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ACTIVATION OF GUANYL CYCLASE AND ADENYL CYCLASE BY SECRETIN

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SUMMARY

Properties of rat liver guanyl cyclase and adenyl cyclase and the effects of hormones on the activity of these enzymes have been investigated.

1. Secretin ($2 \cdot 10^{-7}$–$60 \cdot 10^{-7}$ M) stimulates guanyl cyclase activity of 18 000 $\times g$ supernatants of rat liver homogenates with no change in the Michaelis–Menten constant for GTP ($3.8 \cdot 10^{-5}$ M).

2. Insulin, glucagon, and hydrocortisone *in vitro* were found to have no effect on guanyl cyclase activity in the presence or absence of secretin.

3. *p*-Chloromercuriphenyl sulfonate inhibits guanyl cyclase activity, and *p*-chloromercuriphenyl sulfonate inhibition is reversed by dithiothreitol. *p*-Chloromercuriphenyl sulfonate–dithiothreitol treatments have no effect on the sensitivity of the enzyme to secretin stimulation. These studies indicate that sulfhydryl groups may be involved in the regulation of the activity of liver guanyl cyclase.

4. Adenyl cyclase activity of rat liver homogenate is stimulated by secretin to approximately 50% of the stimulation caused by glucagon. Using hormone concentrations that maximally stimulate homogenate adenyl cyclase, experiments with combinations of glucagon, secretin, and isoproterenol indicate that only secretin and isoproterenol do not have additive effects. Propranolol blocks stimulation due to isoproterenol, but does not affect stimulation by secretin.

5. Insulin has no effect on either glucagon- or secretin-stimulated liver homogenate adenyl cyclase activity.

INTRODUCTION

Our previous studies of the metabolism of cyclic nucleotides indicated in a preliminary fashion that secretin stimulated rat liver guanyl cyclase activity *in vitro*[1]. Since little information has been obtained on factors influencing guanyl cyclase

Abbreviation: PCMPS, *p*-chloromercuriphenyl sulfonate.

activity, we have used secretin stimulation to study some of the properties of this enzyme.

Although the adenyl cyclase activity of liver membrane preparations, as studied by other investigators, was not stimulated by secretin[2,3], our studies of liver homogenate enzyme activity indicated that secretin activates this preparation of adenyl cyclase[1]. We have used this observation to study more details of the actions of insulin, glucagon, and catecholamine in relation to adenyl cyclase activity of rat liver.

Since nothing is known of the action of secretin on rat liver, the physiological consequences of these experiments remains obscure. We have, however, previously hypothesized that certain hormones may act by utilizing cyclic 3′,5′-guanosine monophosphate (cyclic GMP) as well as cyclic 3′,5′-adenosine monophosphate (cyclic AMP)[4]. Because of the similarities of secretin and glucagon structure and amino acid sequence[5], studies of the action of these hormones on rat liver cyclic nucleotide synthesizing enzymes were conducted.

MATERIALS

The following radioactive materials were purchased and treated as described previously[1]: [α-^{32}P]GTP (spec. act. 2.38–31 Ci/mmole), [α-^{32}P]ATP (spec. act. 3.8–12.3 Ci/mmole), cyclic [G-^{3}H]GMP (spec. act. 4.47 Ci/mmole) (New England Nuclear); and cyclic [8-^{3}H]AMP (spec. act. 20.8 Ci/mmole) (Schwarz-Mann). The following materials were used without being further purified: DL-isoproterenol, *p*-chloromercuriphenyl sulfonic acid (PCMPS), dithiothreitol, creatine phosphokinase, creatine phosphate, cyclic AMP and cyclic GMP (Sigma); hydrocortisone acetate (Merck, Sharp and Dohme); propranolol–HCl (Ayerst); glucagon (Lilly), secretin (pure porcine, G.I.H. Research Inst., Karolinska Institutet, Stockholm, courtesy of Dr Jorpes); bovine serum albumin (Nutritional Biochemical Corp., Fraction V) and mangenese oxide (powder, Mallinckrodt).

