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ADENYLATE CYCLASE IN THE RAT PANCREAS PROPERTIES AND STIMULATION BY HORMONES

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SUMMARY

1. In view of evidence for a role of cyclic AMP in the secretin-stimulated water and electrolyte secretion and the pancreozymin-stimulated enzyme secretion by the pancreas, the occurrence of adenylate cyclase in the pancreas has been investigated.

2. The presence of the enzyme in a particulate fraction of rat pancreas has been demonstrated. Basal activity is very low under standard conditions (less than 3 pmoles/min per mg protein), but 10 mM NaF stimulates the enzyme to 15–40 pmoles/min per mg protein.

3. Properties of the enzyme have been determined in the presence of 10 mM NaF. The apparent K_m for ATP, derived from a Lineweaver–Burk plot is 0.3 mM. At ATP concentrations above 1.5 mM there is substrate inhibition. The optimal pH is 7.4. The enzyme requires Mg^{2+} for its activity, concentrations of 5 mM and higher giving maximal activity. Ca^{2+} in concentrations above 0.1 mM inhibits the enzyme.

4. The enzyme is stimulated by a synthetic secretin preparation (half maximally activating concentration $1.5 \cdot 10^{-8}$ M) as well as by a purified pancreozymin preparation (half maximally activating concentration $1.5 \cdot 10^{-6}$ M). The latter activation is not due to a contamination of the pancreozymin preparation by secretin. The secretin-stimulated enzyme has the same optimal pH of 7.4 and shows the same Ca^{2+} inhibition as the NaF-stimulated enzyme.

5. The enzyme is not stimulated by adrenalin and its analogues, by acetylcholine and its analogues, and by the hormones glucagon and gastrin (synthetic).

6. Specific enzyme activity (per mg protein) in enzyme preparations obtained from rats with free access to food and stimulated *in vivo* by secretin and pancreozymin is twice as high as that in enzyme preparations from starved animals.

7. The physiological significance of these findings is discussed.

INTRODUCTION

In mammals the pancreas has an exocrine and endocrine function, which are morphologically separate. The exocrine part of the organ usually covers more than 90 % of the tissue. In the exocrine part, one cell type is responsible for fluid secretion (*i.e.* water and electrolyte secretion) and another for enzyme secretion. The latter

process appears to occur separately from the fluid secretion process, as shown by the fact that Ridderstap and Bonting[1] have been able to inhibit fluid secretion by the isolated rabbit pancreas by two thirds with ouabain without affecting enzyme secretion. This functional difference is also indicated by the different hormonal and neural regulation of both processes. The gastrointestinal hormone secretin stimulates water and electrolyte secretion, while pancreozymin does the same for the enzyme secretion. Enzyme secretion can also be elicited by stimulation of the vagus nerve or by application of acetylcholine or its analogues.

A mediating role of cyclic AMP in the stimulation of fluid secretion by secretin has been suggested by Case *et al.*[2], who observed a stimulation of flow by cyclic AMP in the isolated cat pancreas. Evidence for the involvement of the nucleotide in the stimulation of enzyme secretion by pancreozymin has been reported by Ridderstap and Bonting[3]. Both cyclic AMP and theophylline (which inhibits the breakdown of cyclic AMP by phosphodiesterase) have the same effect as pancreozymin on the secretion of enzymes by the isolated rabbit pancreas; secretion of water and electrolytes occurs at nearly maximal rate. Kulka and Sternlicht[4] showed a similar effect in mouse pancreas slices incubated *in vitro*. More recently, Bauduin *et al.*[5] induced enzyme release from pieces of rat pancreas incubated *in vitro* with dibutyryl cyclic AMP.

In view of these findings, the occurrence and properties of adenylate cyclase, the enzyme responsible for the formation of cyclic AMP, have been studied in rat pancreas. The enzyme's activity is stimulated very considerably by secretin and pancreozymin.

