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GRADUATE COLLEGE

ADENOSINE 3', 5'-MONOPHOSPHATE SYSTEM IN ISLETS OF LANGERHANS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

BY

WU-NAN KUO

Oklahoma City, Oklahoma

1972

ADENOSINE 3', 5'-MONOPHOSPHATE SYSTEM IN ISLETS OF LANGERHANS

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LIST OF ABBREVIATIONS

Cyclic AMP	Adenosine 3', 5'-monophosphate
Cyclic CMP	Cytidine 3', 5'-monophosphate
Cyclic GMP	Guaninosine 3', 5'-monophosphate
Cyclic IMP	Inosine 3', 5'-monophosphate
Cyclic TMP	Thymidine 3', 5'-monophosphate
Cyclic TuMP	Tubercidin 3', 5'-monophosphate
Cyclic UMP	Uridine 3', 5'-monophosphate
5'-AMP	Adenosine 5'-monophosphate
ATP	Adenosine triphosphate
EGTA	Ethyleneglycol-bis(β-aminoethyl ether)-N, N'-tetraacetic acid
I. U.	International Unit
PEP	Phosphoenolpyruvate
PRPP	5-phosphoribosyl-1-pyrophosphate
ACTH	Adrenocorticotropic hormone
TSH	Thyroid-stimulating hormone
PG	Prostaglandin
mg	Milligram
g	Gram
nmole	Nanomole
Tris	Tris (hydroxymethyl) amino methane HCl
Km	Michaelis constant

LIST OF ABBREVIATIONS- -Continued.

v/v	Volume to volume
cm	Centimeter
min	Minute
mM	Millimolar
µM	Micromolar

ADENOSINE 3', 5'-MONOPHOSPHATE SYSTEM IN ISLETS OF LANGERHANS

CHAPTER I

INTRODUCTION

Adenosine 3', 5'-monophosphate (cyclic AMP), which is seen below (Fig. 1), was identified chemically by Sutherland *et al.* (1-3).

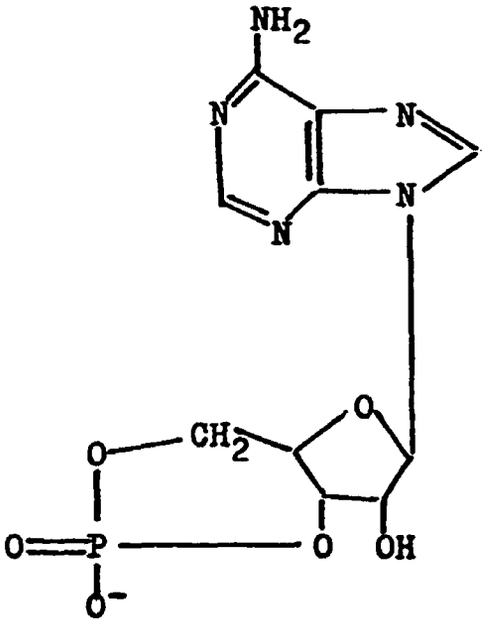

Fig. 1 - Adenosine 3', 5'-monophosphate.

The cellular levels of cyclic AMP are controlled by adenylate cyclase, a membrane bound enzyme (4) which catalyzes its synthesis from adenosine triphosphate (ATP),

and phophodiesterase which mediates its degradation (5).

Since the discovery that cyclic AMP mediates its hyperglycemic effects by the stimulation of glycogen phosphorylase, (1, 6, 7), a great many regulatory functions of cyclic AMP in various tissue have been proved. A model of the protein component of membrane adenylate cyclase, which consists of at least two subunits, a regulatory subunit (R) and a catalytic subunit (C), has been postulated by Robison et al. (8) and is as follows (Fig. 2) :

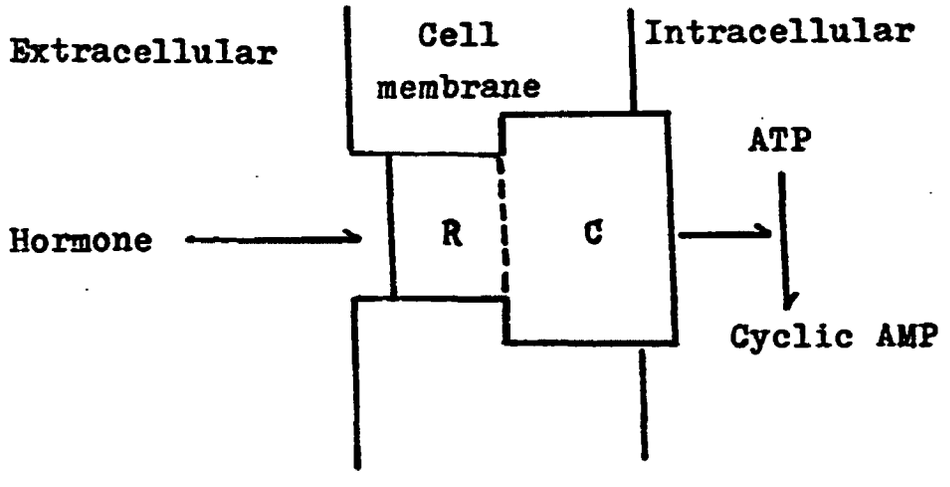

Fig. 2 - Adenylate cyclase system.

In more detail, the regulator subunit is possibly composed of several different parts. The adrenergic α-receptor is in part connected to the catalytic subunit such that interaction with a catecholamine leads to a decrease in adenylate cyclase activity. In contrast, the adrenergic β-receptor mediates an increase in the enzyme activity. However, receptors for other hormones could be located on the same or different subunits (9).

Many hormones, which themselves are the "first messengers" (illustrated on the following page), have been demonstrated to act on their target cells by utilizing cyclic AMP as a "second messenger", the intracellular signal, which may undergo amplification in different ways leading to the ultimate physiological responses (10).

Kuo and Greengard have proposed that all of the varied effects of cyclic AMP results from the phosphorylation of proteins with ATP by cyclic nucleotide dependent protein kinases (11, 12). This hypothesis is supported by recent reports that some protein kinases have a greater affinity for cyclic AMP than cyclic GMP (13), and that histones (14), hormone-sensitive lipase from fat cells (15), microtubular protein from brain (16), glycogen phosphorylase kinase from muscle (17, 18), as well as glycogen synthetase from muscle (19) are phosphorylated by cyclic AMP dependent protein kinase.

Panceatic insulin, a very important polypeptide hormone, serves to decrease hepatic intracellular cyclic AMP levels, while increasing such things as carbohydrate metabolism, glycogen storage, fatty acid synthesis, amino acid uptake, and protein synthesis. Insulin is known to be synthesized by and secreted from the β-cells of multicellular pancreatic islets. Normally, islets only comprise 1-2% of whole pancreatic mass. A method to isolate these is-

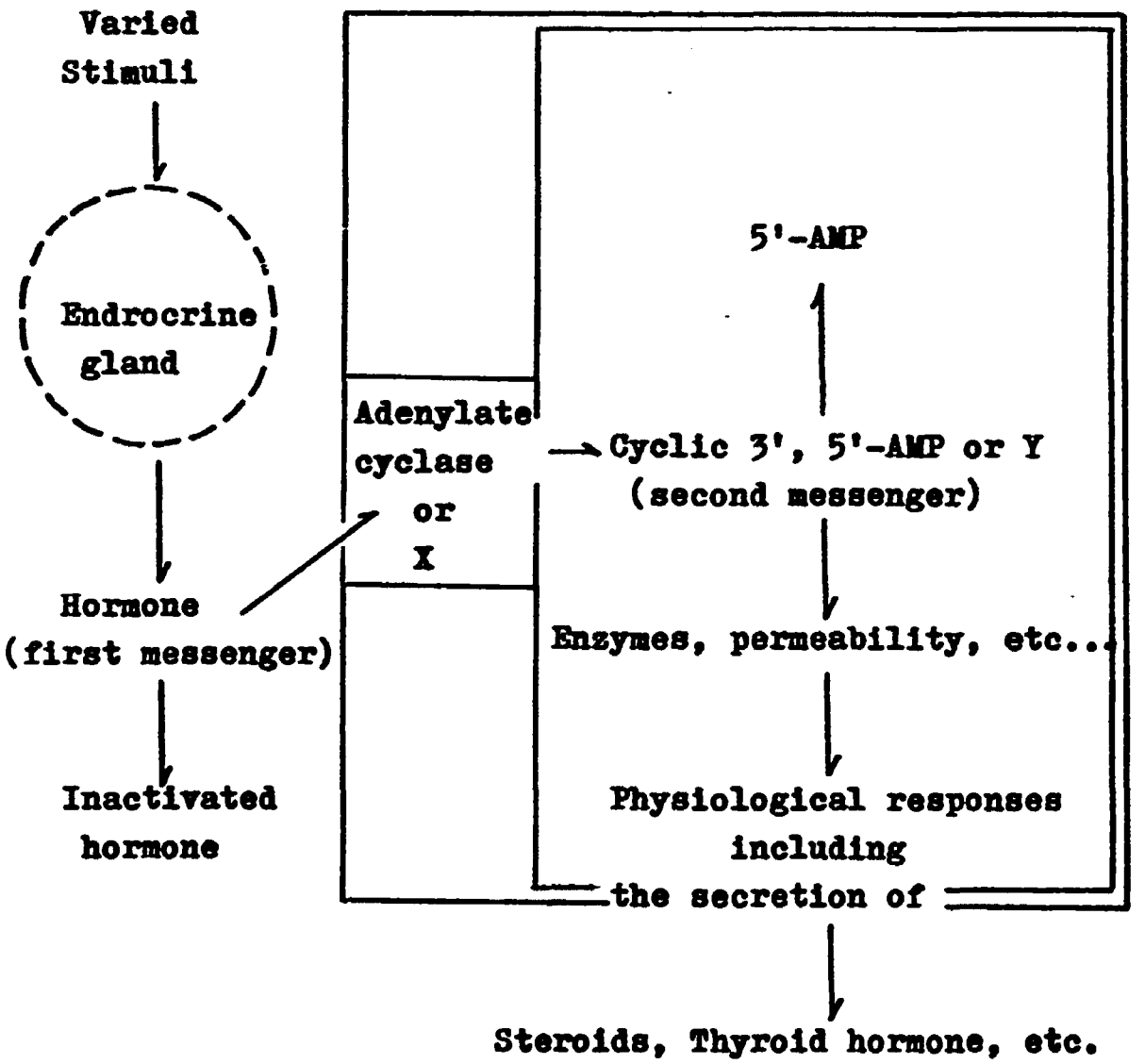

Fig. 3 - Second messenger concept.

lets in sufficient quantities for the study of their physiological and biochemical properties has been elucidated (20). It is based on disruption of the acinar parenchyma by injecting buffer solution into the common bile duct. The gelatinous mass is cut into small pieces and incubated with crude collagenase. After complete digestion, islets are released, and islet-rich fractions can be separated from incubation mixtures by sedimentation. The free, round (or ovoid), greyish-white and intact islets can be recognized easily under a dissecting microscope when viewed against a black background. Insulin is secreted *in vitro* from these isolated islets which appear normal by electron microscopy even after 1.5 hour incubation periods.

Various techniques used for the immunoassay of insulin, and the study of the regulation of insulin secretion both *in vivo* and *in vitro* have been disscussed by Mayhew *et al.*(21). Metabolizable sugars, certain amino acids, enteric hormones, and sufonylureas may initiate insulin secretion. The catecholamines, glucagon, adrenal corticoids, adrenocorticotropin, growth hormone, thyroid hormone, TSH, oxytocin, and vasopressin are known to be endocrine regulators of insulin secretion. However, "neurogenic" control of insulin secretion is another type of regulation.

Although an important role for cyclic AMP in insulin secretion has been suggested by recent studies, its actual meachanism remains obscure. The administration of

cyclic AMP enhances insulin secretion from perfused pancreas (22). Turtle et al. (23) have shown that in isolated islets, the changes in the levels of cyclic AMP correlate well with the pattern of insulin secretion in response to theophylline, epinephrine, adrenergic blocking agents, and glucagon. Many other hormones have been found to increase intracellular levels of cyclic AMP in other tissues. Of further interest, is the finding that many of the enzymes involved in glycolysis, the hexose monophosphate shunt, the Krebs cycle, and in glycogen synthesis are all in islet tissue (24-29). Cyclic AMP has been found to be involved in regulation of glycogen synthesis, glucose oxidation, gluconeogenesis and permeability in certain tissues (30). Several studies indicate that oxidative metabolism of glucose and energy production is important for insulin secretion (30, 32). From these observations, it can be concluded that the secretory mechanism of insulin is very complicated and that under certain conditions, cyclic AMP may be involved.

In this study, the cyclic AMP system in islets of Langerhans includes adenylate cyclase, phosphodiesterase, cyclic AMP dependent kinase, and protein kinase modulator.

CHAPTER II

MATERIALS AND METHODS

Materials

A. Hormones

The sample of prostaglandins was the kind gift of Dr. J. E. Pike, Upjohn. Leuteinizing hormone was obtained from Schwarz/Mann. Isoproterenol (L and DL form), DL-epinephrine, L-norepinephrine, insulin (24 I.U./mg), ACTH (100 I.U./mg), TSH (1 I.U./mg), pancreozymin, and secretin were purchased from Sigma.

B. Drugs

The following drugs were obtained from commercial sources and include: KÖ 592 (1-3-methylphenoxyl-2-hydroxy 3-isopropylpropranolamine), C. H. Boehringer and Sohn; phentolamine, Ciba Pharmaceuticals; MJ 1999 (sotalol), Mead Johnson; pilocarpine hydrochloride (4% isoptocarpine), Alcon Laboratory; D-INPEA (N-isopropyl-p-nitrophenyl-ethanolamine), Selvi e C., Italy; DL-propranolol, Sigma.

