long-term changes in rates of secretion seen under conditions of starvation or pregnancy (Howell *et al.*, 1973; Green *et al.*, 1973). We have now extended this work to include an investigation of the role of cyclic GMP in islets of Langerhans and report here measurements of guanylate cyclase activity, of cyclic GMP concentrations in islets, and of the effects of cyclic GMP on insulin biosynthesis.

Materials and Methods

Reagents

The following reagents were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.: phosphocreatine, creatine phosphokinase, Trizma base, crystalline bovine serum albumin, adrenaline, noradrenaline, glucagon, neutral alumina (type WN 3). Dibutyl cyclic AMP and dibutyl cyclic GMP were obtained from Boehringer Corp., Uxbridge, Middx., U.K. [α - 32 P]-GTP (sp. radioactivity 0.7–1.7 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Secretin and pancreozymin-cholecystokinin were a gift from Professor J. E. Jorpes,

Karolinska Institute, Stockholm, Sweden. Prostaglandins E_1 and E_2 were a gift from Upjohn Ltd., Kalamazoo, Mich., U.K.; glibenclamide was a gift from Hoechst Pharmaceuticals, Hounslow, Middx., U.K. 3-Isobutyl-1-methylxanthine was a gift from G. D. Searle Ltd., Chicago, Ill., U.S.A. Other reagents were of A.R. grade or the purest commercially available.

Methods

Isolation of islets of Langerhans. Islets were isolated from the pancreas of male guinea pigs of Duncan-Hartley strain or male Sprague-Dawley rats by collagenase digestion as described by Howell & Taylor (1966).

Guanylate cyclase assay. Guanylate cyclase activity was determined by a procedure slightly modified from that of White & Zenser (1971). The reaction mixture consisted of 50 μ l of a solution containing 50 mM-Tris-HCl, pH 7.3, 1 mg of albumin/ml, 1.25 mM-MnCl₂, 1 mM-isobutyl-1-methylxanthine, 10 μ M-cyclic GMP and 50 μ M- $[\alpha$ - 32 P]GTP, together with a regenerating system composed of 20 mM-phosphocreatine, 1 mg of creatine phosphokinase/ml and 10 μ M-cyclic GMP. [The use of this regenerating system in guanylate cyclase assay procedures has also been reported by White & Zenser (1971), Bensinger *et al.* (1974) and Goridis *et al.* (1973).] The reaction was started by addition of 50 μ l of a homogenate of guinea-pig islets

prepared in 50mM-Tris-HCl-1mM-EDTA, pH7.3, in a Kontes all-glass homogenizer to give protein concentrations in a range 70–100 µg/50 µl of homogenate, and was continued for up to 20 min at 37°C. After boiling for 3 min the mixtures were transferred to columns (0.5 cm × 4.0 cm) of alumina which were eluted with 10mM-Tris-HCl, pH7.4. Recovery of added cyclic [³H]GMP from alumina columns in these conditions was greater than 96%.

Assay of guanosine 3':5'-cyclic monophosphate. Cyclic GMP concentrations in islets extracts were determined by a specific radioimmunoassay (Steiner *et al.*, 1972) utilizing reagents commercially available from Collaborative Research Inc., Waltham, Mass., U.S.A. The detection limit of this assay was 0.05 pmol of cyclic GMP. The antibody used in this assay shows no cross-reactivity with non-cyclic nucleotides; cyclic AMP is the only naturally occurring cyclic nucleotide which reacts and this is only when it is present at a concentration 1×10^4 greater than that of cyclic GMP.