MATERIALS AND METHODS

Tissue preparation

Male Wistar rats, 2–3 months old with free access to food and water, are killed by a blow on the head. The pancreas is removed and placed immediately in an ice-cold solution, containing 10 mM Tris, 2.5 mM $MgCl_2$, 2.5 mM EDTA and 2 mg/ml trypsin inhibitor at pH 7.4. After removal of fat and connective tissue, the tissue is minced and homogenized gently by hand in 4 vol. of the same solution as above, using a loosely fitting Teflon pestle. The homogenate is filtered through medical gauze and centrifuged at 4000 $\times$ g_{max} for 10 min at 0 °C. The pellet is washed twice with 5 vol. and then suspended in 3 vol. of the same solution. The resulting suspension is used immediately for enzyme assays and is called enzyme preparation. Storage of the preparation at 0 °C or in the frozen state always leads to decreased activities.

Adenylate cyclase assay

The incubation medium is prepared by mixing equal volumes of the following solutions: 0.4 M Tris–HCl (pH 7.4); 50 mM $MgCl_2$; 0.1 M theophylline; 50 mM phosphoenolpyruvate trisodium salt; 2 mg/ml pyruvate kinase in 40 mM Tris buffer (pH 7.4); 4 mM ATP containing $2 \cdot 10^5$ cpm/μl as [α-^{32}P]ATP. To this mixture a double volume of water, in which any further reagents are dissolved, is added. After preincubation of 40 μl of this medium in microtest tubes for 3 min at 37 °C, the reaction is started by the addition of 10 μl enzyme preparation. Thus, the following final concentrations are maintained: Tris–HCl, 46 mM; ATP, 0.4 mM; Mg^{2+}, 5.5 mM;

EDTA, 0.5 mM; theophylline, 10 mM; phosphoenolpyruvate, 5 mM; pyruvate kinase, 0.2 mg/ml; trypsin inhibitor, 0.4 mg/ml; final pH 7.4. Incubations are, unless otherwise stated, carried out at 37 °C for 15 min. The reaction is terminated by placing the tubes for 2 min in boiling water, whereafter they are placed in ice; 5 μl of a solution of cyclic AMP (5 mg/ml in water) is then added and the tubes are centrifuged.

The separation of the radioactive product from the substrate and from other radioactive metabolites is carried out by thin-layer chromatography according to the method of Woods and Waitzman[6]. A 30-μl aliquot from the clear supernatant of each tube is placed on thin-layer chromatographic medium (Chromar-Sheet 500). After developing the thin layers with a mixture of 2-propanol, ethyl acetate and 13 M ammonia (59:25:16, by vol.), the cyclic AMP spots are visualized under 254 nm ultraviolet light. This development system leaves other adenosine phosphates (ATP, ADP and AMP) and also inorganic phosphate and pyrophosphate on the starting point, while cyclic AMP has an R_F of approximately 0.40. The spots are then cut out and put on the bottom of a scintillation vial. After addition of 10 ml Bray scintillation solution radioactivity is counted in a liquid scintillation spectrometer (Packard Tri-Carb; Model 3380). The amount of cyclic AMP formed during the incubation is calculated from the specific activity of [α-^{32}P]ATP in the reaction mixture and the amount of cyclic [^{32}P]AMP formed *minus* the reaction blank, which after proper development of the thin layer is about 0.01 % of the radioactivity of the initially present ATP. All determinations are performed in duplicate and the results, which usually agree within 5 %, averaged. Enzyme activity is expressed in pmoles cyclic AMP formed per mg protein per min of incubation time. Protein is measured by the method of Lowry *et al.*[7], using bovine serum albumin as a standard.

Materials

The following materials and reagents have been used with the source indicated in parentheses. Chromar Sheet 500 (Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.). ATP disodium salt, adenosine 3′:5′-monophosphoric acid (cyclic AMP) and pyruvate kinase, crystalline suspension, 10 mg/ml (Boehringer Mannheim, Germany). Phosphoenolpyruvate trisodium salt and soybean trypsin inhibitor, chromatographically prepared (Sigma Chemical Co., St. Louis, Mo., U.S.A.). Pentagastrin (Peptavlon, ICI, Macclesfield, England). Glucagon (Eli Lilly and Co., Indianapolis, Ind., U.S.A.). Bovine serum albumin, dried purified (Behringwerke A.G., Marburg/Lahn, Germany). Clinical preparations of secretin and pancreozymin have been obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. and from Boots pure Drug Co. Ltd, Nothingham, England.