C. Enzymes and Amino Acids

The following materials were purchased from Sigma:

L-arginine, L-leucine, and pyruvate kinase. Collagenase (crude) was obtained from Worthington. Cyclic AMP dependent protein kinase from bovine heart was prepared by the procedure of Kuo et al. (12, 33).

D. Radioactive Chemicals

The following radioactive chemicals were purchased from commercial sources and include: [8-^{14}C] ATP (0.33 Ci/mole) and [2-^{3}H] adenine (25 Ci/mole), Schwarz/Mann; adenosine-H^{3}(G) 3', 5'-cyclic phosphate (5 Ci/mole), New England Nuclear. [γ-^{32}P] ATP was prepared by the procedure of Post and Sen (34).

E. Others

AG-50WX8 (100-200 mesh, hydrogen form) was purchased from BioRad. Phosphoenolpyruvate and all non-radioactive nucleotides were obtained from Sigma. Other chemicals used were all reagent grade.

Buffers

Three kinds of buffer were employed in digestion or incubation. Hanks' solution (35), without glucose, contained per liter at pH 7.4:

NaCl	8000.0 mg
KCl	400.0 mg
$Na_2HPO_4 \cdot 2H_2O$	60.0 mg
KH_2PO_4	60.0 mg
$MgSO_4 \cdot 7H_2O$	100.0 mg

$CaCl_2$.....140.0 mg

$MgCl_2.6H_2O$.....100.0 mg

$NaHCO_3$.....350.0 mg

Krebs-Ringers bicarbonate buffer, saturated with 95% O_2- 5% CO_2, v/v, contained per liter:

NaCl.....7250.0 mg

KCl.....373.0 mg

$NaHCO_3$.....2180.0 mg

KH_2PO_4.....169.0 mg

$MgSO_4.7H_2O$.....320.0 mg

$CaCl_2$.....89.0 mg

glucose.....2000.0 mg

The buffer used most often for collagenase digestion was Rodbell's Tris-salts solution (35) at pH 7.4 and contained per liter:

NaCl.....7192.0 mg

KCl.....298.0 mg

KH_2PO_4.....272.0 mg

$CaCl_2.2H_2O$.....147.0 mg

$MgCl_2.6H_2O$.....305.0 mg

Tris-HCl.....3026.0 mg

Isolation of Pancreatic Islets (For Adenylate Cyclase Assay)

Three to seven male Sprague-Dawley rats (about 200 g each) were used for each experiment. Each rat was intraperitoneally injected with 0.3 ml of 4% pilocarpine just

three hours before surgery. The surgical procedure and cannulation of the common bile duct was similar to that of Lacy (20), except that 10 ml of Rodbell's Tris-salts buffer, pH 7.4 (36), was injected into the pancreas instead of Hanks' solution (35). The pancreas was then removed, trimmed, chopped into small pieces, washed several times with the Tris-salts buffer and allowed to settle. Ten mg of collagenase for each pancreas was added to the sediment after removal of the supernatent fluid and the mixture was stirred at 37° for 20 min.

Following this digestion, the mixture was poured onto a 180 mesh silk cloth and washed with 200 ml of cold Tris-HCL (40 mM) -$MgSO_4$ (5 mM), pH 7.4. The cloth was then inverted and the islet-rich tissue washed into a tube with 200-300 ml of the same solution. After several min the islets had settled to the bottom of the tube and the supernatant fluid was discarded. The islets were then transferred to a cold Petri dish and viewed with microscopic aid (10 x). Large islets were siphoned through an 18 gauge metal needle into a test tube or glass homogenizer. The islets were homogenized with four or five strokes of a tight fitting ground glass homogenizer. A Lowry protein assay (37) was performed for each experiment and the protein content estimated by comparison with bovine serum albumin. The protein content was consistently 1.7 ± 0.1 μg per islet.

Adenylate Cyclase Assay

Basically, the assay system involves the following reactions (Fig. 4):

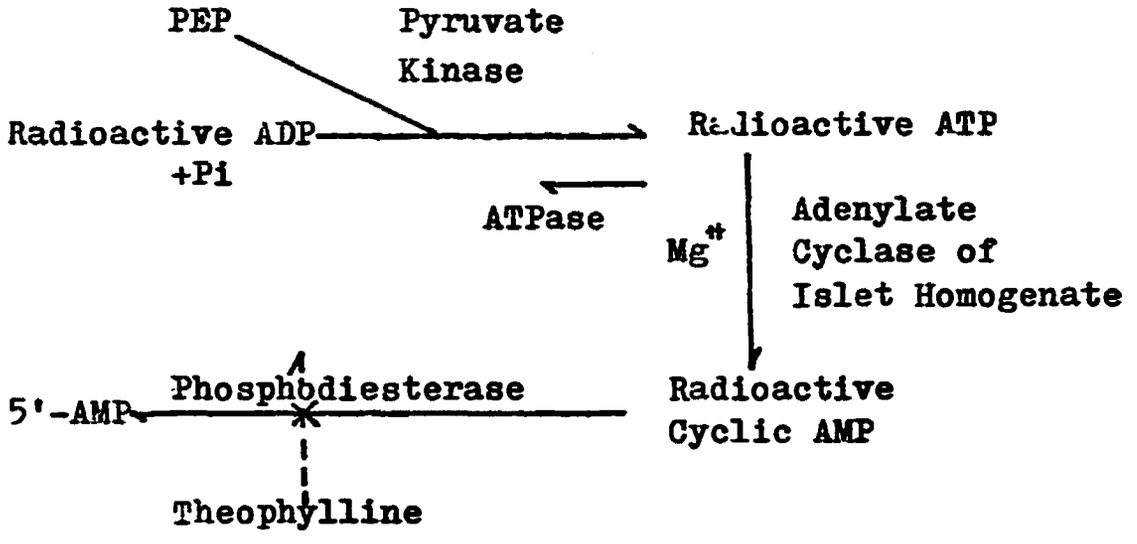

Fig. 4 - Reactions involved in adenylate cyclase assay.

The standard incubation mixture, in a final volume of 0.95 ml, consisted of the following: 40 mM Tris-HCl containing 5 mM $MgSO_4$ (adjusted to pH 7.4 with 5 N NaOH); 0.5 mM cyclic AMP; 1 mM [8-^{14}C] ATP (0.33 Ci/mole); 10 mM theophylline; 5 mM phosphoenolpyruvate; 9 I.U. of pyruvate kinase; 85 to 100 µg islet protein; and test substances where indicated. This mixture was incubated at 30° for 10 min. Unless otherwise stipulated, all experiments, including the controls in which no islet homogenate was added, were performed by incubating the islet samples in triplicate for each type of treatment. Each incubated sample was then separately assayed for the amount of radioactive cyclic AMP synthesized.

The reactions were terminated by the addition of 0.1 ml 50% trichloracetic acid, followed by centrifugation and neutralization of the supernatant solution with 0.1 ml of 2.5 M Tris base. Next, 0.05 ml $ZnSO_4$ (5%) and 0.05 ml $Ba(OH)_2$ (2.56%) were added to the supernatant solution and the mixture was centrifuged (5,000 x g, 10 min). The supernatant fluid was then lyophilized overnight. The dried samples were dissolved in 0.5 ml water and applied to columns (0.5 x 2.5 cm) of AG-50WX8 (Bio-Rad, hydrogen form, 100-200 mesh). The columns were then eluted with water, and the third and fourth ml of eluate used as the cyclic AMP fraction. The optical density at 260 mμ of this fraction was monitored during the first 40 individual enzyme assays (Figs. 7 and 8) and found to consisently reflect a cyclic AMP recovery of 75-80%. The recovery of cyclic AMP was also estimated to be 72-78% when 1 nmole of adenosine-H^3(G) 3', 5'-cyclic phosphate (containing about 2 x 10^6cpm) was added to the incubated islet samples immediately following the addition of trichloracetic acid for the purpose of determining the recovery of the cyclic nucleotide. In all succeeding experiments the value of 75% recovery was used. The entire cyclic AMP fractions (2 ml each) were then mixed with 18 ml of Aquasol (New England Nuclear) and the radioactivity was counted in a liquid scintillation spectrometer. In all cases, with 85 to 100 μg islet protein (representing 50-60 islets) the control experiments produced about 2

nmoles of cyclic AMP per min per mg protein after subtraction of the negligible zero time control (0.1-0.2 nmole). This procedure for assaying adenylate cyclase activity is a modification of that developed by Krishna et al. (38), and was found to result in minimal contamination of the cyclic AMP fractions with the radioactive ATP. The authenticity of cyclic AMP in the AG-50WX8 eluate was verified by the following experiment. The cyclic AMP sample obtained by the above procedure was chromatographed with standard cyclic AMP on a thin layer plate (SilicAR TLC-7GF, Mallinckrodt), using a solvent system consisting of isopropyl alcohol-H_2O-29% NH_4OH (7:3:1, v/v). The radioactivity recovered from the cyclic AMP spot on the developed plate represented the total radioactivity originally present in the cyclic AMP sample.

The results of all experiments are presented as the means and standard deviation for the triplicate samples of each set of treatments.

Prelabeling of Pancreatic Islets

Intact islets were used in this procedure. The principle of prelabeling is based on (Fig. 5):

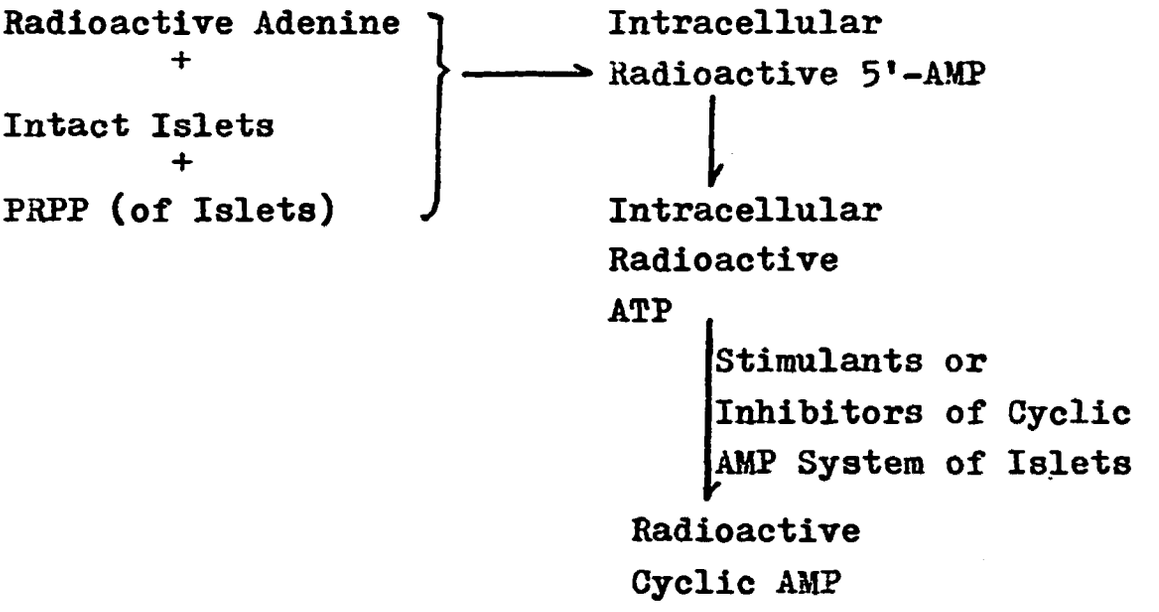

Fig. 5 - Reactions involved in prelabeling method.

Two methods were employed for prelabeling the islets. The first method (Figs. 10-12) consists of simultaneously digesting pancreatic pieces with crude collagenase and prelabeling the islets. Pancreatic pieces obtained from 2 male Sprague-Dawley rats (about 200 g each) were incubated, with stirring, at 37° in 2 ml of Rodbell's Tris-salts solution (36) in the presence of 20 mg of crude collagenase and 36 μ Ci of $[2\text{-}^{3}H]$ adenine for 30 minutes. The digested tissue was then treated, and intact islets isolated, according to the procedures described previously. The collected islets were washed once with 30 ml of cold Kreb-Ringer bicarbonate buffer (previously saturated with 95% O_2-5% CO_2, v/v, and containing no glucose), and were finally suspended in an appropriate volume of the same buffer to yield a suspension containing 30-50 islets per ml.

The second method (Tables 8-11) consists of digesting the pancreatic tissue followed by prelabeling the isolated islets from five rats. The procedures used to digest pancreatic pieces and isolate islets were exactly the same as described before. About 2,000 islets were suspended in 4 ml of Krebs-Ringer bicarbonate buffer (previously saturated with 95% O_2- 5% CO_2, v/v) containing 50 µCi of [2-^{3}H] adenine. The islets were incubated for 20 min at 37°, with shaking, at the end of the prelabeling period, 30 ml of cold Krebs-Ringer buffer (containing no glucose) was added and the mixture was centrifuged at 2,000 r.p.m. for 3 minutes. The supernatant solution was then discarded. The washing procedure was repeated 5 times, and the islets were finally transferred to an appropriate volume of same buffer to yield a suspension containing 20-40 islets per ml.

In experiments (Table 12) in which the absolute levels of total cyclic AMP were measured by the protein kinase catalytic method (33), the islets were isolated as in the second method, with the omission of the prelabeling step. The islets were suspended in an appropriate volume of Krebs-Ringer bicarbonate buffer to yield a suspension containing 45 islets per ml.