Crystalline porcine insulin (Burroughs Wellcome) was further purified as previously described[6].

Neutral aluminum oxide (Activity I, E. Merck) was washed for use in guanyl cyclase assay[1], or stored in an evacuated desiccator and not further treated for use in adenyl cyclase assay.

METHODS

Male Wistar rats (80–100 g), fed Purina lab chow *ad libitum*, were used in all experiments. Rats were sacrificed by decapitation after a blow to the cervical vertebrae. Liver tissue was rapidly excised, rinsed in 0.32 M sucrose (4 °C), weighed, and homogenized (0.5 g/3 ml) in 0.32 M sucrose (4 °C) for guanyl cyclase assay, or in 33 mM Tris–Cl^{-} (pH 7.0) for adenyl cyclase assay. A Duall-type glass homogenizing vessel was used on ice, and four strokes of a motor-driven Teflon pestle were applied.

Liver homogenate was diluted with 33 mM Tris–Cl^{-} (pH 7.0) for immediate use as the adenyl cyclase preparation. For guanyl cyclase enzyme preparation, liver homogenate was centrifuged at 18 000 $\times$ *g* for 20 min, and the supernatant was frozen in aliquots (—20 °C). These preparations contained 83% of the guanyl cyclase

activity of the original homogenate. Up to 2 months storage at −20 °C did not affect total guanyl cyclase activity.

Guanyl cyclase and adenyl cyclase were assayed as previously described[1]. Guanyl cyclase assay reaction constituents included [α-^{32}P]GTP (5–100 μM; $1.2 \cdot 10^6$ cpm), 24 mM caffeine, 5.0 mM $MnCl_2$, 2.0 mM cyclic GMP, 10 mM Tris–Cl$^-$ (pH 7.4), and 0.05 ml enzyme ($\approx$ 120 μg protein)[7] in a final volume of 0.2 ml. Reactions were initiated by addition of enzyme at 4 °C, incubated at 30 °C in a shaking water bath, and terminated by immersion of the reaction tubes in a dry ice–acetone bath until frozen (15 s), following by immersion in boiling water (30 s). The reaction mixture was cooled in an ice bath and 0.8 ml of 2.0 mM sodium pyrophosphate (pH 7.4) containing approximately 10 000 cpm of cyclic [^{3}H]GMP added. Alumina chromatography was used to isolate cyclic [^{32}P]GMP formed in the reaction. Liquid scintillation techniques were employed to measure ^{3}H and ^{32}P radioactivity.

Adenyl cyclase assay reaction constituents included [α-^{32}P]ATP (50 μM; $1.2 \cdot 10^6$ cpm), 0.2 mg creatine phosphokinase, 15.6 mM phosphocreatine, 7.5 mM theophylline, 5 mM $MgCl_2$, 0.5 mM cyclic AMP, 0.09% bovine serum albumin, 8 mM Tris–Cl$^-$ (pH 7.4), and 0.05 ml enzyme (158–580 μg protein)[7] in a final volume of 0.20 ml. Reactions were initiated by addition of enzyme at 4 °C, incubated at 30 °C in a shaking water bath, and terminated by immersion of the reaction tubes in a dry ice–acetone bath until frozen (15 s) and subsequent immersion in boiling water (3 min). The reaction mixture was cooled in an ice bath and diluted to 1 ml with 50 mM Tris–Cl$^-$ (pH 7.4), containing approximately 10 000 cpm of tracer cyclic [^{3}H]AMP. After adding 1 g MnO_2, the mixture was vortexed and centrifuged (4000 $\times$ g) for 8 min and cyclic [^{32}P]AMP was isolated by alumina chromatography. Liquid scintillation techniques were used to measure ^{32}P and ^{3}H.

The validity of assay methods was previously established using thin-layer and paper chromatographic methods, specific cyclic AMP and cyclic GMP antibody binding, anion-exchange resin precipitation, and ion-exchange chromatography[1]. All reaction velocities were linear with respect to time of incubation and protein concentration.