Synthetic secretin (Pansecrin, activity 3 Clinical Units per μg) is a gift from Dr M. Ondetti, The Squibb Institute for Medical Research, New Brunswick, N.J., U.S.A. Highly purified natural cholecystokinin–pancreozymin (activity 3 Ivy Dog Units per μg), further called pancreozymin, is a gift from Prof. V. Mutt of the Karolinska Institute, Stockholm, Sweden. All other reagents are commercial preparations of the highest obtainable purity.

RESULTS

Basal and fluoride-stimulated activity

Basal activity of the enzyme in the various preparations is low and ranges from

undetectable (< 0.5 pmoles/min per mg protein) to about 3 pmoles/min per mg protein.

NaF has a strongly activating affect on the enzyme. Fig. 1 shows the activity as a function of the fluoride concentration. Maximal activity occurs at 10–40 mM NaF. The fluoride (10 mM)-stimulated activity of the various enzyme preparations ranges from 15 to 40 pmoles/min per mg protein. The possible cause of this variability will

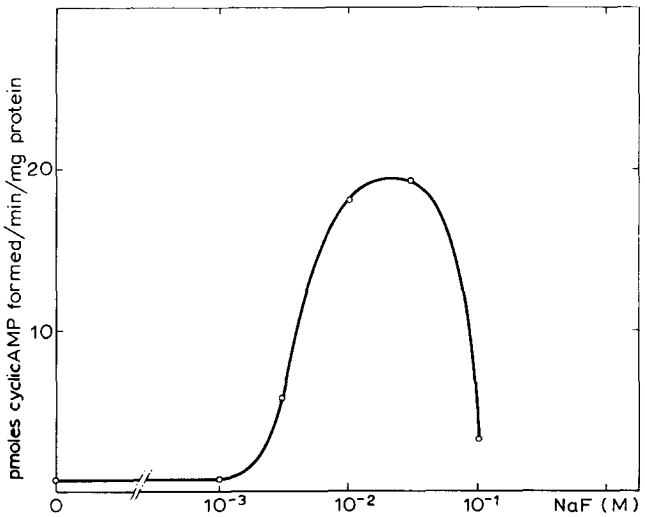

Fig. 1. Effect of NaF on adenylate cyclase activity. Freshly prepared enzyme preparation was incubated for 15 min with varying concentrations of NaF.

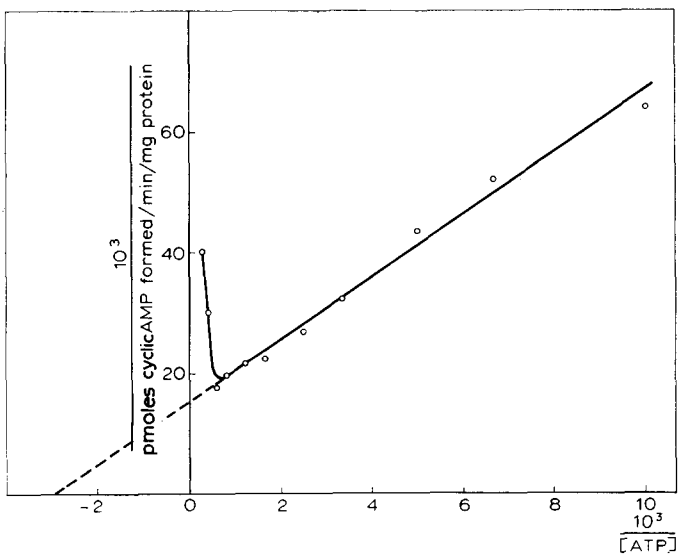

Fig. 2. Lineweaver–Burk plot for the relation between NaF-stimulated adenylate cyclase activity and ATP concentration. Freshly prepared enzyme preparation was incubated for 15 min with varying ATP concentrations in the presence of 10 mM NaF.

be discussed later in this paper. The 4000 × *g* particulate fraction contains about 2/3 of the total pancreatic enzyme activity.