Incubation of Islets and Measurement of Cyclic AMP Levels

In experiments (Figs. 10-12; Tables 8-11) in which the relative levels of radioactive cyclic AMP in islet

cells were measured, appropriate volumes of the islet suspension were incubated at 37° for different periods in the presence of various agents, as indicated in the legends to the figures and the tables. At the end of the incubation period, one-tenth volume of 50% trichloracetic acid was added to the incubation mixture followed by the addition of 0.02 ml of 1 μM cyclic AMP as a carrier. The islet suspension was then sonicated for 0.5 min, then centrifuged at 2,000 r.p.m. for 5 min. The precipitate was removed and the supernatant fluid was then neutralized with 1 M Tris. The radioactive cyclic AMP in the supernatant fluid was purified by the same procedure described in the adenylate cyclase assay. One ml of the pooled cyclic AMP fraction was assayed for radioactivity.

The absolute amounts of cyclic AMP (Table 12) were measured by cyclic AMP dependent protein kinase catalytic method (33). The experimental procedure was based on the following reaction (Fig. 6):

$$\left.\begin{array}{l}\text{Histone}\\ +\\ [\gamma\text{-}^{32}\text{P}]\ \text{ATP}\end{array}\right\} \xrightarrow[\text{Cyclic AMP Dependent Protein Kinase}]{Mg^{++},\ \text{Cyclic AMP}} \text{Histone}\text{—}^{32}\text{P}+\text{ADP}$$

Fig. 6 - Reaction involved in cyclic AMP dependent protein kinase catalytic method.

The incubation mixture used for the assay contained, in a final volume of 0.2 ml; sodium acetate buffer, pH 6.0,

10 μmoles; magnesium acetate, 2 μmoles; histone mixture, 40 μg; [γ-^{32}P] ATP, 1 nmole, containing about 2.1 x 10^6cpm; heart protein kinase, 3 μg. Incubations were carried out at 30° for 5 min in a shaking water bath. The reaction was terminated by addition of 2 ml of 5% trichloracetic acid containing 0.25% sodium tungstate, pH 2.0 (39); 0.1 ml of 0.63% bovine serum albumin was added as a carrier protein. After standing at 0° for 5 min, the mixture was centrifuged, and the supernatant solution removed by aspiration. The precipitate was dissolved in 0.1 ml of 0.5 N NaOH, and 2 ml of 5% trichloracetic acid-0.25% sodium tungstate solution, pH 2.0, was added. This mixture was centrifuged followed by aspiration of the supernatant described before. The above procedure was repeated once more. The protein was finally collected by centrifugation and dissolved in 0.1 ml of 0.5 N NaOH, and the radioactivity was measured in a liquid scintillation counter. The amount of cyclic AMP present in the samples was determined from standard curves with known quantities of cyclic AMP.

CHAPTER III

RESULTS

Isolation of Pancreatic Islets

The effects of pilocarpine usage on the yield of large islets as compared to other procedures, are presented in Table 1. In author's hands, treatment of rats with pilocarpine facilitated more than a 10-fold increase in the yield of pancreatic islets, i.e. from 25-35 islets per pancreas from the untreated rats to 350-500 islets from the treated rats. When the amount of collagenase used was increased 6-fold and the volume of the digestion mixture was increased 4- to 8-fold by the addition of Hanks' solution, the yield of islets was less than one-half of our usual value. The procedure described here, to the author's knowledge, yields the greatest amount of islets per pancreas, and moreover, the islets isolated have been shown to possess adenylate cyclase responsive to various agents which are known to stimulate insulin secretion.

Another method to isolate islets by discontinous dextran density gradients (2.5%, 12%, and 22%; 2.5%, 8%, 14%, 16%, 18.5%, and 22%. at 1000xg centrifugation for 10

TABLE 1

THE EFFECT OF PILOCARPINE PRETREATMENT ON THE YIELD OF ISLET ISOLATED FROM RAT PANCREAS

Pretreatment	Buffer used with collagenase digestion	Digestion time	Collagenase concentration	Yield of islets
		min	mg/ml	number of islets/pancreas
None	4 ml Hanks' solution	15	60	80-150
None	4 ml Rodbell's Tris-salts solution	15	60	80-150
None	None	20	10	25-35
Pilocarpine	None	20	10	350-500

Method for isolation of pancreatic islets was as described under "Experimental Proceedure" except for some modifications as indicated in the table.

minute) was also tried. Although islets could be isolated between the 12% and 22% density layers (or between the 16% and 18.5% density layers), the total yield of islets was quite poor. Unfortunately, islets could not be frozen and stored to accumulate large quantities for study. Only most of phosphodiesterase activity was found to be stable, while adenylate cyclase activity was rapidly lost (data not shown).

Adenylate Cyclase Activity In Isolated Pancreatic Islets

A. Dependence of Cyclic AMP Production on Incubation Time and Islet Protein Concentration

Fig. 7 illustrates the time course of the reaction at five and ten minutes in the presence and absence of the polypeptide enteric hormone, secretin. The control reponse is roughly linear with time while the stimulated enzyme further increases its activity in the second 5 min. period, i.e., a 49% stimulation at 5 min. and 130% at 10 min.

The dependence of cyclic AMP production upon islet protein concentration is demonstrated in Fig. 8. A linear relationship was noted both in the presence and absence of secretin. Similar linearity was also observed with experiments in which the adenylate cyclase preparation was incubated with glucagon and ACTH (data not shown).

Fig. 7. The dependency of cyclic AMP production upon incubation time. The assays were performed under standard conditions with 0.102 mg protein/ml, in the absence (•) or presence (o) of 20 µg/ml secretin.

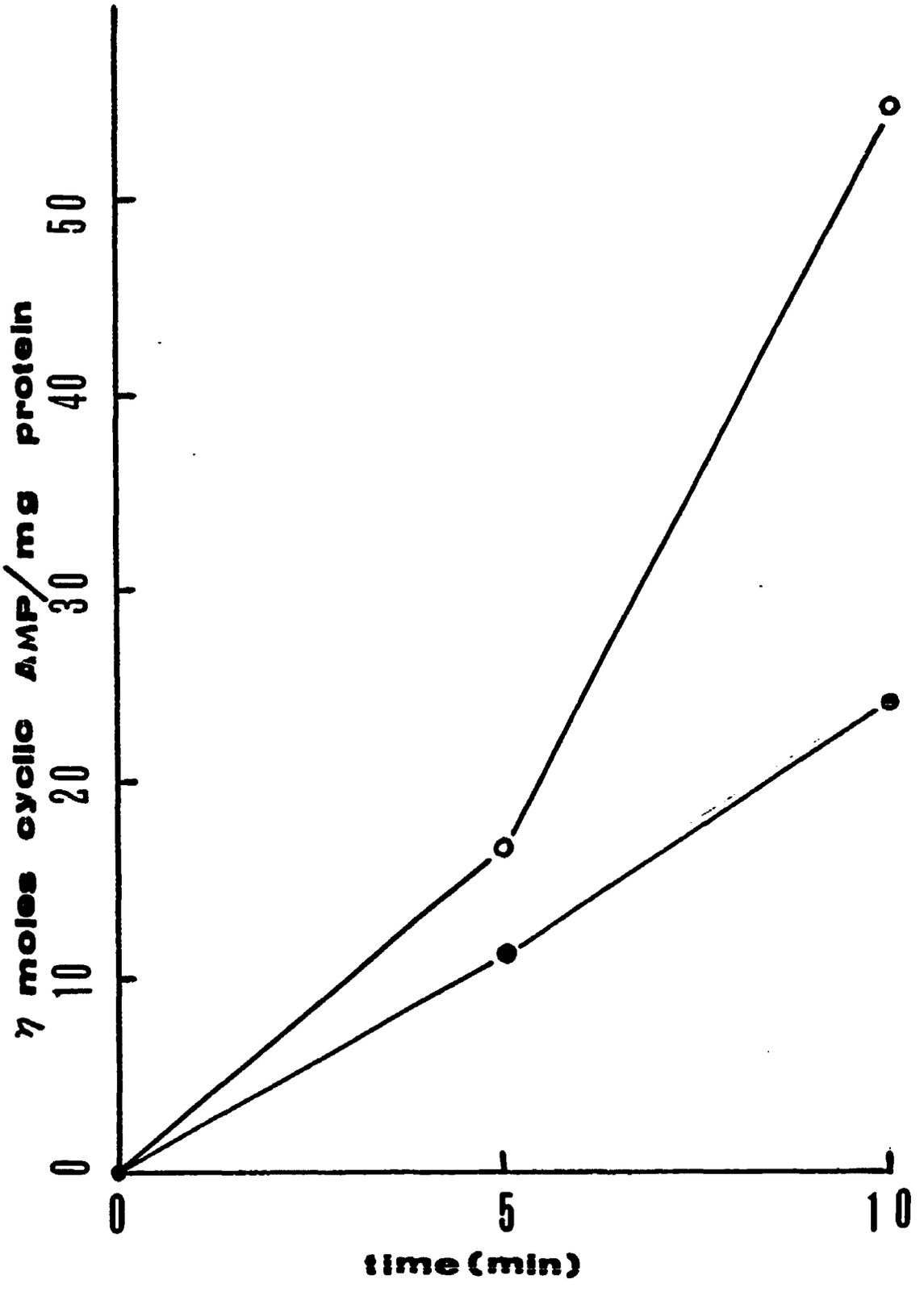

Fig. 7 - The dependency of cyclic AMP-production upon incubation time.

Fig.8 . The dose-dependency of cyclic AMP production as a function of the amount of adenylate cyclase. The assays were performed under the standard conditions except for the variation of enzyme amount, in the absence (•) and presence (o) of secretin (20 μg/ml). Each point shown represents mean of the values obtained with triplicate samples.

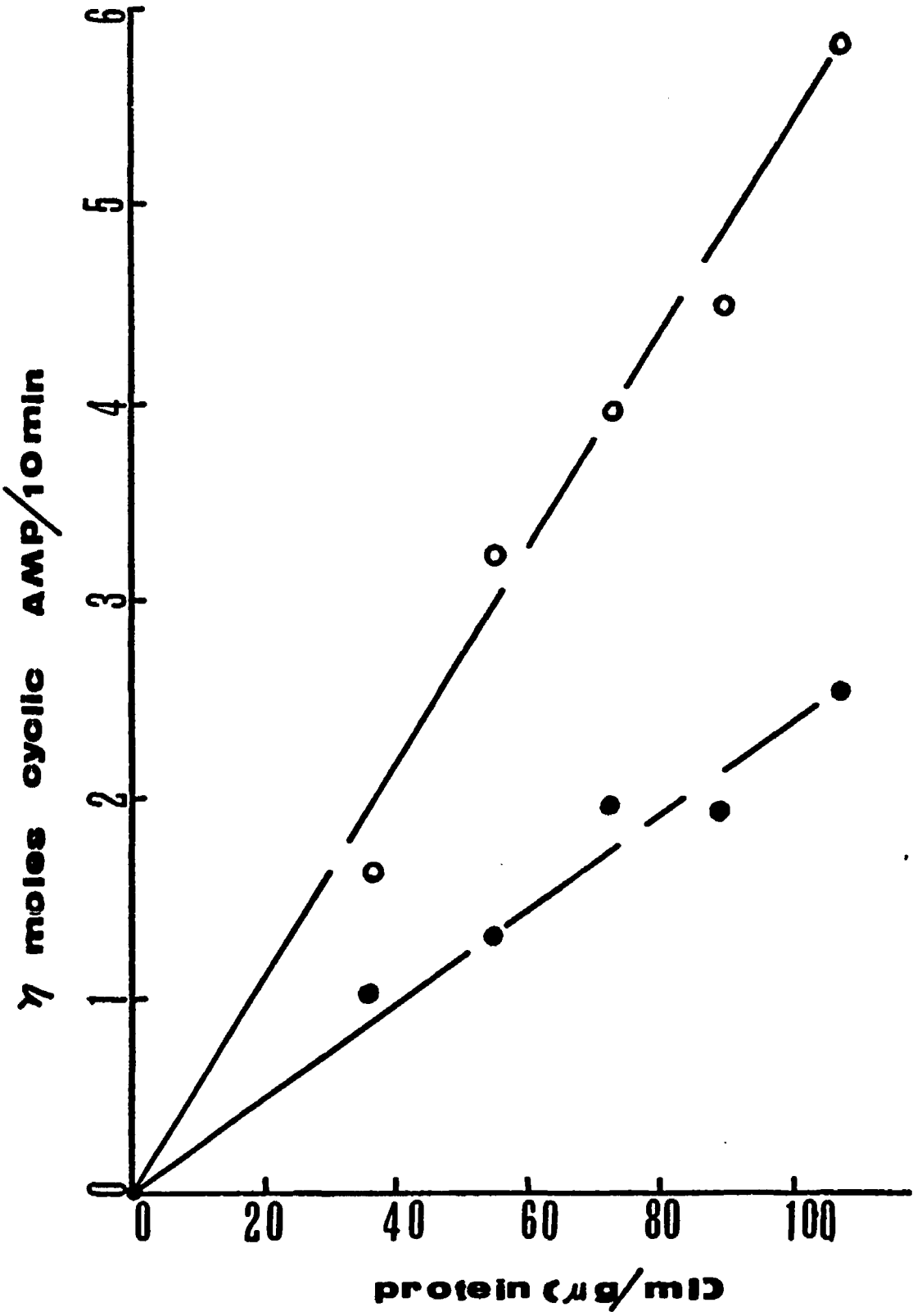

Fig. 8 - The dose-dependency of cyclic AMP production as a function of the amount of adenylate cyclase.