Preparation of islet tissue for assay. Groups of 100–200 islets were incubated in 2 ml of bicarbonate-buffered medium (Gey & Gey, 1936) which contained glucose alone or was supplemented with 0.5 mM-3-isobutyl-1-methylxanthine or 10 µM-acetylcholine. After incubation for 30 min at 37°C the incubation medium was removed from the islets, which were immediately frozen in a solid CO₂-ethanol mixture. The islet tissue was disrupted ultrasonically for 15 s in 1 ml of 6% (v/v) HClO₄ and then centrifuged to remove the precipitated proteins. The cyclic GMP in the supernatant was separated from cyclic AMP (see below) on a column of Dowex AG-1 (X8) and the precipitated protein was dissolved in 0.1 M-NaOH and assayed for protein by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Purification of samples of islet cyclic GMP. The HClO₄ extract containing cyclic GMP was neutralized with 4 M-KOH to pH7.0 (pH checked with BDH universal indicator paper) and 0.1 ml of 1 M-Tris-HCl, pH7.4, added to ensure neutrality of the extract. The extract was centrifuged to remove the precipitated KClO₄ and the supernatant was applied to a column (4.5 mm × 250 mm) of Dowex AG-1 (X8; 100–200-mesh; formate form). The column was washed with 10 ml of water followed by 10 ml of 1 M-formic acid to elute the cyclic AMP. The cyclic GMP was then eluted with 10 ml of 4 M-formic acid. Samples (1 ml) of HClO₄ alone and HClO₄ containing known amounts of cyclic GMP were carried through the entire procedure to give 'blank' and 'standard' samples for the assay. Blanks, standards and islet cyclic GMP samples were freeze-dried and then reconstituted in 0.5 ml of assay buffer (0.05 M-sodium acetate, pH6.2) and used directly in the assay procedure.

Insulin biosynthesis. Groups of 20 isolated rat islets were incubated in a bicarbonate-buffered

medium (Gey & Gey, 1936) containing 5.5 mM-glucose and 10 µCi of L-[³H]leucine/ml of specific radioactivity 46 Ci/mmol (The Radiochemical Centre, Amersham, Bucks., U.K.). After 60 min incubation at 37°C in the presence or absence of dibutyl cyclic GMP the islets were homogenized in 10% (w/v) trichloroacetic acid, followed by the addition of 50 µg of unlabelled bovine insulin. The precipitate was washed repeatedly in 5% trichloroacetic acid and then redissolved in 0.02 M-HCl before separation of the proteins by polyacrylamide-gel electrophoresis in the system of Davis (1964). Sections of the gels containing respectively the proinsulin and both rat insulin bands (Clark & Steiner, 1969) were dissolved in H₂O₂ by incubation at 60°C overnight and radioactivity was determined in a liquid-scintillation spectrometer with Insta-gel (Packard Instruments Ltd., Wembley, Middx., U.K.) as scintillant.

Subcellular fractionation. Groups of 250 islets were homogenized in a buffer containing 0.3 M-sucrose-10 mM-sodium phosphate, pH6.0, this and all subsequent procedures being performed at 4°C. A nuclei-and-debris fraction was prepared by centrifugation of the homogenate at 600g for 5 min, the supernatant being removed and re-centrifuged at 24000g for 10 min to provide a fraction rich in mitochondria, storage granules and heavy (rough) microsomal vesicles. The supernatant from this centrifugation was separated into light microsomal and supernatant fractions by a final centrifugation at 105000g for 60 min.

Fig. 1. Effect of cyclic GMP and of a GTP-regenerating system on recovery of cyclic [³²P]GMP in the guanylate cyclase assay

A homogenate of guinea-pig islets of Langerhans was incubated for periods of 10, 20 or 30 min with a solution containing the basic reaction mixture and [α -³²P]GTP together with: ●, 1 mg of creatine phosphokinase/ml, 20 mM-phosphocreatine and 10 µM-cyclic GMP; □, 1 mg of creatine phosphokinase/ml, and 20 mM-phosphocreatine; ■, 10 µM-cyclic GMP only; ○, nothing.

Results

Guanylate cyclase in islets of Langerhans

Preliminary experiments were designed to test the linearity of the guanylate cyclase assay in the presence of 1mM or 10μM unlabelled cyclic GMP and of a GTP regenerating system consisting of 20mM-phosphocreatine and 1mg of creatine phosphokinase/ml, and were performed in the presence of 1.25mM-MnCl₂. Fig. 1 shows that the assay remained linear for 20min provided that a GTP regenerating system was present. Decrease of added cyclic GMP from 1mM to 10μM did not appear to affect the recovery of labelled cyclic GMP in the assay so the concentration of nucleotide added was maintained at 10μM in future experiments. The assay was linear over a range of tissue homogenate concentration of 50–120 μg/50 μl of homogenate (results not shown); the pH optimum of the enzyme was investigated with the results shown in Fig. 2; a pH of 7.3 was used in all future experiments. Requirements for Ca²⁺, Mg²⁺ and Mn²⁺ are shown in Fig. 3; activity of guanylate cyclase was undetectable

Fig. 2. pH-dependence of guanylate cyclase activity in guinea-pig islets of Langerhans

Homogenates of guinea-pig islets were incubated in the conditions described in the text for 20min, in media whose pH was adjusted to the values shown.