Properties of the NaF-stimulated enzyme

The very low basal activity of the enzyme makes it impossoble to determine the properties of the enzyme in the non-stimulated condition. Hence, we have chosen to study these properties in the presence of 10 mM NaF. The enzyme rate under our assay conditions was constant up to 30 min incubation and up to at least 150 μg of enzyme protein per tube. Hence, all further experiments are carried out with 10- or 15-min incubation periods and with less than 150 μg protein to ensure linear assay conditions.

Fig. 2 represents a Lineweaver–Burk plot for the relation between enzyme activity and substrate concentration. The value for K_m is approximately 0.3 mM ATP. A concentration of 0.4 mM ATP is used in all subsequent enzyme assays. Although this concentration is suboptimal, there are valid reasons for this choice: firstly, the need to maintain a high specific radioactivity of the substrate and thus of the radioactive cyclic AMP formed, and secondly the occurrence of substrate inhibition at ATP concentrations above 1.5 mM ATP.

The relation between enzyme activity and pH of the incubation medium is

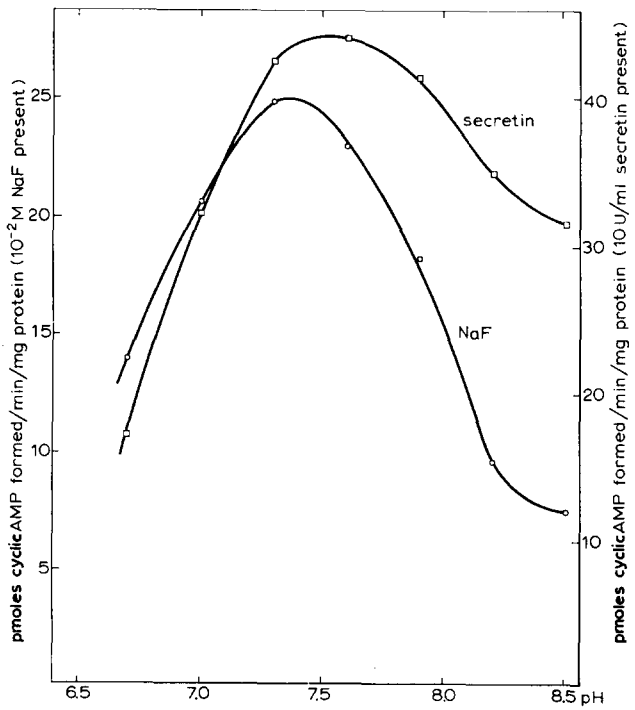

Fig. 3. Effect of pH on NaF-stimulated (○—○) and secretin-stimulated (□—□) adenylate cyclase activity. Both curves were made with different freshly prepared enzyme preparations, which were incubated in media prepared with 30 mM Tris/30 mM HEPES buffers. With synthetic secretin the incubation time was 10 min.

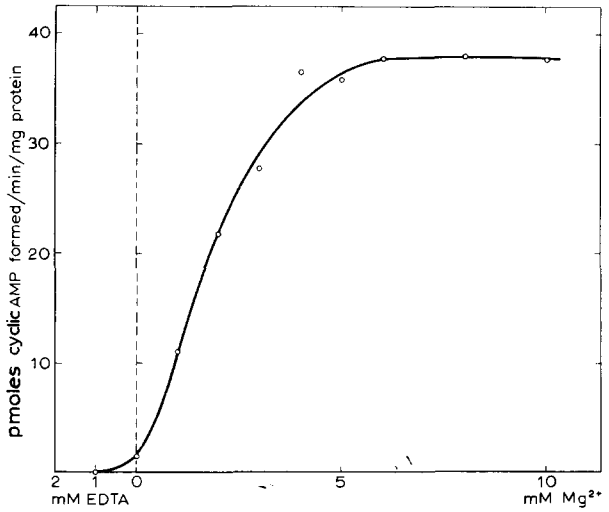

Fig. 4. Effect of Mg^{2+} and EDTA on NaF-stimulated adenylate cyclase. Freshly prepared enzyme preparation was incubated in the presence of varying $MgCl_2$ concentrations or of 1 mM EDTA. Due to the Mg^{2+} and EDTA contained in the enzyme preparation the incubation medium always contained 0.5 mM Mg^{2+} and EDTA additionally.

shown in Fig. 3. The pH optimum of the NaF-stimulated adenylate cyclase activity is 7.4.