B. The Effect of Polypeptide Hormones

The main purpose of the present study was to ascertain the relationship between the activation of islet adenylate cyclase and the stimulation of insulin secretion. Glucagon, previously found to stimulate insulin secretion in isolated islets (40) and other pancreatic tissue preparations (41, 42), stimulated islet adenylate cyclase. Maximal stimulation of islet adenylate cyclase was found to occur at about 30 μM glucagon (Fig. 9) compared with 2 μM for adenylate cyclase of adipocytes (43) and 0.1 μM for that of liver cells (44). Turtle and Kipnis (23), using intact rat islets, have reported that glucagon, in the presence of theophylline, causes an increase in islet cyclic AMP content. Their results are consistent with our observations, although the intact cells are apparently more sensitive to hormone than is the adenylate cyclase of islet homogenates.

In view of the recently published observations by Rodbell et al. (45) that guanosine phosphates augment the effect of glucagon in stimulating liver cell adenylate cyclase. We studied the effects of GTP and GDP on the islet enzyme. While glucagon alone (1 μM) had virtually no effect on enzyme activity and 5 μM GDP alone gave a 51% stimulation, the combination of the two agents resulted in a 118% stimulation (Table 2). A ten fold increase in GDP concentration (50 μM) resulted in a 146% stimulation of adeny-

Fig. 9. The effect of glucagon upon islet adenylate cyclase activity. The assays were performed under standard condition at the indicated glucagon concentrations. The basal level of activity (with no added glucagon), which amounts to 2.3 nmoles cyclic AMP produced per min per mg islet protein, was normalized to 100. Each point shown represents a value obtained from a single incubation.

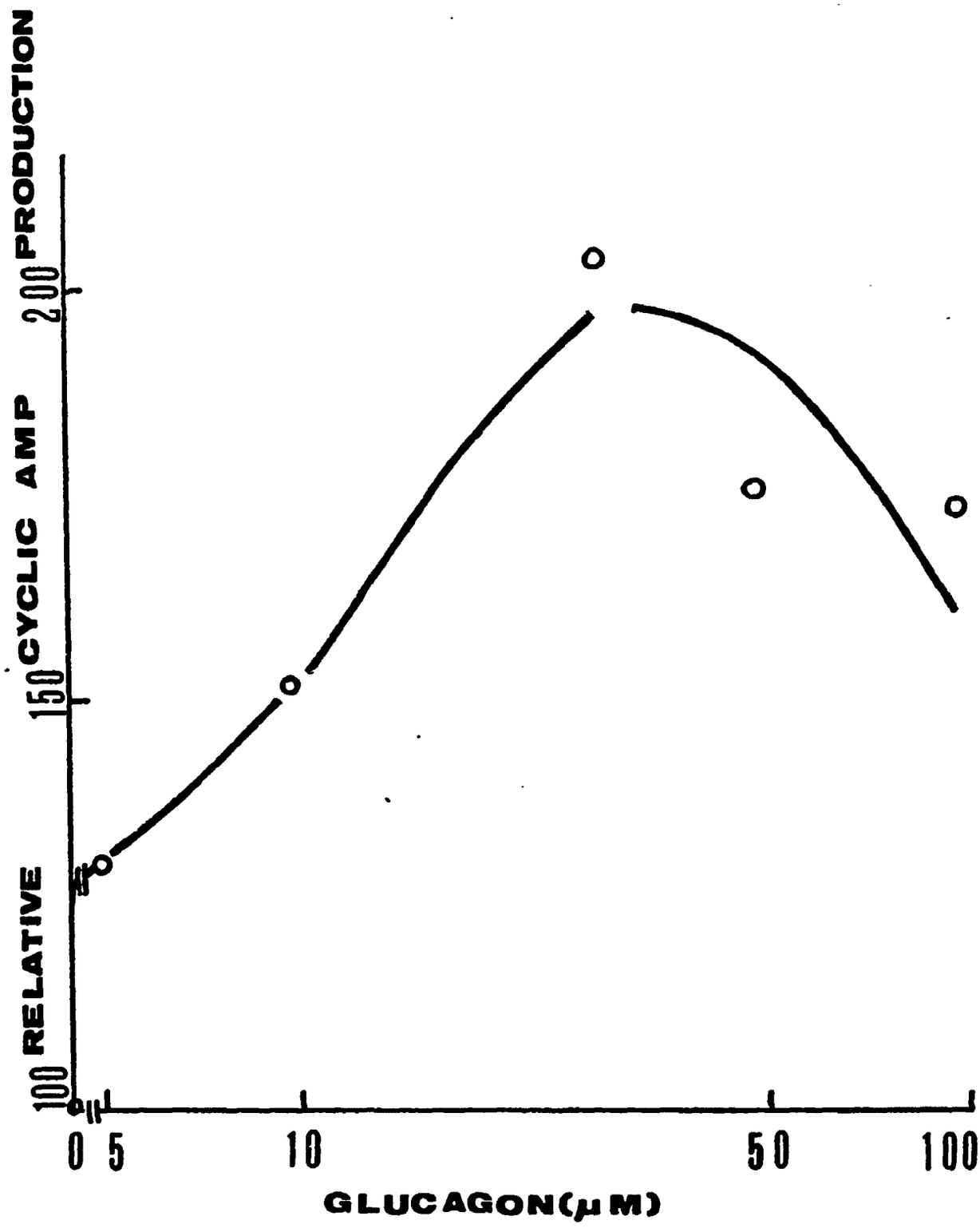

Fig.9 -The effect of glucagon upon islet adenylate cyclase activity.

Table 2. Effects of glucagon, GDP, GTP, and EGTA, present singly or in combination on the islet adenylate cyclase activity. The incubation conditions were as described under "Experimental Procedure". The control (without added agent) value, represents 2.01 ± 0.05 nmoles cyclic AMP per min per mg islet protein. Each value shown represents mean ± standard deviation of triplicate samples.

TABLE 2

EFFECTS OF GLUCAGON, GDP, GTP, AND EGTA, PRESENT SINGLY OR IN COMBINATION ON THE ISLET ADENYLATE CYCLASE ACTIVITY

Addition and concentration	Relative cyclic AMP production
None (control)	100.0 ± 2.5
Glucagon (5.0 μM)	129.2 ± 4.3
EGTA (1.0 mM)	121.0 ± 4.6
Glucagon (5.0 μM) + EGTA (1.0 mM)	251.9 ± 8.2
Glucagon (1 μM)	100.0 ± 2.1
GDP (5 μM)	151.2 ± 4.2
Glucagon (1 μM) + GDP (5 μM)	218.5 ± 8.9
GDP (50 μM)	246.8 ± 6.7
GTP (5 μM)	218.9 ±16.6
Glucagon (1 μM) + GTP (5 μM)	181.5 ±20.1

late cyclase activity in the absence of glucagon. GTP, at a concentration of 5 μM, stimulated the enzyme activity 119%, whereas in the presence of 1 μM glucagon, the stimulation was reduced to 81%. Rodbell et al. (45, 46) found that in liver the glucagon-stimulated adenylate cyclase activity is further augmented by both GDP and GTP under their experimental conditions.

At a concentration of 1 mM, the calcium chelating agent EGTA stimulated the basal activity of islet adenylate cyclase by 21%. It was found to synergistically enhance the stimulatory effects of 5 μM glucagon from 20% in its absence to 152% in its presence (Table 2). Similar results have been obtained by Hepp et al. (47) with liver adenylate cyclase. They postulated that the EGTA stimulation is due to the chelation of inhibitory membrane-bound calcium ions.

Other polypeptide hormones were also tested for their effects on islet adenylate cyclase (Table 3). The two gut hormones, secretin (also see Figs. 7 and 8) and pancreozymin, were both found to be effective stimulants. These hormones have been reported to stimulate insulin release from pieces of rabbit pancreas (48, 49), but are ineffective in stimulating insulin release from isolated rat islets (50) and other pancreatic tissue preparations (51, 52). Neither of the hormone preparation used in the

experiments was pure; the secretin (Sigma, Type II) containing 25-30 units of pancreozymin per 100 units of secretin, and the pancreozymin (Sigma, Grade II), containing about 10% secretin. The results obtained with the two hormone preparations could be due to the action of either one of them, or of both. Further elucidation of this point requires the use of pure hormone preparations.

ACTH activated islet adenylate cyclase to a greater extent than did any other hormone (Table 3). This hormone has been found to enhance insulin secretion from isolated rat pancreas (22, 42) and increase plasma insulin levels in mice (53). TSH, previously shown to augment the glucose-induced insulin secretion from pieces of rat pancreas (42), was also found to activate islet adenylate cyclase. Somatotropin also increased the activity of islet adenylate cyclase. This hormone has been found to elevate canine serum levels of insulin *in vivo* (54), but has not been shown to increase insulin secretion *in vitro* (31, 55). Luteinizing hormone was not active at the concentration tested. Insulin at 20 μg per ml significantly inhibited islet adenylate cyclase, suggesting that insulin may regulate the islet cyclic AMP levels (and thus insulin secretion from the organelles) through a feed-back control mechanism.

Table 3. Effects of polypeptide hormones on islet adenylate cyclase activity. Experimental conditions were as under "Experimental Procedure". The control (without added agent) value, represents 2.35 ± 0.11 nmoles cyclic AMP produced per min per mg islet protein. Each value shown represents mean ± standard deviation of triplicate sample, except that the value obtained with luteinizing hormone was from a simple sample.

TABLE 3

EFFECTS OF POLYPEPTIDE HORMONES ON ISLET ADENYLATE CYCLASE ACTIVITY

Additions and concentrations	Relative cyclic AMP production
None (control)	100.0± 4.7
ACTH (46 I.U./ml)	361.3±23.2
ACTH (23 I.U./ml)	508.9±16.3
Somatotropin (100 µM)	181.8± 6.6
Somatotropin (50 µM)	224.3±14.3
TSH (20 µg/ml)	204.2±14.9
Pancreozymin (20 µg/ml)	186.7± 4.7
Luteinizing Hormone (20 µg/ml)	101.2
Insulin (20 µg/ml)	74.8± 6.1
Insulin (10 µg/ml)	87.7± 8.2

C. Effects of Amino Acids, Alcohol, Acetylcholine and NaF

Several amino acids have been found to stimulate insulin release from pieces of rabbit pancreas. Milner (56) has shown, for example, that arginine enhances glucose-induced insulin secretion and that leucine, even in the absence of glucose, causes insulin release. Theophylline was found to enhance leucine-induced but not arginine-induced secretion. In the present study, neither of these amino acids were found to stimulate islet adenylate cyclase (Table 4). In fact, they slightly inhibited enzyme activity, suggesting that cyclic AMP may not mediate their insulinogenic effect.

Ethanol was found to stimulate islet adenylate cyclase (Table 4). The lowest level tested (0.05% or 8.6 mM) was found to give a 59% stimulation, whereas the stimulation was reduced at higher ethanol concentrations. Various alcohols have been found to stimulate adenylate cyclase of both rat liver and hamster insulinoma (insulin producing islet adenoma) (57). Ethanol, for example, was found to stimulate the hepatic enzyme but was not tested with the insulinoma. 2-Propanol, which stimulated insulinoma adenylate cyclase, also enhanced the glucagon response of this enzyme. Clinical observations have indicated that ethanol administration (oral or intravenous) results in an increase in the plasma insulin response to

Table 4 . Effects of leucine, ethanol and acetylcholine on islet adenylate cyclase activity. The incubation conditions were as described under "Experimental Procedure". The control (without added agent) value, represents 1.88 ± 0.07 nmoles cyclic AMP produced per min per mg islet protein. Each value shown represents mean ± standard deviation of triplicate samples.

TABLE 4

EFFECTS OF LEUCINE, ETHANOL AND ACETYLCHOLINE ON ISLET ADENYLATE CYCLASE ACTIVITY

Addition and concentration	Relative cyclic AMP production
None (control)	100.0 ± 3.7
L-leucine (5.0 mM)	73.1 ± 3.3
L-leucine (2.5 mM)	80.5 ± 3.6
L-arginine (5.0 mM)	50.8 ± 3.2
L-arginine (2.5 mM)	64.7 ± 1.9
Ethanol, 0.5% (v/v)	105.9 ± 2.9
Ethanol, 0.1% (v/v)	128.7 ± 5.1
Ethanol, 0.05% (v/v)	159.5 ± 2.6
Acetylcholine (0.1 mM)	279.1 ±12.3
NaF (10.0 mM)	405.5 ±28.7

glucose. Ethanol alone, however, did not cause an elevation of plasma insulin levels (58, 59). Thus, ethanol stimulation of islet adenylate cyclase may enhance glucose-initiated insulin secretion.

Cholinergic drugs have been found to stimulate the release of insulin from slices of rat pancreas (60, 61). Acetylcholine has been shown to stimulate the release of insulin from isolated rat islets in the presence of suboptimal glucose levels (1.5 mg/ml) (40). The finding (Table 4) that acetylcholine stimulates islet adenylate cyclase is consistent with these observations. To the author's knowledge, adenylate cyclase stimulation by acetylcholine has not been found with other tissues. Sodium fluoride greatly stimulated islet adenylate cyclase (Table 4), as noted with many other tissue preparations.

D. Effects of Glucose and Hypoglycemic Drugs

Glucose was found to have a slight inhibitory effect upon islet adenylate cyclase at 0.5-5.0 mM (Table 5), concentrations known to initiate insulin secretion (44). The data from the present study are similar to those reported by Atkins and Matty with mouse islets (62). These results are consistent with an intracellular mechanism of secretory stimulation by this metabolite, especially since glucose freely permeates islet cells (63, 64).

Tolbutamide, 1-butyl-3-(p-tolylsulfonyl) urea, a

Table 5. Effects of glucose and hypoglycemic drugs on islet adenylate cyclase activity. Experimental conditions were as described under "Experimental Procedure". The control (without added agent) value, represents 2.26 ± 0.06 nmoles cyclic AMP produced per min per mg islet protein. Each value shown represents mean ± standard deviation of triplicate samples.