Table 1. Subcellular distribution of guanylate cyclase in homogenates of guinea-pig islets, with or without pretreatment with Triton X-100

Guinea-pig islets were homogenized in 0.3M-sucrose–10mM-sodium phosphate buffer, pH6.0, at 4°C and separated by differential centrifugation into the fractions shown. Each pellet was resuspended in similar sucrose solution and separated into two parts, one part being treated with 0.1% Triton X-100. Untreated and Triton X-100-treated fractions were then assayed for guanylate cyclase activity by the methods described in the text. Results are means±s.e.m. of six observations and are given as percentages of the total activity of the untreated homogenate which was present in each fraction.

	% of total recovered from untreated homogenate				
	Homogenate	Nuclei+debris (600g for 5min)	Mitochondria-granules-heavy microsomal fraction (24000g for 10min)	Light microsomal fraction (105000g for 60min)	Supernatant
Without Triton X-100	100	46±7	17±3	7±3	30±6
With 0.1% Triton X-100	230±20	115±12	55±7	21±5	39±5

in the absence of added cations, and of those cations tested Mn²⁺ was by far the most effective; a concentration of 1.25mM was optimal and was used in subsequent studies.

Fig. 3. Effect of Ca²⁺, Mg²⁺ or Mn²⁺ on guanylate cyclase activity of guinea-pig islets

Homogenates of guinea-pig islets were incubated in the complete reaction mixture as described in the Materials and Methods section, except for the omission of Mn²⁺. Bivalent cations were added at the concentrations shown and the reaction was continued for 20min at 37°C as described in the text. ■, Mn²⁺; ○, Ca²⁺; ●, Mg²⁺.

The distribution of guanylate cyclase between four impure subcellular fractions prepared by differential centrifugation is shown in Table 1. Each fraction contained some activity, about one-half of the total being present in the nuclei and debris (600g for 5 min) pellet and approximately one-third in the final supernatant. Treatment of each fraction with 0.1% Triton X-100 resulted in a 2–3-fold increase in overall enzyme activity, the relative recovery of activity being increased to a similar extent in each particulate fraction. The specific activity of guanylate cyclase/mg of protein was highest in the mitochondria-granule-heavy microsomal (24000g for 10 min) fraction.

The effects of a range of hormones known to affect islet-cell adenylate cyclase and/or insulin secretion were tested with the results shown in Table 2. Significant stimulation of activity was observed in the presence of secretin, pancreozymin and acetylcholine, whereas glucagon, insulin, prostaglandins E_1 and E_2 were without effect. Adrenaline and noradrenaline inhibited guanylate cyclase activity: the time-course of this inhibition was linear over a 20 min period of incubation.

Addition of glucose (2 or 20 mM), glibenclamide (10 μ g/ml), diazoxide (0.1 mg/ml), arginine (5 mM) or NaF (10 mM) showed each agent to be without signifi-

cant effects on the enzyme at the concentrations used. Addition of ATP (0.1 mM) resulted in a significant ($27 \pm 4\%$) inhibition of guanylate cyclase activity.

Effect on guanylate cyclase of incubation of islets in the presence of 5 mM- or 20 mM-glucose

The recent observation that incubation of isolated islets for 2–7 h in a medium containing 20 mM-glucose increased their adenylate cyclase activity (Howell *et al.*, 1973) suggested that it might be worth while to investigate the possible occurrence of comparable long-term effects of glucose on guanylate cyclase. The results suggested that the glucose concentration of the incubation medium during a 4 h period of incubation has no influence on the guanylate cyclase activity which is subsequently observed in homogenates.

Cyclic GMP amounts in guinea-pig islets of Langerhans

Table 3 shows that 3-isobutyl-1-methylxanthine and acetylcholine (10 μ M) increase the intracellular concentration of cyclic GMP in islets of Langerhans, although raising the glucose concentration of the incubation medium from 2 mM to 20 mM was without effect on islet cyclic GMP concentrations.