Fig. 4 shows the effect of varying Mg^{2+} concentrations on the fluoride-stimulated activity. The rate of cyclic AMP formation reaches a maximal value at a concentration of 5 mM Mg^{2+}. When no Mg^{2+} is added some residual activity is left, which can be abolished by addition of 1 mM EDTA. This suggests that the residual activity in the absence of added Mg^{2+} is due to the presence of some bivalent cation in the enzyme preparation.

Some incubations have been carried out in the absence of theophylline in order to determine whether this substance has any inhibitory effect on adenylate cyclase, stimulated by 10 mM NaF. To minimize hydrolysis of the reaction product by phosphodiesterase an excess unlabeled cyclic AMP (0.5 mg/ml, corresponding to approximately 1.5 mM) is added. The activities in these experiments are low and vary considerably, and otherwise non-observable fluorescent spots appear on the thin-layer plate, presumably due to the action of phosphodiesterase and other phosphatase activities. Since no increase in adenylate cyclase activity is found, theophylline has been included in all other assays to inhibit breakdown of cyclic AMP.

Trypsin inhibitor is added to the assay medium in order to preclude the possible action of trypsin from the zymogen granules on the enzyme. An effect of trypsin on hormone-stimulated adenylate cyclase in fat cells has been reported (Rodbell *et al.*[8]). In control experiments the presence of this inhibitor has no adverse influence on rat brain adenylate cyclase activity. Therefore, even though omission of trypsin inhibitor in some pancreatic adenylate cyclase assays shows no clear decrease in enzyme activity, it has been added routinely to the assay medium.

Effect of secretin and pancreozymin on adenylate cyclase activity

Rat pancreatic adenylate cyclase, in the absence of NaF, is stimulated by the

hormone secretin (Fig. 5). Secretin in final concentrations of 0.01 to 10 units/ml causes a progressive increase in activity, which remains constant at still higher concentrations. The maximal activity is slightly less than the maximal NaF-stimulated activity. The pH optimum for the secretin-stimulated activity is virtually the same as for the NaF-stimulated activity although the curve for secretin is flattened out on the alkaline side (Fig. 3). The secretin (10 units/ml)-stimulated enzyme rate is constant for at least 15 min of incubation and up to 150 μg enzyme protein per tube. The effect of secretin on the adenylate cyclase activity is not additive to that of 10 mM NaF; lack of additivity is commonly observed for this enzyme with hormone and fluoride in other tissues. Half-maximal activation occurs at a secretin concentration of 0.15 unit/ml, which at an activity of 3 units/μg and a molecular weight of 3056 is equivalent to $1.5 \cdot 10^{-8}$ M.

The enzyme, in the absence of NaF, is also stimulated by pancreozymin (Fig. 6). A similar curve as with secretin is obtained for highly purified pancreozymin in concentrations up to 150 units/ml. Although higher concentrations could not be tested due to the limited amount of material available, the activity seems to reach a maximum at about the same level as for secretin, and slightly less than the maximal NaF-stimulated activity. For pancreozymin also, its effect on the adenylate cyclase activity is not additive to that of 10 mM NaF. Half-maximal activation occurs at a pancreozymin concentration of approximately 20 units/ml, which at an activity of 3 units/μg and a molecular weight of 3915 is equivalent to $1.5 \cdot 10^{-6}$ M. This half-maximally activating concentration is 100 times higher than that for secretin. The enzyme activity is not further increased in the presence of maximally activating concentrations of each hormone, *i.e.* there is no additive effect of the two hormones.

It occurred to us that the high half-maximal activating concentration of pancreozymin and the non-additivity of the hormone effects might mean that the purified pancreozymin preparation was contaminated by a small amount of secretin.