TABLE 5

EFFECTS OF GLUCOSE AND HYPOGLYCEMIC DRUGS ON ISLET ADENYLATE CYCLASE ACTIVITY

Addition and concentration (mM)	Relative cyclic AMP production
None (control)	100.0 ± 2.7
D-glucose (5.0 mM)	94.3 ± 2.4
D-glucose (1.0 mM)	97.8 ± 2.7
D-glucose (0.5 mM)	100.1 ± 2.5
Tolbutamide (0.5 mM)	273.7 ±20.1
Tolbutamide (0.1 mM)	234.6 ±26.2
Phenformin (0.1 mM)	105.6 ± 2.3

common clinically utilized drug for the treatment of diabetes, was found to effectively stimulate islet adenylate cyclase; 0.1 and 0.5 mM concentrations of the hypoglycemic drug gave 135 and 174% stimulation, respectively (Table 5). This drug has been found to stimulate insulin secretion both *in vitro* with isolated rat islets (65) and *in vivo* (66, 67). Levey *et al.* have recently reported that tolbutamide stimulates adenylate cyclase of rabbit heart (68) and islet cell adenoma (69). Tolbutamide has also been shown to inhibit phosphodiesterase activity in an insulin-secreting islet cell tumor of Syrian golden hamster (70) and increase cyclic AMP level in fat cells (71). However, Hellman *et al.* (72) have presented evidence that tolbutamide does not enter the β-cells and therefore must have an extracellular site of action.

Another hypoglycemic drug, phenformin (1-phenethylbiguanidine), thought to act intracellularly and differently from tolbutamide (73), had no effect on islet adenylate cyclase (Table 5).

E. Effects of Prostaglandins and Adrenergic Agents

Prostaglandins E_1, E_2, A_1, and $F_{1\alpha}$, each tested at a concentration of 20 µg/ml, significantly enhanced the production of cyclic AMP (Table 6). *In vivo* insulin secretion is induced by the prostaglandins (74), although prostaglandin E_1 (1 µg/ml), in the presence of 1.5 mg/ml

glucose, fails to stimulate the secretion of insulin by isolated rat islets (40).

Atkins and Matty (62) have presented evidence that epinephrine and isoproterenol, in the presence of phentolamine (an α-adrenergic blocker), increase adenylate cyclase activity of microdissected mouse islet tissue. They reported that the stimulation by epinephrine in the presence of α-blocker is inhibited by propranolol (a β-adrenergic blocker). They suggested that stimulation of islet adenylate cyclase is a β-adrenergic function while inhibition of the cyclase activity is an α-adrenergic function.

Montague and Cook (44) have reported that epinephrine inhibits glucose-induced insulin secretion and lowered the intracellular cyclic AMP content of isolated islets. Epinephrine and norepinephrine were also shown to inhibit insulin secretion in vitro with a hamster pancreas preparation (75). Turtle and Kipnis (23) have observed that epinephrine plus phentolamine, but not epinephrine plus propranolol, increses of cyclic AMP levels in intact islets.

In the present studies with rat islet adenylate cyclase, epinephrine and norepinephrine were both found to inhibit its activity by about 14-17%; this inhibition was increased to about 35-37% in the presence of KÖ 592, a β-adrenergic blocking agent (Table 6). Conversely, both catecholamines, in the presence of phentolamine, activated

Table 6. Effects of epinephrine, norepinephrine, isoproterenol, KÖ 592, phentolamine, and prostaglandins, present singly or in combination on islet adenylate cyclase activity. Experimental conditions were as described under "Experimental Procedure". The control (without added agent) value, represents 2.14 ± 0.04 nmoles cyclic AMP synthesized per min per mg islet protein. Each value shown represents the mean ± standard deviation of triplicate samples.

TABLE 6

EFFECTS OF EPINEPHRINE, NOREPINEPHRINE, ISOPROTERENOL, KÖ 592, PHENTOLAMINE, AND PROSTAGLANDINS ON ISLET ADENYLATE CYCLASE ACTIVITY

Addition and concentration	Relative cyclic AMP production
None (control)	100.0 ± 1.9
DL-epinephrine (0.1 mM)	83.0 ± 3.6
DL-epinephrine (0.1 mM) + KÖ 592 (0.1 mM)	63.2 ± 8.0
DL-epinephrine (0.1 mM) + phentolamine (0.1 mM)	446.6 ±19.8
L-norepinephrine (0.1 mM)	86.1 ± 4.8
L-norepinephrine (0.1 mM) + KÖ 592 (0.1 mM)	65.1 ± 6.7
L-norepinephrine (0.1 mM) + phentolamine (0.1 mM)	394.5 ±11.5
L-isoproterenol (0.1 mM)	452.1 ±27.7
L-isoproterenol (0.1 mM) + KÖ 592 (0.1 mM)	151.6 ±25.0
PG E_1 (20 µg/ml)	212.2 ±32.7
PG E_2 (20 µg/ml)	167.1 ±18.5
PG A_1 (20 µg/ml)	150.3 ±16.8
PG $F_{1\alpha}$ (20 µg/ml)	160.7 ±28.4

adenylate cyclase by about 300%. Isoproterenol alone greatly activated the enzyme and this activation was largely abolished by KÖ 592, indicating that the activation by isoproterenol was characteristically a β-effect.

F. Effects of 3', 5'-Cyclic Nucleotides

Cyclic AMP, cyclic UMP, and cyclic IMP were found to have inhibitory effects on islet adenylate cyclase at concentrations higher than 2 mM. Cyclic GMP, even when tested at a concentration of 0.1 mM, tremendously inhibited adenylate cyclase activity. It could be due to the inhibition of ATP binding. However, both cyclic TMP and cyclic CMP did not significantly inhibit the production of cyclic AMP (Table 7).

Regulation of Cyclic AMP Levels in Intact Pancreatic Islets

Glucagon, isoproterenol, ACTH, and acetylcholine, agents known to stimulate insulin release, all increased the accumulation of radioactive cyclic AMP in intact rat pancreatic islets incubated in the presence of 10 mM theophylline (Fig. 10). The accumulation of cyclic AMP in all cases was reasonably proportional to the incubation time. It has been shown that these hormones also stimulated adenylate cyclase in islet homogenates. The cyclic AMP level for the control experiment (in which no stimulatory

Table 7. Effects of 3', 5'-cyclic nucleotides on islet adenylate cyclase activity. Experimental conditions were as described under "Experimental Procedure". The control (without added agent) value, represents 1.88 ± 0.99 nmoles cyclic AMP synthesized per min per mg islet protein. Each value shown represents the mean ± standard deviation of triplicate samples.

TABLE 7

EFFECTS OF 3', 5'-CYCLIC NUCLEOTIDES ON ISLET ADENYLATE CYCLASE ACTIVITY

Addition and concentration	Relative cyclic AMP production
None (control)	100.0 ± 4.5
Cyclic AMP (2 mM)	86.6 ± 2.7
Cyclic AMP (3 mM)	67.7 ± 3.1
Cyclic GMP (0.1 mM)	0.0 ± 2.5
Cyclic UMP (2 mM)	75.8 ± 3.4
Cyclic IMP (2 mM)	71.4 ± 9.5
Cyclic IMP (3 mM)	57.9 ±11.0
Cyclic TMP (2 mM)	102.8 ±14.0
Cyclic TMP (3 mM)	92.8 ± 6.4
Cyclic CMP (3 mM)	100.6 ± 5.8

Fig. 10. Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of incubation time in the presence of a high concentration of theophylline. About 20 islets were incubated for varying time intervals in 0.5 ml of Krebs-Ringer bicarbonate buffer containing 10 mM theophylline. The radioactive cyclic AMP found in the zero time sample was about 3200 cpm per mg protein. X, control; □ , glucagon (0.1 mM); ■ , DL-isoproterenol (0.1 mM); ○ , corticotropin (0.05 mM); ● , acetylcholine (0.1 mM).

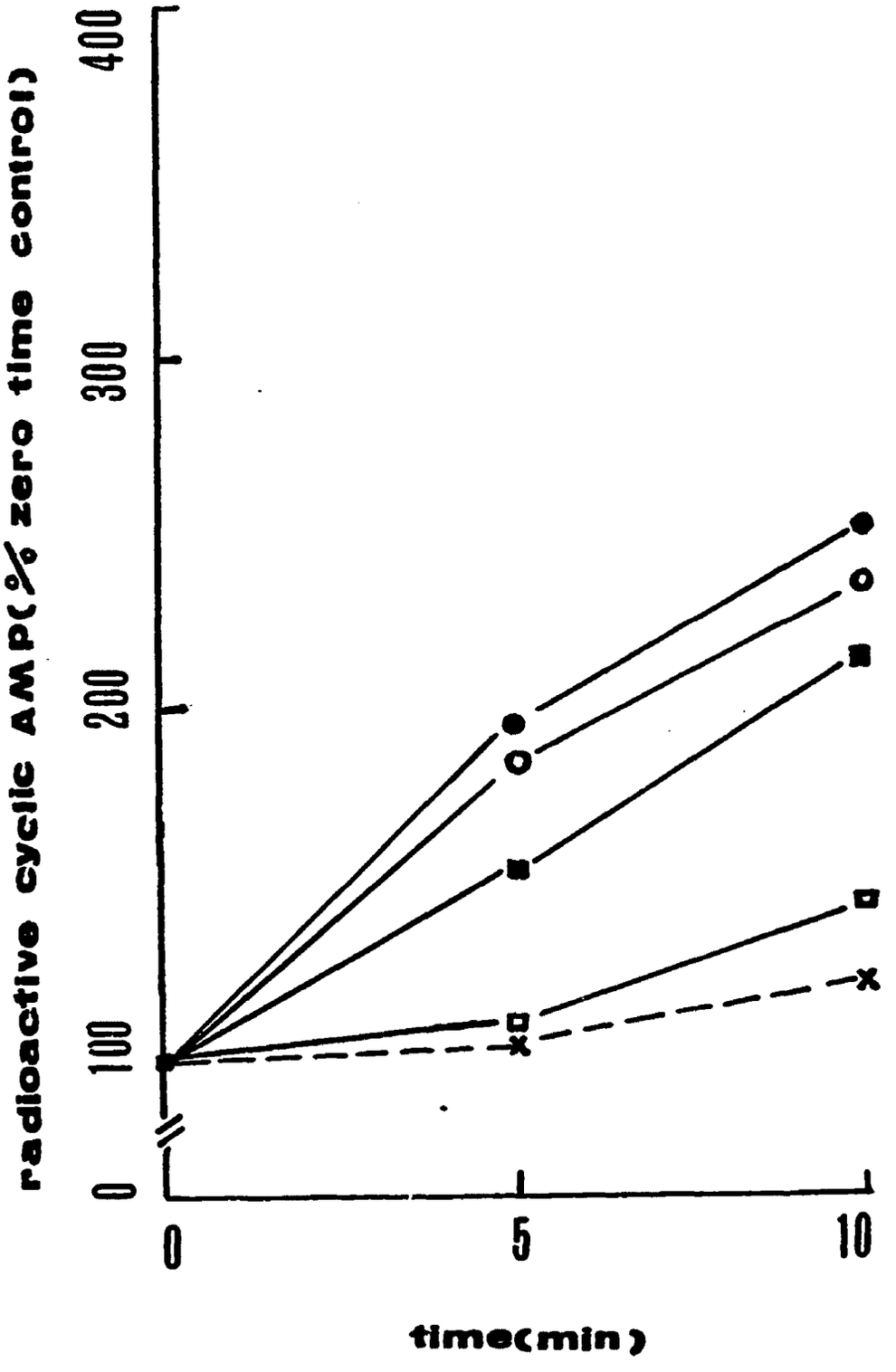

Fig. 10 - Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of incubation time.

agents were added) at the end of the 10-min incubation was higher than the zero-time value. With similar experiments in which the islets were incubated in the presence of 3 mM theophylline, the levels of cyclic AMP found in the control and the hormone-treated islet samples were considerably lower than the zero-time value (Fig. 11), suggesting an extremely high phosphodiesterase activity in rat islets. In order to insure a positive increment in the cyclic AMP level during the incubations. The islets were incubated with 10 mM theophylline in all experiments.

The dose-dependent stimulation by glucagon, acetylcholine, isoproterenol and ACTH of cyclic AMP accumulation in the islets is illustrated in Fig. 12. EGTA, which slightly increased the cyclic AMP level when present alone, acted synergistically with glucagon to greatly increase the cyclic AMP levels, confirming these observations of their synergistic effect on adenylate cyclase activity of islets.

The effects of some other agents on the islet cyclic AMP levels are presented in Table 8. Secretin and prostaglandins (E_1, E_2, A_1 and $F_{1\alpha}$), which have been reported to have an insulin-releasing action (48, 49, 74), were all capable of elevating cellular cyclic AMP levels. Thyroid-stimulating hormone, with an unclear insulinogenic effect, was also active. It is interesting that tolbutamide, an oral hypoglycemic drug, caused an elevation of cyclic AMP in islets. The level of islet cyclic AMP was

Fig. 11. Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of incubation time in the presence of a low concentration of theophylline. About 20 islets were incubated for varying time intervals in 0.5 ml of Kreb-Ringer bicarbonate buffer containing 3 mM theophylline. The radioactive cyclic AMP found in zero time sample was about 3200 cpm per mg protein. X, control; □, glucagon (0.1 mM); ■, DL-isoproterenol (0.1 mM); ○, acetylcoline (0.1 mM).