RESULTS

Progressive increase in guanyl cyclase activity were produced by secretin from $3.9 \cdot 10^{-7}$ to $62.6 \cdot 10^{-7}$ M (Fig. 1). Secretin ($125 \cdot 10^{-7}$ M) was inhibitory. All of the increased ^{32}P measured in the guanyl cyclase assay in the presence of secretin could be precipitated with cyclic GMP-specific antibody and migrated with cyclic GMP on paper chromatography. Comparable concentrations of secretin had no effect on rat liver cyclic AMP phosphodiesterase or cyclic GMP phosphodiesterase activity.

The dose–response curve obtained (Fig. 1) indicated secretin stimulation of guanyl cyclase activity over a narrow range. Lineweaver–Burk kinetic analysis indicated that secretin ($7.5 \cdot 10^{-7}$ M) increased the maximum velocity of the enzyme with no alteration of the apparent affinity for GTP (K_m $3.8 \cdot 10^{-5}$ M) (Fig. 2).

Neither insulin ($1.8 \cdot 10^{-8}$ M) nor glucagon ($5 \cdot 10^{-9}$ M) *in vitro* affected basal guanyl cyclase activity (Fig. 3). Moreover, insulin (10^{-12}–10^{-7} M) and glucagon (10^{-9}–10^{-6} M) had no significant effect on secretin-stimulated guanyl cyclase activity (data not shown).

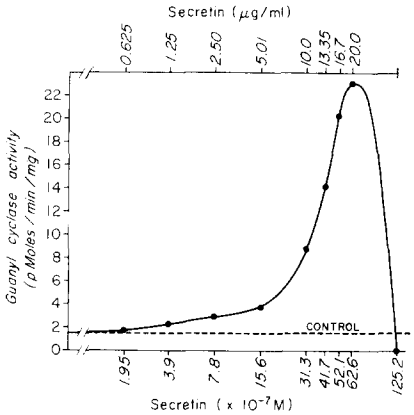
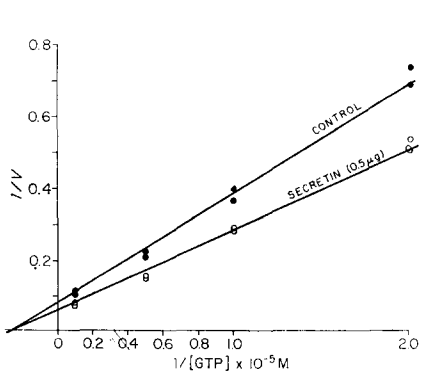


Fig. 1. Secretin stimulation of rat liver guanyl cyclase. Guanyl cyclase activities were measured as described. GTP concentration used was $1 \cdot 10^{-5}$ M. Assays were incubated 7 min at 30 °C. Values are expressed as the mean of triplicates with control value ± S.D. shown as a dashed line. All values except $1.95 \cdot 10^{-7}$ M secretin are statistically significant ($P < 0.05$) from control.

In previous reports[4] we have demonstrated increased guanyl cyclase activity in liver, lung, heart, and skeletal muscle homogenates of adrenalectomized rats. However, hydrocortisone acetate (75 or 300 μM) had no effect *in vitro* on basal or secretin-stimulated hepatic guanyl cyclase activity (Table I).

Dithiothreitol has previously been shown by others to inhibit the slight inactivation caused by dialysis of a partially purified lung guanyl cyclase preparation and to reverse inactivation caused by 10 mM $ZnCl_2$, $CdCl_2$, and $HgCl_2$ (see ref. 9). We found no effect on basal liver guanyl cyclase activity of dithiothreitol, cysteine, glutathione, or *N*-ethylmaleimide (10^{-5} or 10^{-3} M). However $4 \cdot 10^{-5}$ M *p*-chloro-



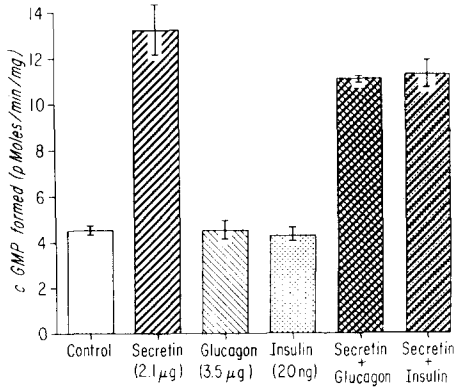


Fig. 2. Kinetics of secretin stimulation of guanyl cyclase. Assay conditions are those given in Fig. 1. In each assay, 134 μg protein were used. Data are plotted according to the method of Lineweaver and Burk[8].