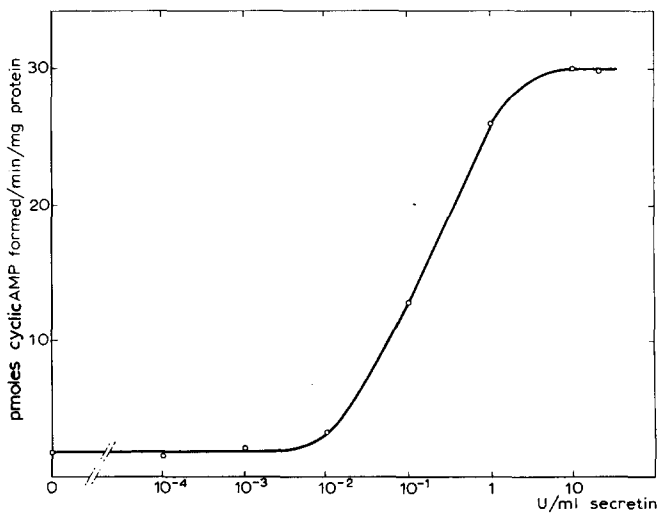

Fig. 5. Effect of synthetic secretin on adenylate cyclase activity. Freshly prepared enzyme preparation was incubated for 10 min in the presence of varying concentrations of synthetic secretin.

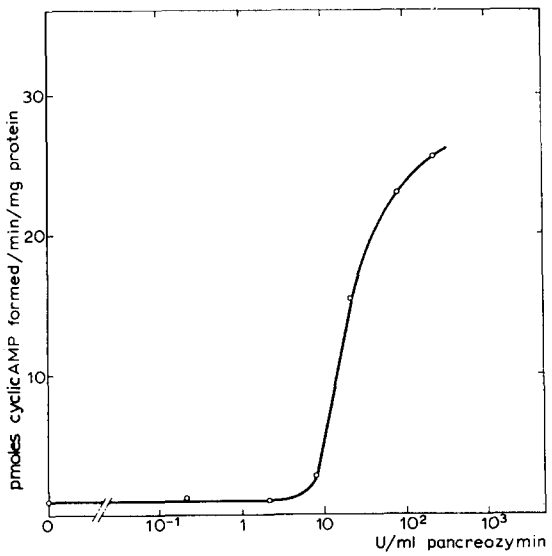

Fig. 6. Effect of highly purified pancreozymin on adenylate cyclase activity. Freshly prepared enzyme preparation was incubated for 10 min in the presence of varying concentrations of purified pancreozymin.

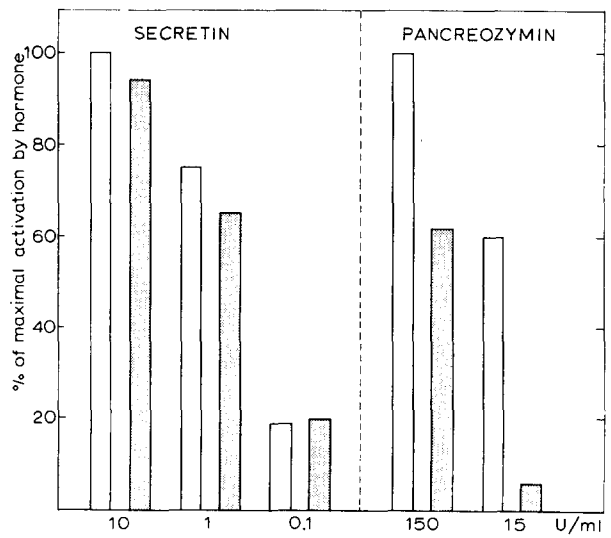

Fig. 7. Effect of oxidation of synthetic secretin and purified pancreozymin on their activation of adenylate cyclase. To solutions of 10 units synthetic secretin and 150 units highly purified pancreozymin in 50 μl water 50 μl of a solution of 0.6% H_2O_2 in 1 M acetic acid was added. After 45 min at room temperature each solution was diluted with 500 μl distilled water and then lyophilized. Freshly prepared enzyme preparation was incubated for 10 min in the presence of different concentrations of the oxidized and non-oxidized hormones. Other conditions were as described in Materials and Methods. The results were expressed as percentage of the maximal obtainable activation by the relevant hormone. Open bars: non-oxidized hormones. Shaded bars: oxidized hormones.