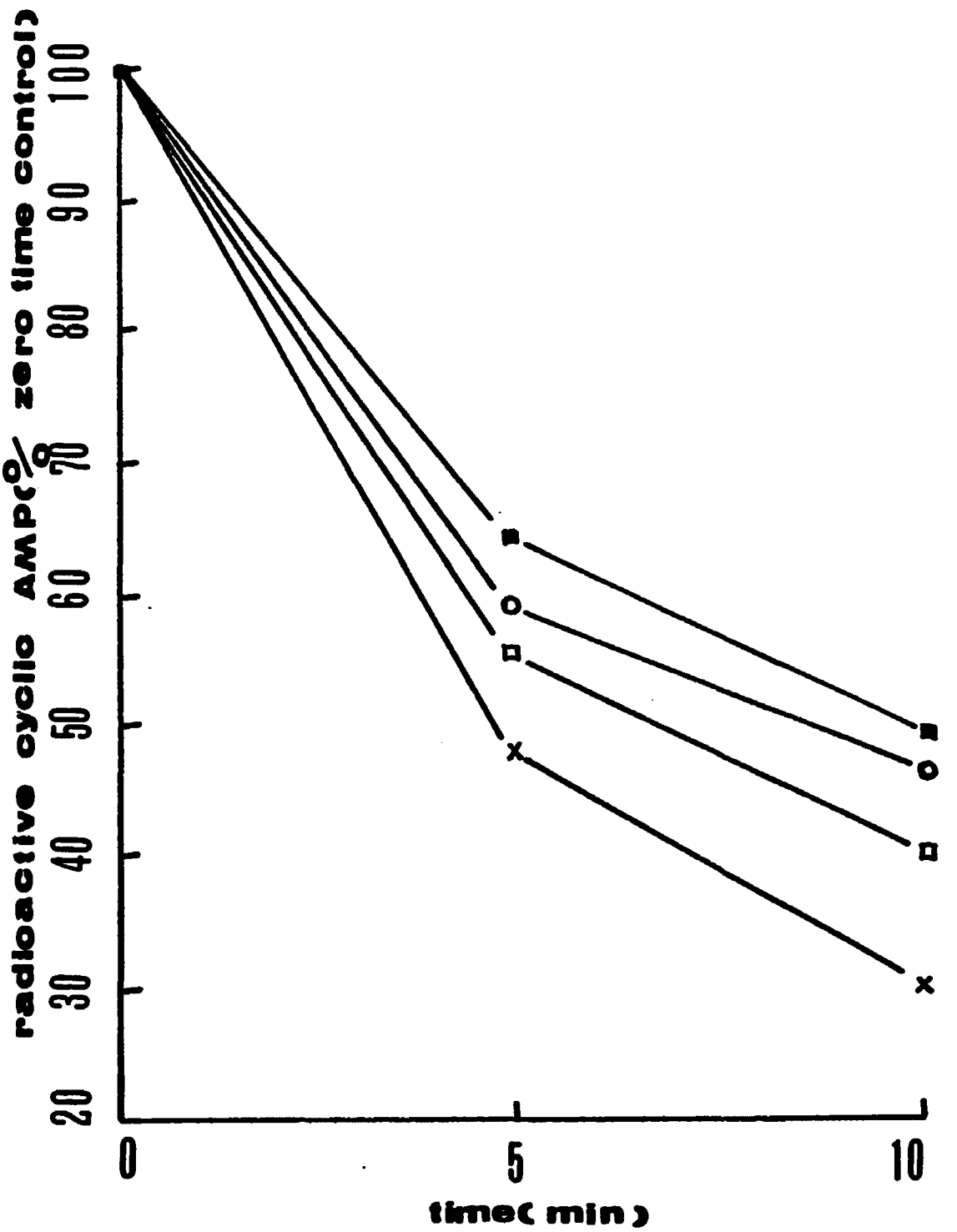

Fig. 11 - Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of incubation time.

Fig.12. Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of hormone concentration. About 20 islets were incubated, in the presence of varying the concentration of hormones, for 10 min in 0.5 ml of Krebs-Ringer bicarbonate buffer containing 10 mM theophylline. The basal value (found in the absence of added hormones) of radioactive cyclic AMP at the end of 10 min incubation was about 3000 cpm per mg protein. ○, glucagon; ●, glucagon + EGTA (2 mM); ⊛, DL-isoproterenol; □, corticotropin; X, acetylcholine.

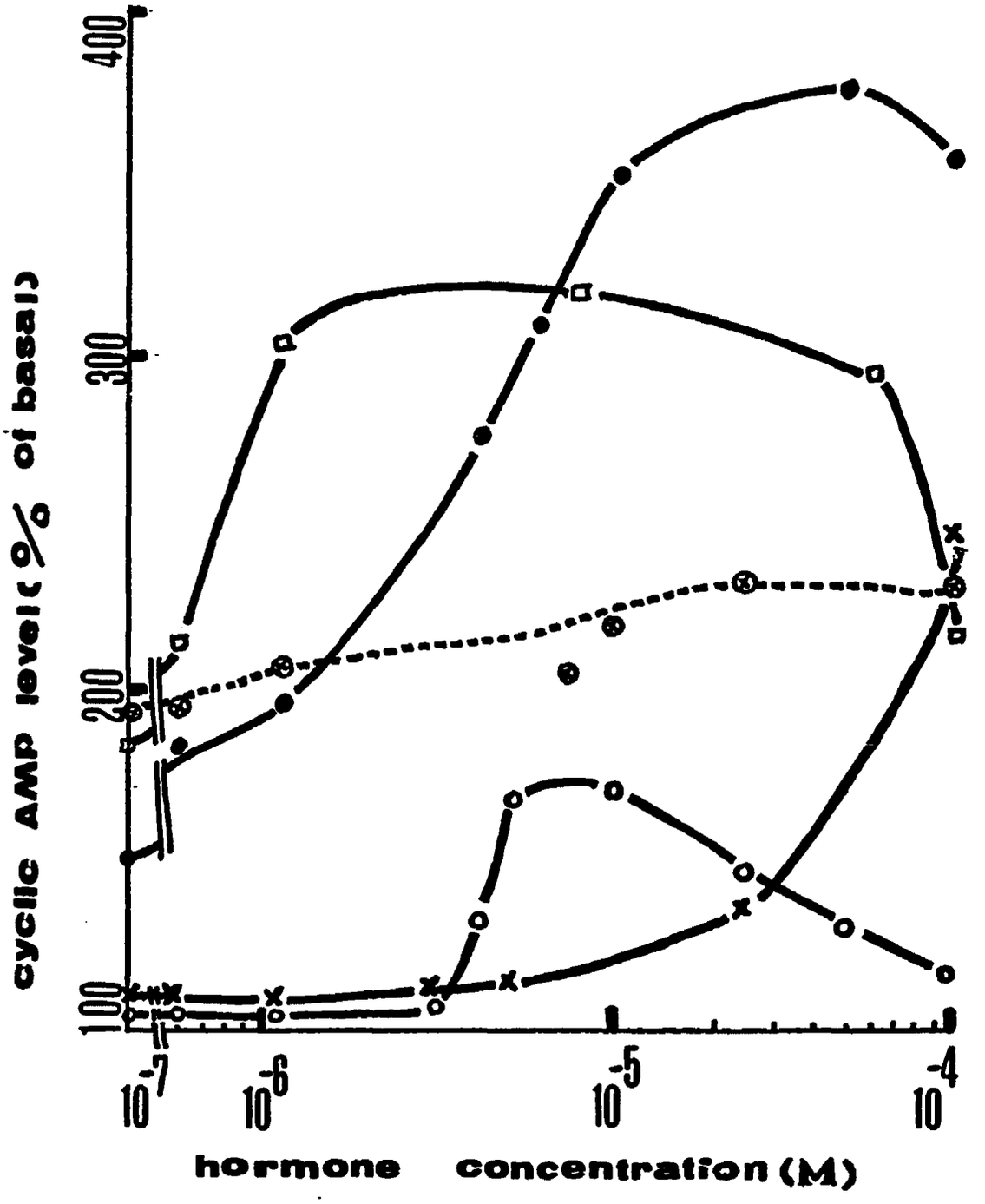

Fig. 12 - Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of hormone concentration.

Table 8 . Effects of prostaglandins, secretin, thyroid-stimulating hormone, tolbutamide, leucine and glucose on cyclic AMP levels in pancreatic islets. About 70 islets were incubated in 2.5 ml of Krebs-Ringer bicarbonate buffer containing 10 mM theophylline and various additives, at 37° for 10 min, with shaking. Each incubation was performed at least in duplicate and the mean and the individual value (in parentheses) are presented.

TABLE 8

EFFECTS OF PROSTAGLANDINS, SECRETIN, TSH, TOLBUTAMIDE, LEUCINE AND GLUCOSE ON CYCLIC AMP LEVELS IN PANCREATIC ISLETS

Additive	Cyclic AMP found
	cpm x 10^2/mg protein
None (control)	21.3 (22.0;20.5)
Prostaglandin E_1 (10 µg/ml)	61.9 (57.7;66.0)
(20 µg/ml)	72.0 (66.2;78.0;81.8;61.8)
(30 µg/ml)	79.9 (85.0;74.8)
Prostaglandin E_2 (20 µg/ml)	26.5 (23.4;29.6)
(30 µg/ml)	81.0 (77.1;84.9)
Prostaglandin A_1 (20 µg/ml)	49.3 (38.3;60.3)
(30 µg/ml)	62.4 (70.1;54.7)
Prostaglandin $F_{1\alpha}$ (20 µg/ml)	96.3 (86.3;106.2)
(30 µg/ml)	46.8 (44.3;49.2)
Secretin (20 µg/ml)	43.1 (40.7;44.2)
TSH (20 µg/ml)	35.9 (32.2;39.6)
Tolbutamide (0.1 mM)	37.5 (27.5;49.3;40.2;33.0)
Leucine (5 mM)	23.4 (22.6;24.3)
Glucose (5 mM)	21.5 (22.1;20.8)

not significantly increased by leucine or glucose, suggesting that the action of these metabolites to stimulate insulin secretion may not be mediated through a mechanism involving cyclic AMP. The effects of these agents on islet cyclic AMP levels confirmed the findings previously made with adenylate cyclase in the islet homogenates.

It has been shown (23, 62) that rat pancreatic islets contain both α-and β-adrenergic receptors. Interaction of agents with the α-receptor causes a reduction in adenylate cyclase activity and consequently a decrease in cellular cyclic AMP. Interaction of agents with the β-receptor, on the other hand, results in an activation of adenylate cyclase and an increase in cyclic AMP levels. The effect of isoproterenol in increasing islet cyclic AMP level was effectively antagonized by all four of the β-adrenergic blockers (propranolol, MJ 1999, KÖ 592, and D-INPEA) tested (Table 9). It is clear that activation of adenylate cyclase and the consequent stimulation of insulin secretion (76) by isoproterenol is a typical β-adrenergic effect. Epinephrine and norepinephrine caused reductions in islet cyclic AMP levels (Table 10), in agreement with the known effect of these hormones in inhibiting insulin release (31, 44). In the presence of α-adrenergic blocking agents, such as phenoxybenzamine and phentolamine, the inhibition by these two hormones was overcome, and pronounced increases in cyclic AMP due to these hormones were ulti-

Table 9. Inhibition by β-adrenergic blocking agents of increase in islet cyclic AMP. About 90 islets were incubated in 2.5 ml of Krebs-Ringer buffer containing 10 mM theophylline and various additives at 37°, with shaking, for 10 min. Each incubation was performed in duplicate and the mean and the individual values (in parentheses) are presented.

TABLE 9

INHIBITION BY β-ADRENERGIC BLOCKING AGENTS OF INCREASE IN ISLET CYCLIC AMP

Additive	Cyclic AMP found	Effect of agent
	cpm x 10^2/mg protein	% control
None (control)	19.5 (20.6; 18.9)	100
DL-Isoproterenol (0.2 mM)	417.3 (344.5; 490.0)	2141
DL-Isoproterenol (0.2 mM) + DL-propranolol (0.08 mM)	98.1 (100.0; 96.1)	503
DL-Isoproterenol (0.2 mM) + MJ 1999 (20 µg/ml)	101.7 (93.6; 109.8)	522
DL-Isoproterenol (0.2 mM) + KÖ 592 (0.1 mM)	19.8 (21.5; 18.1)	102
DL-Isoproterenol (0.2 mM) + D-INPEA (0.4 mM)	108.7 (83.9; 133.4)	557

Table 10. Comparison of effect of epinephrine and norepinephrine on islet cyclic AMP levels in the absence and presence of α-adrenergic blocking agents. About 65 islets were incubated in 2.5 ml Krebs-Ringer bicarbonate buffer containing 10 mM theophylline. Each incubation was performed in duplicate and the mean and the individual values (in parentheses) are presented.

TABLE 10

COMPARISON OF EFFECT OF EPINEPHRINE AND NOREPINEPHRINE ON ISLET CYCLIC AMP LEVELS IN THE ABSENCE AND PRESENCE OF α-ADRENERGIC BLOCKING AGENTS

Additive	Cyclic AMP found
	cpm x 10^2/mg protein
None (control)	20.1 (19.0;21.2)
DL-Epinephrine (0.05 mM)	1.9 (3.4;0.4)
DL-Epinephrine (0.05 mM) + Phenoxybenxamine (0.03 mM)	89.9 (79.8;99.9)
DL-Epinephrine (0.05 mM) + Phentolamine (0.04 mM)	87.3 (82.2;92.4)
L-Norepinephrine (0.05 mM)	5.3 (8.0;2.5)
L-Norepinephrine (0.05 mM) + Phenoxybenzamine (0.03 mM)	26.9 (30.7;23.0)
L-Norepinephrine (0.05 mM) + Phentolamine (0.04 mM)	101.5 (87.5;115.5)

mately noted. It seems that the action of epinephrine and norepinephrine in the absence of phenoxybenzamine and phentolamine is predominantly an α-adrenergic effect, while the action of these hormones becomes a typically β-adrenergic one in the presence of the α-adrenergic blockers. The results obtained with the present study employing intact islets confirmed both the findings of Turtle and Kipnis (23) with intact islets, and the studies of islet adenylate cyclase.