Effect of dibutyryl cyclic GMP on rates of proinsulin biosynthesis

Effects of 1 mM-dibutyryl cyclic GMP and 1 mM-dibutyryl cyclic AMP on rates of incorporation of [3 H]leucine into proinsulin in rat islets were examined in these experiments, which were performed in the presence of 5.5 mM-glucose in the incubation medium. Addition of dibutyryl cyclic AMP to the medium during a 1 h period of incubation resulted in a stimu-

Table 2. *Effects of hormones on guanylate cyclase activity in homogenates of islets of Langerhans*

Guanylate cyclase activity of islet homogenates was determined as described in the text, in the presence or absence of hormones at the concentrations shown. Each result is the mean $\pm$ S.E.M. of triplicate observations in four separate experiments, and is expressed as a percentage of the activity of homogenates assayed in the absence of added hormone. * Indicates a result significantly different from control (no added hormone) experiments ($P < 0.05$).

Hormone	Concentration	% of activity observed in the absence of added hormone
Secretin	1 μ g/ml	126 $\pm$ 7*
	10 μ g/ml	141 $\pm$ 7*
Pancreozymin-cholecystokinin	1 μ g/ml	119 $\pm$ 4*
	10 μ g/ml	135 $\pm$ 6*
Glucagon	1 μ g/ml	102 $\pm$ 5
	10 μ g/ml	104 $\pm$ 4
Adrenaline	10 μ M	81 $\pm$ 4*
	0.1 mM	69 $\pm$ 6*
Noradrenaline	10 μ M	72 $\pm$ 5*
	0.1 mM	58 $\pm$ 7*
Prostaglandin E_1	10 μ M	94 $\pm$ 7
Prostaglandin E_2	10 μ M	95 $\pm$ 6
Acetylcholine	10 μ M	132 $\pm$ 6*
	0.1 mM	109 $\pm$ 5

Table 3. *Cyclic GMP concentrations in islets of Langerhans incubated in vitro*

Groups of 100–200 islets were incubated for 30 min 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 the cyclic GMP was purified and then measured as described in the Materials and Methods section. Each value is the mean $\pm$ S.E.M. of the number of observations shown in parentheses. * Values significantly different from controls ($P < 0.05$).

Addition to medium	Cyclic GMP (pmol/mg of islet protein)
2 mM-Glucose	2.0 $\pm$ 0.3 (6)
6 mM-Glucose	1.9 $\pm$ 0.4 (6)
20 mM-Glucose	2.2 $\pm$ 0.4 (6)
6 mM-Glucose + 0.5 mM-3-isobutyl-1-methylxanthine	22.2 $\pm$ 2.3* (6)
6 mM-Glucose + 10 μ M-acetylcholine	3.6 $\pm$ 0.3* (3)

lation of incorporation of 1.8 ± 0.4 -fold (mean $\pm$ S.E.M. of 10 observations), an increase comparable with that observed by Schatz *et al.* (1973) in experiments utilizing mouse islets and 10mM-dibutyryl cyclic AMP. Addition of dibutyryl cyclic GMP at a concentration of 1 mM resulted in a stimulation of incorporation of 2.3 ± 0.6 -fold (10 observations), an increase not significantly different from that seen in the presence of dibutyryl cyclic AMP. Both nucleotides also caused smaller increases in rates of incorporation into other proteins present in the islet extracts.

Discussion

This paper demonstrates for the first time the presence of guanylate cyclase activity and of cyclic GMP in guinea-pig islets of Langerhans, the concentration of the nucleotide being apparently one-quarter that of cyclic AMP determined in islets under similar conditions.

Guanylate cyclase activity in islet homogenates shares many of the properties of the enzyme reported in other tissues. Thus a pH optimum close to 7.3 and a dependence on bivalent cations, especially Mn^{2+} , were noted by Hardman & Sutherland (1969). Stimulation of activity on exposure to secretin has been reported in liver by Thompson *et al.* (1973), and acetylcholine raises cyclic GMP amounts in thyroid (Yamashita & Field, 1972) and heart (George *et al.*, 1970). A marked (2.3-fold) increase of activity was noted after treatment of particulate fractions with Triton X-100, the magnitude of this increase being similar to that reported in intestine by Ishikawa *et al.* (1969) and in retinal photoreceptors by Bensinger *et al.* (1974).