This possibility has been tested by utilizing the different effects of oxidation on the two hormones. Small amounts of each hormone are subjected to oxidation by H_2O_2 according to Mutt[9] before incubation. This treatment destroys 90 % of the physiological effects of pancreozymin in the rat, on enzyme secretion[9] as well as on fluid secretion[10], while secretin is not affected by it. The effect of prior oxidation of the hormones on their activation of rat pancreatic adenylate cyclase is shown in Fig. 7. Both maximally and submaximally stimulating concentrations are used. The activation caused by secretin (0.1, 1 and 10 units/ml) is virtually unaffected at any concentration. Pancreozymin in submaximally activating (15 units/ml) and nearly maximally activating concentration (150 units/ml) demonstrated 90 % and 40 % losses in activation after oxidation. Both losses represent approximately 90 % inactivation of the hormone by oxidation, as can be seen from the activating effect of 15 units/ml non-oxidized pancreozymin shown in Fig. 7.

Since the purified hormone preparations were available only in limited amounts, clinical preparations were also tested. Secretin preparations from two sources stimulated pancreatic adenylate cyclase, but the maximally obtainable activity was much lower than in the case of the pure preparation. Furthermore, after reaching a maximum with increasing amounts of the hormone, the activity fell again to a lower level at still higher concentrations of secretin (4 units/ml, Sigma). Addition of 4 units/ml of this secretin preparation to an assay medium containing 10 mM NaF lowered the NaF-stimulated enzyme activity by 90 % (Fig. 8). These effects were not observed with the synthetic preparation, suggesting that the clinical preparation contains an inhibitor of adenylate cyclase. With pancreozymin (Sigma) no enzyme activation was observed, while with pancreozymin (Boots) a similar behaviour as with secretin (Sigma) was observed except that the fluoride activation was not decreased.

Effects of other hormones and autonomic stimulants

In view of the structural similarity of secretin and glucagon on the one hand, and pancreozymin and gastrin on the other, the effects of preparations of glucagon

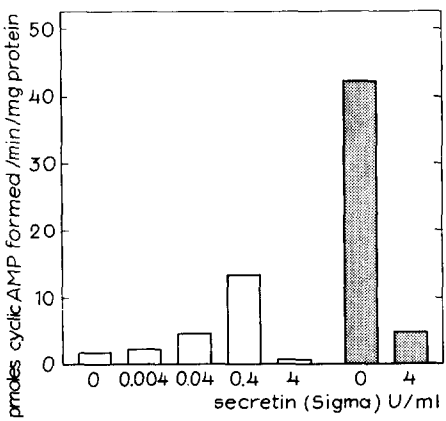

Fig. 8. Effect of a commercial secretin preparation on basal activity and NaF-stimulated activity. Freshly prepared enzyme preparation was incubated with varying concentrations of secretin (Sigma). In some incubations 10 mM NaF was also present. Open bars: no NaF present. Shaded bars: 10 mM NaF present.

and gastrin on the enzyme have also been tested. With glucagon no activation is found, which also is true for pentagastrin, a synthetic analogue of gastrin.

Since pancreatic enzyme output is known to be stimulated by acetylcholine and carbachol, these compounds have also been tested. Neither compound, in concentrations ranging from 10^{-6} to 10^{-2} M, causes accumulation of cyclic AMP above the basal level.

The same observation is made when epinephrine, norepinephrine or isoprenaline are tested in this concentration range. No increase of the basal activity is obtained. Neither does epinephrine change the activation of the enzyme caused by 10 units/ml secretin.