Fig. 3. Insulin and glucagon on secretin activation of guanyl cyclase. Assay conditions are those given in Fig. 1. GTP concentration used was $2 \cdot 10^{-5}$ M. Assays were incubated 8 min at 30 °C. Hormone quantities are amounts per assay (0.2 ml).

TABLE I

EFFECTS OF HYDROCORTISONE AND SECRETIN ON RAT LIVER GUANYL CYCLASE

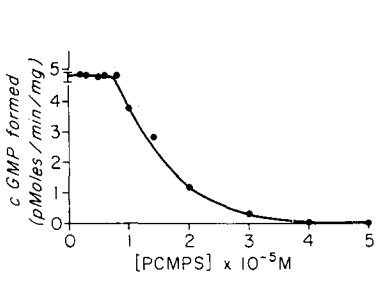
Guanyl cyclase assays were performed using rat liver enzyme preparations as described. In each assay, 125 μg of protein[7] was used. Assays were incubated for 8 min at 30 °C using $2 \cdot 10^{-5}$ M GTP. Values are the mean of duplicates ± S.D.

Conditions	*Guanyl cyclase activity (pmoles/min/mg)*
Control	3.45 ± 0.17
Control + secretin (1.58 μg)	7.09 ± 0.02
Control + hydrocortisone (6 μg)	3.35 ± 0.01
Control + hydrocortisone (25 μg)	3.46 ± 0.12
Control + secretin (1.58 μg) + hydrocortisone (6 μg)	7.01 ± 0.44
Control + secretin (1.58 μg) + hydrocortisone (25 μg)	7.75 ± 0.49

mercuriphenyl sulfonate completely inhibited enzyme activity (Fig. 4). Dithiothreitol when added to enzyme inhibited by $5 \cdot 10^{-5}$ M PCMPS completely restores activity to control values (Fig. 5). Cysteine was less effective in restoring guanyl cyclase activity. These data imply sulfhydryl group participation either in the active site of guanyl cyclase or in the structural integrity of this enzyme.

Since secretin stimulation of guanyl cyclase did not appear to involve the active site, we tested the effects of secretin with PCMPS and dithiothreitol on enzyme activity (Table II). Secretin-stimulated basal and PCMPS–dithiothreitol-treated enzyme to a similar degree. Secretin also restored PCMPS-inhibited enzyme to control levels, and at higher concentration increased it above control levels. Since 75 units of secretin contain 1 mg cysteine, the latter effects may be partially due to the cysteine and may not indicate direct secretin displacement of PCMPS.

Liver homogenate adenyl cyclase response to secretin and glucagon is shown in Fig. 6. Glucagon stimulation of homogenate adenyl cyclase was of the same magnitude as found by others[10,11] with half-maximal stimulation occurring at $5.9 \cdot 10^{-9}$ M.



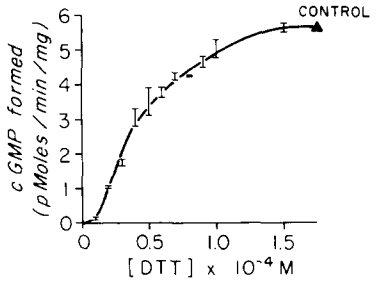


Fig. 4. PCMPS effects on liver guanyl cyclase. Assay conditions are those given in Fig. 1. GTP concentration used was $2 \cdot 10^{-5}$ M. Assays were incubated 7 min at 30 °C. PCMPS was added just before enzyme addition. Values are expressed as means of duplicate determinations, with control values (quadruplicate) ± S.D. shown on the ordinate.