We have not attempted a precise localization of the enzyme to elements within particulate fractions, nor

have we been successful in preliminary attempts at cytochemical localization of guanylate cyclase by using the GTP analogue guanylyl imidodiphosphate and methods analogous to those previously described for localization of adenylate cyclase (Howell & Whitfield, 1972).

Comparison of adenylate and guanylate cyclase activities in islets of Langerhans

A comparison of the relative activities of adenylate cyclase and guanylate cyclase in islets in the presence of a variety of agents which are known to affect insulin secretion is of interest, and is shown in Table 4. Activities of both enzymes are stimulated by secretin, pancreozymin or acetylcholine and both are inhibited in the presence of adrenaline or noradrenaline, the inhibition of guanylate cyclase being more pronounced than that of adenylate cyclase (Howell & Montague, 1973). These observations suggest that guanylate cyclase and adenylate cyclase may both play a role in mediating the effects of those hormones on islet cells. However, the role of guanylate cyclase would seem in some respects to be less important than that of adenylate cyclase since glucagon and prostaglandins E_1 and E_2 , which have marked effects on insulin release, stimulated adenylate cyclase but were without effect on guanylate cyclase. Moreover, no agent was found which would alter guanylate cyclase without also altering adenylate cyclase activity.

Addition of 0.1 mM-ATP to the reaction mixture resulted in inhibition of guanylate cyclase, whereas addition of 0.1 mM-GTP to the assay for adenylate cyclase causes activation of the enzyme (Howell & Montague, 1973). Competitive inhibition of liver guanylate cyclase by ATP has been reported by Thompson *et al.* (1973).

Table 4. Effects of some insulin secretagogues on islet-cell adenylate cyclase and guanylate cyclase activities

For details see the text. References are: * Howell & Montague (1973): rat islets; † Kuo *et al.* (1973): pilocarpine-pretreated rat islets; ‡ Davis & Lazarus (1972): mouse islets. 0 = No effect; + = stimulation; - = inhibition.

Insulin secretagogues	Adenylate cyclase activity	Guanylate cyclase activity	Effect on secretion
Glucose	0*†‡	0	+
Glibenclamide	0*	0	+
Arginine	0*†	0	+
Leucine	0*†	0	+
Glucagon	+*†‡	0	+
Prostaglandins E_1 and E_2	+*†	0	+
Acetylcholine	+†	+	+
Secretin	+‡	+	+
Pancreozymin	+†	+	+
Diazoxide	0*	0	
Adrenaline	-*†	-	
Noradrenaline	-*†	-	

Role of cyclic GMP in islets of Langerhans

Addition of cyclic GMP or dibutyryl cyclic GMP to incubation media during incubation of intact islets of Langerhans resulted in a significant stimulation of rates of proinsulin biosynthesis, the magnitude of the effect being comparable with that observed with cyclic AMP or its dibutyryl derivative. Dibutyryl GMP has been shown to stimulate protein synthesis in thyroid slices (Macchia & Varrone, 1971) and in a cell-free system from rat liver by Varrone *et al.* (1973), and a stimulant effect of dibutyryl cyclic GMP on insulin secretion from rat islets has been observed by Voyles *et al.* (1973). The effects of cyclic GMP on insulin secretion appear to be dependent on the presence of a stimulatory concentration of glucose in the incubation media (I. C. Green, D. Perrin & W. Montague, unpublished work), suggesting that cyclic GMP, like cyclic AMP, may serve to modulate the secretory responses of the β -cell to a primary stimulus such as glucose.

No evidence was obtained in the present study that glucose, the major physiological stimulus of insulin synthesis and secretion, causes either activation of guanylate cyclase or elevation of cyclic GMP concentrations within islets, either acutely or during a 4h period of incubation, although from the present study it is not possible to exclude the possibility of changes in cyclic GMP amounts at very short time-intervals after exposure to glucose stimulation. Thus there seems on present evidence to be no compelling reason to consider guanylate cyclase or cyclic GMP as major components of the system responsible for the physiological regulation of rates of insulin secretion. The nucleotide may, however, play a role in mediating the effects of those hormones (secretin, pancreozymin, acetylcholine, adrenaline, noradrenaline) which apparently influence activity of adenylate cyclase and of guanylate cyclase in a similar way.

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