It has been shown that GTP enhances glucagon-stimulated adenylate cyclase activity in rat liver plasma membrane preparations (45) and rat islet homogenates, and that GTP itself also stimulates the enzyme activity in these two systems (45). In the present study with intact islet cells, GTP was found to cause increases in islet cyclic AMP levels; the stimulation seen with 10 μM GTP being much higher than that seen with 5 μM GTP (Table 11). The stimulatory effect of 5 μM GTP and 5 μM glucagon was additive. The stimulation due to 10 μM GTP, on the other hand, was inhibited by 10 μM glucagon. The cyclic AMP level was, however, still higher in the presence of both GTP and glucagon than in the presence of glucagon alone, the results shown here with 10 μM GTP are the same is seen with 5 μM GTP.

Most of the agents whose effects on islet cyclic AMP levels were studied with the prelabeling method (which measures the relative cyclic AMP levels) were also re-

TABLE 11

STIMULATORY EFFECT OF GLUCAGON AND GTP, PRESENT SINGLY OR IN COMBINATION, ON THE ACCUMULATION OF CYCLIC AMP IN PANCREATIC ISLETS

Additive	Cyclic AMP found	Effect of agent
	cpm/mg protein	% control
None (control)	19.7 (19.2;20.1)	100
Glucagon (5 μM)	47.8 (43.2;52.3)	243
GTP (5 μM)	25.4 (21.8;28.9)	129
Glucagon (5 μM) + GTP (5 μM)	57.7 (57.2;58.2)	293
Glucagon (10 μM)	40.9 (43.6;38.2)	208
GTP (10 μM)	310.4 (300.1;320.7)	1576
Glucagon (10 μM) + GTP (10 μM)	73.7 (67.1;80.2)	374

About 80 islets were incubated in 2.5 ml of Krebs-Ringer bicarbonate buffer containing 10 mM theophylline and various additives, at 37° for 10 min, with shaking. Each incubation was performed in duplicate and the mean and the individual values (in parentheses) are presented.

examined with the protein kinase catalytic method (which measures the absolute cyclic AMP levels) (Fig. 13). The results, presented in Table 12, confirm all the findings made with the prelabeling method. It is worth mentioning that EGTA, showing a small stimulatory effect itself, synergistically enhanced the stimulation evoked by glucagon. The potentiation of glucagon action by EGTA was also seen with the prelabeling experiments (Fig. 12), and also with adenylate cyclase in islet homogenates. The basal level of cyclic AMP in isolated islets (49.0 pmoles/mg protein, see footnote to Table 12), about 3-to 10-fold higher than the value found in many other tissues (33), was comparable to that reported by Turtle and Kipnis (23). Enhancement of intracellular cyclic AMP levels were as follows: DL-isoproterenol, 491%; acetylcholine, 203%; corticotropin, 330%; EGTA, 41%; glucagon + EGTA, 375%; glucagon, 130%; ouabain, 214%; prostaglandin E_1, 825%; TSH, 134%. The extracellular levels of cyclic AMP from the same islet incubation were also measured (Table 12). Some parallelism between the intra- and extra-cellular cyclic AMP levels as influenced by various agents was noted.

Separation of Cyclic AMP from Other Nucleotides

The column technique (Table 13) utilized was slightly different from that of Krishna *et al.* (38). Theophylline was separated so well from cyclic AMP that it could not in-

Fig. 13. Standard curve for the measurement of cyclic AMP with a partially purified preparation of bovine heart cyclic AMP dependent protein kinase. Assay conditions were as described under "Experimental Procedure". The activity of the heart enzyme was proportional to cyclic AMP amount within 10 pmoles.

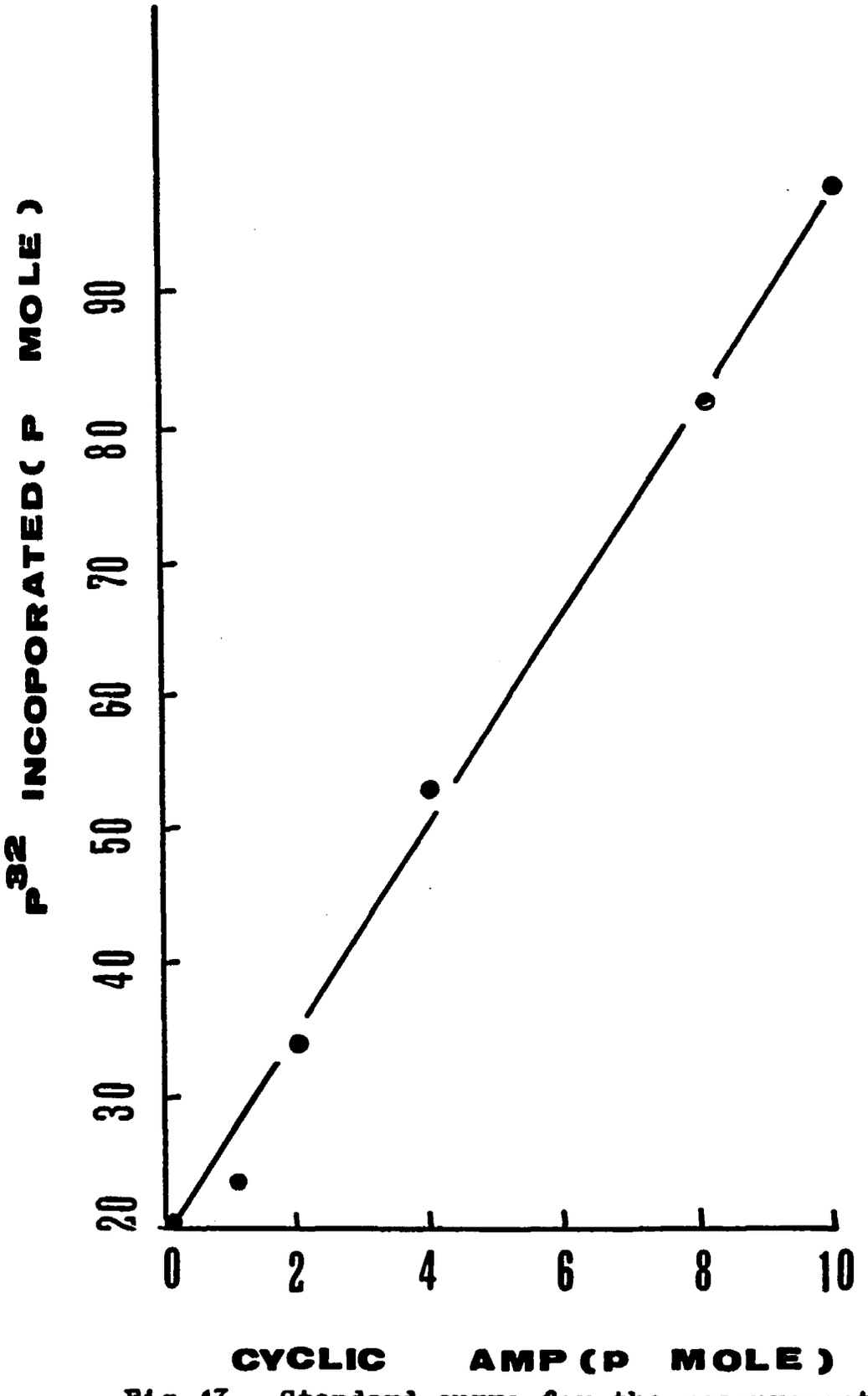

Fig. 13 - Standard curve for the measurement of cyclic AMP with cyclic AMP dependent protein kinase.

Table 12. Effects of various agents on cyclic AMP levels in pancreatic islets. Isolation and incubation of islets and measurements of the amount of total cyclic AMP by the protein kinase catalytic method were as described under "Experimental Procedure". About 225 islets were present in each incubation tube of 5 ml Krebs-Ringer bicarbonate buffer containing 10 mM theophylline. Each tube was incubated for 10 min at 37° in a shaking water bath. Each incubation was performed in duplicate and the mean is presented. Each sample from the individual incubations was assayed for cyclic AMP in triplicate. Values presented in the parentheses represent the means of such triplicate assays.

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[a]Absolute level found in islets after 10 min incubation.

[b]Values corrected for cyclic AMP level found in islets before incubation (zero time control), which amounted to 49.0 pmoles/mg protein.

[c]Absolute level found in the incubation medium (free of islets) after 10 min incubation.

TABLE 12

EFFECTS OF VARIOUS AGENTS ON CYCLIC AMP LEVELS IN PANCREATIC ISLETS

Agent and concentration	Intracellular cyclic AMP		Extracellular cyclic AMP (total)[c]
	Total[a]	Change[b]	
	pmoles/mg islet protein		pmoles/mg islet protein
None (control)	61.9 (60.0;63.7)	12.9	4.4
DL-Isoproterenol (0.1 mM)	125.3 (123.4;127.2)	76.3	11.2
Acetylcholine (0.1 mM)	88.1 (82.8;93.4)	39.1	13.3
Corticotropin (0.05 mM)	91.6 (89.7;98.4)	42.6	8.9
Glucagon (0.01 mM)	78.7 (74.9;82.4)	29.7	6.9
Thyroid-stimulating hormone (20 µg/ml)	79.2 (76.0;82.4)	30.2	8.9
Prostaglandin E_1 (20 µg/ml)	118.4 (158.7;178.0)	119.4	14.1
EGTA (2 mM)	67.3 (67.3;67.3)	18.3	6.8
Glucagon (0.01 mM) + EGTA (2 mM)	110.4 (108.5;112.3)	61.4	3.4

TABLE 13

PURIFICATION OF CYCLIC AMP WITH AG-50WX8 COLUMNS
(0.5 X 2.5 cm)

mls of eluate collected	nucleotides, etc.
0.5 ml of sample loaded + first 2 ml of eluate	ATP, ADP, Theophylline
3rd and 4th ml	Cyclic AMP

0.5 ml of samples were loaded, and eluted with water. 5'-AMP did not appear in the first 4 mls of collected eluate. The separation between different nucleotides was identified by optical density (260 mμ) and radioactive counts of ATP and cyclic AMP.

TABLE 14

MIGRATION OF SOME ADENOSINE NUCLEOTIDES ON SILICAR TLC-7GF MALLINCKRODT

Compound	R_F
Cyclic AMP	0.91
5'-AMP	0.55
ADP	0.18
ATP	0.14

Developed with isopropyl alcohol-water-29% NH_4OH (7:2:1, v/v) as solvent for 5 hours. All spots were checked under ultraviolet light.

terfere with the cyclic AMP recovery examined by optical density (260 mμ) in adenylate cyclase assay.

In addition to the observation of R_f values of cyclic AMP and 5'-AMP (77), the R_f values of both ATP and ADP were examined with SilicAR TLC-7GF Mallincrodt (Table 14), and the results confirmed that the thin layer chromatography technique was good enough to examine the purity of cyclic AMP in adenylate cyclase assay.

CHAPTER IV

DISCUSSION

The pancreatic islet isolation procedure is a modified version of that developed by Lacy and Kostianovsky (20). The most significant innovation in the present procedure is the use of pilocarpine, a potent cholinomimetic agent. This drug has been shown to cause a complete or a near-complete depletion of zymogen granules from pancreatic acinar tissue (78). These zymogens, if present, could potentially be released and activated during the collagenase digestion of pancreatic tissue. The active enzymes, especially the proteolytic ones, could interfere with the isolation procedure and destroy the islet plasma membrane structure.

The use of a 180-mesh silk screen to retain islets during the washing of the collagenase-digested pancreatic tissue and the use of an 18-gauge needle attached to a siphon device for picking the islets greatly facilitate the isolation of islets in adequate amounts for the experiments. The islets obtained by this procedure are largely

free of acinar tissue as judged by a microscopic examination. The islet preparations, containing 1.7 ± 0.1 μg protein per islet, compare favorably with the value of about 3 μg protein per islet reported by others (44). Although the islet preparations are undoubtedly contaminated with small amounts of exocrine pancreatic tissue, the extent to which this contributed to the total adenylate cyclase activity may be minimal. The β-cells, which are actually involved in insulin secretion, comprise about 60-90% of the islet cell content (79). The responses of islet adenylate cyclase to many hormones and agents, might also largely represent those of β-cell adenylate cyclase.

The advantages of the pilocarpine pretreatment of rats prior to preparation of the islets are clear. First, it permits an increased yield of islets. Second, adenylate cyclase in the isolated islets, perhaps with its receptors remaining relatively undamaged, retains its responsiveness to a wide variety of hormones. Author has observed with islets obtained from rats not pretreated with pilocarpine that adenylate cyclase activity shows little or no response to glucagon, whereas, under the same experimental conditions, this hormone causes a doubling of the enzyme activity in islets obtained from pretreated rats. These results may be somewhat analogous to the f ndings of Rodbell et al. made with fat cell ghosts (45), wherein tryp-

sin digestion of the plasma membrane causes a loss in the sensitivity of adenylate cyclase to hormones. It is also reasonable to assume that cholinergic stimulation leads to the discharge of insulin (and possibly glucagon) from the islet tissue. Acetylcholine or vagal stimulation have been shown to initiate insulin secretion (60, 80). Pretreatment with pilocarpine could thus increase the responsiveness of islet adenylate cyclase by decreasing endogenous insulin or glucagon in the tissue preparation. This step also helped to assure that the author was not observing the combined effects of endogenous polypeptides and the various test substances investigated. The results of the present study indicate that islet adenylate cyclase is responsive to a great number of stimulants, perhaps reflecting a central role of insulin in many homeostatic mechanisms. In a recently published communication, Rosen et al. described a study concerning adenylate cyclase activity of an insulin-producing islet cell tumor (83). They reported that only glucagon and fluoride, among many substances tested, were capable of stimulating islet tumor adenylate cyclase. The agents tested and found to be ineffective were: epinephrine, norepinephrine, isoproterenol, arginine, glucose, prostaglandin E_1, ethanol, secretin, insulin and ACTH. Their results are obviously at variance with the findings. It should be emphasized, however, that the adenylate cyclase

which the author studied is from normal rats which had been treated with pilocarpine. The purity of some hormones, such as pancreozymin, secretin, and TSH, used in the present study is rather low. The effects seen with these hormone preparations remain to be confirmed when pure hormones become available to the author.