Fig. 5. Dithiothreitol (DTT) on PCMPS-inhibited liver guanyl cyclase. Assay conditions are those given in Fig. 4. Experimental additives were added to the reaction in the order PCMPS, enzyme, and dithiothreitol. PCMPS concentration used was $5 \cdot 10^{-5}$ M. Data are shown as determined in duplicate. Control value is shown as a closed triangle representing the mean of quadruplicate determinations in the upper right portion of the figure.

TABLE II

EFFECTS OF SULFHYDRYL REAGENTS AND SECRETIN ON RAT LIVER GUANYL CYCLASE

Assay conditions are as listed in Table I. GTP concentrations used were $1 \cdot 10^{-5}$ M. The order of addition of additives was: PCMPS, enzyme, dithiothreitol, and secretin. All values are the mean of duplicates ± S.D.

Control Additions	*Guanyl cyclase activity (pmoles/min/mg)*
Control	1.67 ± 0.03
PCMPS ($5 \cdot 10^{-5}$ M)	0
Dithiothreitol ($2 \cdot 10^{-4}$ M)	1.89 ± 0.22
Secretin ($0.78 \cdot 10^{-6}$ M)	2.49 ± 0.12
Secretin ($3.1 \cdot 10^{-6}$ M)	3.55 ± 0.16
PCMPS + dithiothreitol	1.72 ± 0.03
PCMPS + secretin ($0.78 \cdot 10^{-6}$ M)	1.95 ± 0.21
PCMPS + secretin ($3.1 \cdot 10^{-6}$ M)	2.87 ± 0.02
PCMPS + dithiothreitol + secretin ($0.78 \cdot 10^{-6}$ M)	2.50 ± 0.19
PCMPS + dithiothreitol + secretin ($3.1 \cdot 10^{-6}$ M)	3.47 ± 0.51

Secretin caused less stimulation than glucagon with half-maximal stimulation at $3.3 \cdot 10^{-7}$ M.

The actions of secretin in relation to those of glucagon and isoproterenol on rat liver homogenate adenyl cyclase activity are shown in Table III. Maximally-stimulating concentrations of all of the hormones were used. Glucagon (0.15 μM) and secretin (0.42 μM), glucagon and isoproterenol (8 μM), but not secretin and isoproterenol showed stimulation of activity above that seen with each hormone individually. Propanolol (8–300 μM), a β-adrenergic blocker, inhibited isoproteronol (8 μM) stimulation of adenyl cyclase (Table IV) but had no effect on secretin (0.42 μM) and glucagon (0.15 μM) stimulation. Insulin (10^{-12}–10^{-7} M) had no effect on secretin (0.42 μM) or glucagon (0.15 or 0.015 μM) stimulation of liver homogenate adenyl cyclase activity (Table V).

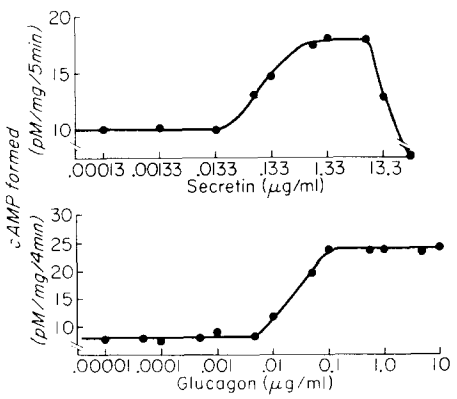


Fig. 6. Rat liver homogenate adenyl cyclase activation by secretin and glucagon. Adenyl cyclase activities were measured as described. 50 μM ATP was used in each assay. For secretin and glucagon activations 659 μg of protein incubated for 5 min and 375 μg incubated for 4 min were used, respectively. All values are the means of duplicates.

TABLE III

ADDITIVE EFFECTS OF LIVER ADENYL CYCLASE STIMULANTS

Enzyme assays of liver homogenate adenyl cyclase activity were performed as described in Methods. Amounts of protein per assay and incubation times were as follows: Expt 1, 211 μg, 10 min; Expt 2, 580 μg, 5 min; Expt 3, 158 μg, 10 min. Hormone concentrations of maximal stimulating capacity were: glucagon, 0.15 μM; isoproterenol, 8 μM; and secretin, 0.42 μM. ATP concentration was $5 \cdot 10^{-5}$ M in duplicate assays.

Additions	*Expt 1 (pmoles/min/mg)*		*Expt 2 (pmoles/min/mg)*		*Expt 3 (pmoles/min/mg)*	
	Activity	*Increase*	*Activity*	*Increase*	*Activity*	*Increase*
Control	2.2 ± 0.3		2.2 ± 0.3		2.9 ± 0.3	
Glucagon	4.8 ± 0.2	2.6	5.5 ± 0.1	3.3	10.0 ± 1.0	7.2
Secretin	4.3 ± 0.4	2.1	4.0 ± 0.3	1.7	6.6 ± 0.9	3.7
Glucagon + secretin	7.6 ± 0.8	5.4	6.7 ± 0.3	4.5	10.6 ± 0.2	7.7
Isoproterenol	3.6 ± 0.4	1.4	4.7 ± 0.6	2.5	6.1 ± 0.4	3.2
Secretin	4.3 ± 0.4	2.1	4.0 ± 0.3	1.7	6.6 ± 0.9	3.7
Isoproterenol + secretin	4.7 ± 0.3	2.5	5.0 ± 0.4	2.8	6.6 ± 0.4	3.7
Glucagon	4.8 ± 0.2	2.6	5.5 ± 0.1	3.3	10.1 ± 1.0	7.2
Isoproterenol	3.6 ± 0.4	1.4	4.7 ± 0.6	2.5	6.1 ± 0.4	3.2
Glucagon + isoproterenol	7.1 ± 0.2	5.0	7.1 ± 0.2	4.9	12.3 ± 0.5	9.4

TABLE IV

EFFECT OF PROPRANOLOL ON STIMULATION OF LIVER ADENYL CYCLASE

Assays were performed using rat liver homogenate as described. In each assay, 200 μg of protein was used. Incubations were for 10 min at 30 °C. ATP concentration was $5 \cdot 10^{-5}$ M. All values are the mean of duplicates ± S.D. expressed as pmoles of cyclic AMP formed/10 min /mg protein.

Additions	*Adenyl cyclase activity (pmoles/10 min/mg)*			
Propranolol (M)	*0*	*$8 \cdot 10^{-6}$*	*$8 \cdot 10^{-5}$*	*$3 \cdot 10^{-4}$*
Basal	17.7 ± 2.4	16.0 ± 0.1	15.2 ± 3.6	12.2 ± 1.1
Isoproterenol (8.1 μM)	26.9 ± 2.4	17.8 ± 2.6	18.8 ± 3.3	15.0 ± 3.3
Secretin (0.42 μM)	40.3 ± 2.1	40.7 ± 1.6	43.8 ± 7.8	38.1 ± 4.5
Glucagon (0.15 μM)	62.6 ± 2.5	66.9 ± 5.7	54.0 ± 2.9	53.8 ± 4.6

DISCUSSION

Secretin stimulation of liver guanyl cyclase is the only reported hormonal effector of this enzyme *in vitro*. The amounts of secretin required for stimulation are much higher than immunoassayable plasma secretin[12] but are in the same range as normally used to elicit physiological responses[13]. Peptide fragments of secretin have not been tested in our assay system, although there are no known fragments of biological activity[14]. The kinetics of activation suggest the possibility of a regulatory site distinct from the active site of liver guanyl cyclase. No interaction of secretin with glucagon or insulin directly on guanyl cyclase is indicated.

TABLE V

EFFECT OF INSULIN ON STIMULATION OF LIVER ADENYL CYCLASE

Assay conditions are as listed in Table IV. All values are the mean of duplicates or triplicates ± S.D. expressed as pmoles of cyclic AMP formed/10 min/mg protein. Insulin was single component insulin used with 0.10% human serum albumin in assay incubations and hormone dilutions.