It has been shown (60, 61, 80) that acetylcholine and vagal stimulation cause an increase in insulin secretion. (The author has also observed that acetylcholine elevates cyclic AMP levels in the intact pancreatic islets prepared from rats pretreated with pilocarpine.) This effect of acetylcholine on insulin secretion may be an indirect one. It may be explained, at least in part, by assuming that acetylcholine interacts with islet α-cells and leads to a release of glucagon. The glucagon, in turn, activates the adenylate cyclase of neighboring β-cells, resulting in elevated cellular cyclic AMP levels. The effect on pancreatic islet cyclic AMP production by both insulin and glucagon has been confirmed, but the major target cells of these two hormones is not absolutely clear. The finding that acetylcholine stimulates adenylate cyclase in the islet homogenates (Table 4) is quite unexpected. Nevertheless, it provides direct evidence supporting the contention that the action of acetylcholine on the organelles (and perhaps β-cells) may be a direct one. The ability of acetylcholine, like many hormones, to activate islet adenylate cyclase may

be of particular physiological significance. It remains to be seen whether acetylcholine also increases cyclic GMP levels in islets. Several lines of recent evidence have suggested that cyclic GMP, but not cyclic AMP, may be involved in cholinergic neurotransmission and that acetylcholine functions to specifically increase the tissue levels of cyclic GMP (84-86).

All hormones, drugs, compounds and metabolites in this study were examined for their possible effects on islet phosphodiesterase assayed in the presence and absence of 10 mM theophylline. At concentrations the same as those employed in the adenylate cyclase studies, none of these hormones and agents had any significant effects on the phosphodiesterase activity[1]. Goldfine et al. (70) reported that islet cell tumor phosphodiesterase is inhibited by tolbutamide which has a K_i of 3 mM. In their study, however, the concentrations of tolbutamide found to significantly inhibit phosphodiesterase were about an order of magnitude higher than those required to activate adenylate cyclase of normal islets as observed in the studies (Table 5) and in islet cell adenoma as reported by Levey et al. (69).

Most of the hormones and agents studied for their

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[1]Kuo, W. N., Hodgins, D. S., Kuo, J. F. Adenylate cyclase in islets of Langerhans, manuscript to be submitted for publication.

effects on adenylate cyclase in islet homogenates were also examined for their effects on the cyclic AMP levels in intact islets, employing either the technique of pre-labeling the cellular ATP pool with radioactive adenine (87, 88) or with the protein kinase catalytic method (33). Most of the results obtained with the broken cell system were confirmed with the intact cell experiments, except for NaF which was not effective in increasing cyclic AMP levels in intact islets. NaF was shown earlier to be a potent stimulator of adenylate cyclase in fat cell ghosts (89) while having no effect on intact fat cells (88). Because of the three different kinds of techniques employed under different conditions, it was not necessary to get exactly the same degree of effect on pancreatic islet cyclic AMP production by various agents. Glucagon, isoproterenol, prostaglandins (E_1, E_2, A_1, $F_{1\alpha}$), ACTH, secretin, acetylcholine, TSH, tolbutamide, EGTA, GTP, and epinephrine or norepinephrine in the presence of α-adrenergic blockers were found to increase cyclic AMP level in rat pancreatic islets. The increase in intracellular cyclic AMP levels evoked by these agents were apparently due to their stimulation of islet adenylate cyclase. In addition to the above mentioned agents, pancreozymin, somatotropin, GDP, ethanol and NaF were also found to stimulate islet adenylate cyclase. Many of these agents are known to have a

physiological role or pharmacological effect in promoting insulin secretion. Another class of insulinogenic agents, such as glucose, arginine, leucine, and phenformin (an oral hypoglycemic drug), whose action is considered to be mediated through an intracellular mechanism (62) did not increase islet cyclic AMP levels. They inhibited slightly or exerted no effect on islet adenylate cyclase. Many of the hormones studied with islets are also known to act on rat fat cells (36, 81, 82). The obvious differences seen in rat fat cells are that prostaglandins, particularly in the presence of catecholamines and other lipolytic hormones, decrease cyclic AMP levels (88, 92), and that epinephrine or norepinephrine (in the absence or presence of α-adrenergic blockers) increase cyclic AMP levels (36, 81, 82), suggesting adrenergic receptors in rat fat cells are functionally of the β-type. It is worthy mentioning that, like rat islets, human fat cells were found to possess both α-and β-adrenergic receptors (93). The acetylcholine-mediated stimulation of adenylate cyclase and increase of cyclic AMP levels in rat islets, to the author's knowledge,have not yet been found in other tissues. Cholinergic agents have been shown to stimulate the release of insulin from slices of rat pancreas (60, 61) and islets (40).

The author has not studied the effects of hormones and other agents on insulin secretion by the islet preparation, since pilocarpine pretreatment of rats may have

depleted the insulin stores of the β-cells.

Cyclic AMP, as has been previously hypothesized, appears to play an important role in the secretion, and possibly the synthesis, of insulin (for example, see 22, 23, 40, 42, 44). The exact mechanism for the action of cyclic AMP, however, remains unclear. Since a complete correlation between adenylate cyclase activation and the stimulation of insulin secretion appears to be lacking, intracellular cyclic AMP levels alone may not be the sole determinants of insulin secretion. It seems at least two separate secretory mechanisms are involved; one being cyclic AMP-mediated and the other being closely associated with the metabolism of glucose and certain amino acids. Another possibility is a mechanism that requires a combination of the effects of both cyclic AMP and metabolites. Thus, increased intracellular cyclic AMP levels may facilitate insulin secretion as elicited by metabolites. However, a cyclic AMP dependent protein kinase, and a protein kinase modulator activity have been found in islet[2]. The islet protein kinase has some catalytic properties common to the enzyme found in many other sources; these include the artificial substrate and cyclic nucleotide specificity,

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[2]Kuo, J. F., Kuo, W. N., Hodgins, D. S., Greengard, P. Cyclic AMP dependent protein kinase from islets of Langerhans, manuscript to be submitted for publication.

and the metal ion requirement. No cyclic GMP dependent protein kinase activity was found. It is reasonable to assume that the phosphorylation catalyzed by cyclic AMP dependent protein kinase inside the β-cells of islet tissue could lead to the decomposition of glycogen and triglyceride, the conformational change of histones, microtubular elements, and membrane proteins. Furthermore, all of above phosphorylation functions could ultimately facilitate both insulin biosynthesis and insulin secretion (Fig. 14). The modulator inhibits or activates cyclic AMP dependent and cyclic GMP dependent protein kinase under certain conditions (90, 91), suggesting a specific role for cyclic AMP in the secretion and possibly synthesis of insulin.

In addition to experiments using whole isolated islets it may be quite possible to study:(a) the cyclic AMP system in α-cells of pancreatic islet tissue by alloxan pretreatment of rats to result in the selective damage of β-cells (94), (b) the secretion of insulinogenic hormones regulated directly or indirectly by insulin mediated through cyclic AMP system, (c) and the effect of gastrin, tetragastrin, glucagon-like hormone, serotonin, and some other analogs of cyclic AMP (tubercidin 3', 5'-monophosphate, 3'-methylene cyclic phosphonate analog, 5'-methylene cyclic phosphonate analog, and dibutyryl analog of cyclic AMP) on pancreatic islet cyclic AMP production.

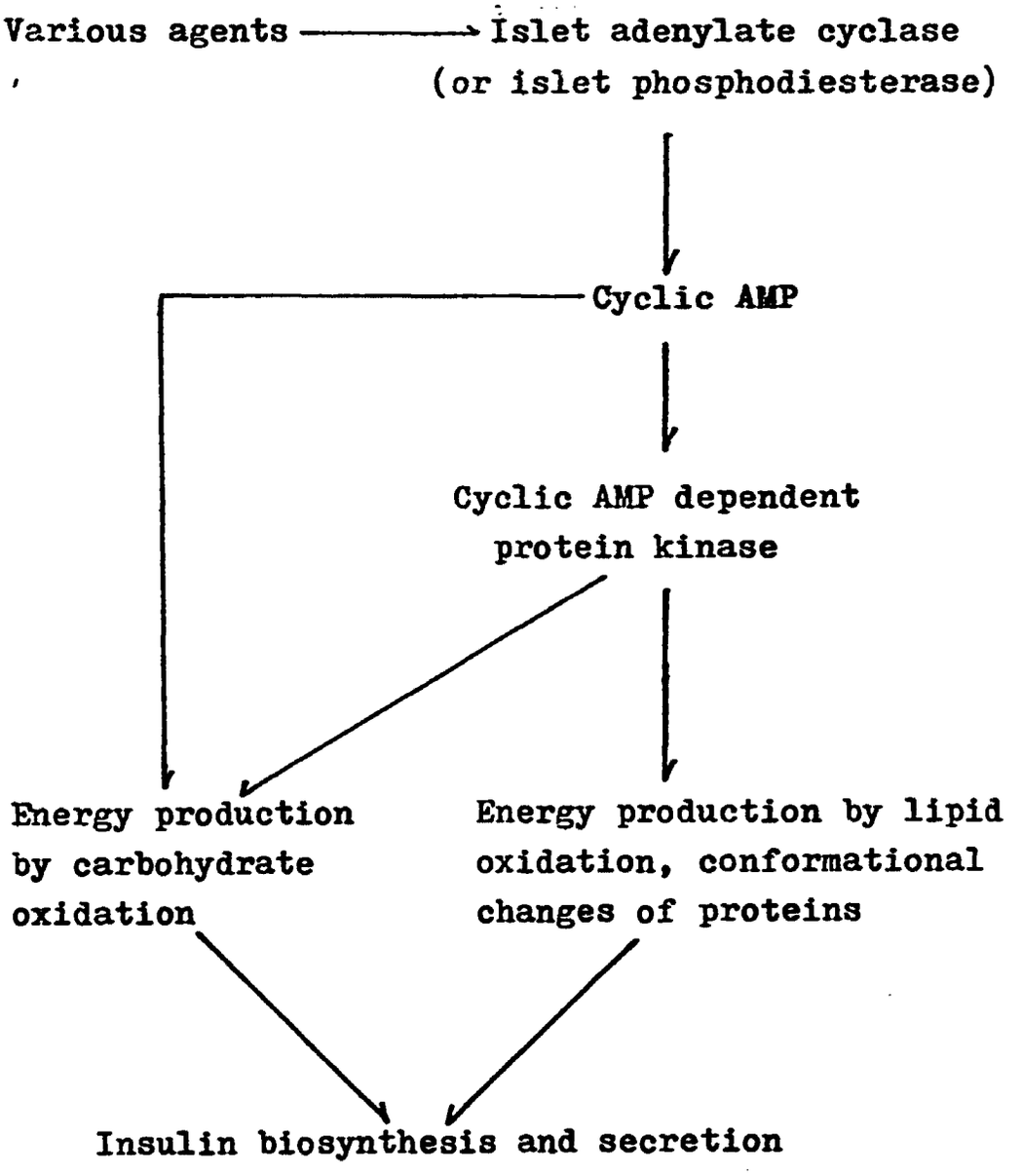

Fig. 14 - Possible mechanism of cyclic AMP system on insulin secretion.

CHAPTER V

SUMMARY

An improved method for the isolation of rat pancreatic islets possessing adenylate cyclase responsive to various hormones is described, The key step in this method, which yielded up to 500 islets per pancreas, involved pretreatment of rats with pilocarpine. This allowed depletion of zymogens from the exocrine pancreatic tissue and thus minimized the destruction of islet membranal structure which could otherwise occur during the collagenase digestion of the tissue.

The adenylate cyclase activity in the islet homogenates was assayed in the presence of a variety of hormones and other agents. With the exception of some insulinogenic metabolites, such as goucose, arginine and leucine, all hormones previously demonstrated by others to stimulate insulin secretion were found to augment the synthesis of cyclic AMP by islet adenylate cyclase. These agents include glucagon, adrenocorticotropic hormone, secretin, pancreozymin, various prostaglandins, and β-adrenergic agents. Acetylcholine, thyrotropin, somatotropin, ethanol, EGTA, GTP, GDP, and NaF

also stimulated cyclic AMP production, but their insulinogenic action has not been clearly demonstrated. Of the two hypoglycemic drugs studied, tolbutamide was found to stimulate the cyclase activity whereas phenformin was without effect. Insulin inhibited the islet adenylate cyclase activity, suggesting a possible feed-back mechanism involved. None of these agents had any effect on the islet phosphodiesterase activity.

Regulation by various agents of cyclic AMP levels in intact islets of rat pancreas was studied, employing either the technique of prelabeling the cellular ATP pool or the protein kinase catalytic method. The results obtained with the intact cell system confirmed most of the findings made with the broken cell system, clearly suggesting a role for cyclic AMP in the action of certain insulinogenic agents.

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