Additions	*Adenyl cyclase activity (pmoles/10 min/mg)*						
Insulin (M)	0	10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}
Control	22 ± 4	21 ± 4		22 ± 2		24 ± 1	
Secretin (0.42 μM)	52 ± 5	60 ± 18	67 ± 22	58 ± 6	60 ± 3	54 ± 2	75 ± 3
Glucagon (0.15 μM)	74 ± 14	68 ± 4	67 ± 1	76 ± 6	67 ± 7	92 ± 37	72 ± 10

Sulfhydryl-binding reagents are reversible inhibitors of liver guanyl cyclase but do not distinguish catalytic site from structural modification by these agents. Because secretin is capable of fully activating the enzyme treated with PCMPS and dithiothreitol, it suggests that the catalytic site of guanyl cyclase may contain sulfhydryl groups, or that sulfhydryl groups are involved in its structural integrity.

Previous studies[4] indicated increased guanyl cyclase activity in rat liver, lung, heart, and skeletal muscle of adrenalectomized rats. Since hydrocortisone has no effect on either basal or secretin-stimulated liver guanyl cyclase activity *in vitro*, it appears that effects of glucocorticoids on guanyl cyclase require intact cell mechanisms.

Previous reports indicated that secretin stimulates adipose tissue membrane preparations of adenyl cyclase[15], but does not stimulate liver membrane preparations of adenyl cyclase[2,3]. Because secretin stimulation is additive with glucagon but not with isoproterenol, the implication is that secretin activates adenyl cyclase through a fragile receptor distinct from that of glucagon which binds both catecholamines and secretin. Secretin stimulation of adenyl cyclase was not blocked by propranolol, suggesting that secretin binds in a region of this receptor separate from that of catecholamines.

However, preliminary investigations indicate that guanyl nucleotide triphosphates (10 μM) enhance glucagon- and catecholamine-stimulated liver homogenate adenyl cyclase, but are without effect on secretin stimulation (0.42 μM). Secretin (0.42 μM) stimulation is more than additive with 10 mM NaF stimulation of adenyl cyclase. These uncharacteristic adenyl cyclase activations suggest that secretin has a molecular mechanism distinct from that of glucagon and the catecholamines. Insulin does not appear to affect basal homogenate adenyl cyclase or activation by glucagon or secretin.

Kinetic analysis, sulfhydryl data, lack of hormonal interaction, and the unusual character of adenyl cyclase activation indicate that the activation of guanyl cyclase by secretin does not involve the active site, may involve sulfhydryl groups, and does require a specific hormone sequence and structure. Our unpublished observations show liver guanyl cyclase to be a relatively large (300 000–600 000 mol. wt) oligomeric protein possibly composed of regulatory subunits as well as catalytic subunits. Further investigation is in progress to extend these preliminary observations.

The physiological significance of these observations is obscure because extragastrointestinal effects of secretin have not been elucidated. Although secretin has close structural and sequential similarity to glucagon[5], secretin is only "glucagon-like" with respect to lipolysis[16], insulin release[17,18], and bile secretion[19]. Interestingly, secretin causes little or no hyperglycemia *in vitro*[12] nor "glucagon-like" effects on perfused liver[20,21]. Our studies suggest that these latter observations may be related to liver guanyl cyclase and regulation of these metabolic processes by cyclic AMP and cyclic GMP in this tissue.

Secretin is known to counteract duodenal acidification by causing the pancreas to secrete bicarbonate and water from either centroacinar or intercalated duct cells[22]. We speculate that exploration of the role of cyclic GMP in relation to cyclic AMP in basic secretory processes with the use of secretin may be beneficial.

Efferent vagal stimulation, histamine, pilocarpine, or pancreozymin are thought to act synergistically with secretin[23], although experimental interpretation is complicated by blood flow problems. Although little is known about parasympathetic relationships and cyclic GMP, cyclic GMP levels are increased by acetylcholine infusions of heart[24] and acetylcholine increases cyclic GMP in thyroid and liver[25]. It is attractive speculation that secretin action may involve cyclic GMP.

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