Effects of Ca^{2+}

Inhibition of adenylate cyclase activity by Ca^{2+} has been reported for several tissues. Therefore, the effect of Ca^{2+} on the pancreatic enzyme preparation has also been investigated. Ca^{2+} added in concentrations ranging from 0.1 to 5 mM progressively inhibits the enzyme activity in the presence of either secretin or fluoride (Fig. 9).

Changes in adenylate cyclase activity after pancreatic stimulation in vivo

The large range in both basal and NaF- or secretin-stimulated adenylate cyclase activities (Table I, column I) led us to measure the enzyme activity in starved rats (column II) and in rats maximally stimulated *in vivo* by feeding and injection of secretin and pancreozymin (column III). While the basal activity is not significantly increased, the NaF- and secretin-stimulated activities are greatly and significantly increased in the latter group of animals. Since the activities are expressed per mg

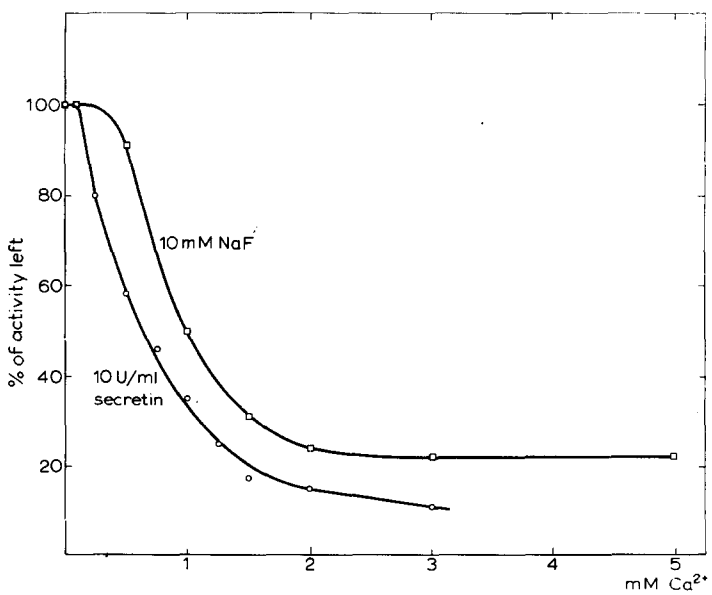

Fig. 9. Inhibition of NaF-stimulated and secretin-stimulated adenylate cyclase by Ca^{2+}. Freshly prepared enzyme preparation was incubated with varying Ca^{2+} concentrations in the presence of 10 mM NaF for 15 min or in the presence of 10 units/ml synthetic secretin for 10 min. Adenylate cyclase activity was plotted as percentage of activity in the absence of Ca^{2+}.

protein present in the preparation, the increased activities could be due to a lowering of the protein content through secretion of the digestive enzymes. This explanation is unlikely for the following reasons. The preparation of the particulate adenylate cyclase involves hypotonic treatment in order to lyse the zymogen granules and to remove soluble proteins. The slightly higher protein yield, which we find in the preparations from starved animals can account for only about a third of the difference of the specific activities in columns II and III.

TABLE I

EFFECT OF EXTENSIVE STIMULATION ON SPECIFIC ADENYLATE CYCLASE ACTIVITY

One group of four rats was starved for 20 h prior to removal of the pancreas (column II). Another group of four rats had free access to food and was intravenously injected with 5 units each of pancreozymin (Boots) and secretin (Boots) 17 h and with 10 units each 1.5 h before sacrifice (column III). Enzyme preparations from each animal were made and assays were carried out in the presence of 10 mM fluoride, 10 units/ml secretin or no addition (10 min incubation). Other conditions were as described in Materials and Methods. Enzyme activity is given in pmoles cyclic AMP formed/min per mg protein. Column I shows for purposes of comparison the range for the specific activity in all animals investigated previously. In columns II and III averages are given with S.E. of the mean.

Addition	*I* *Normal range*	*II* *Starved, no hormones*	*III* *Food, hormones injected*	*Difference between II and III*
No	0–3	1.1 ± 0.4	1.9 ± 0.8	Not significant
10 mM NaF	≈15–40	19.2 ± 1.8	45.0 ± 4.8	$P<0.001$
10 units/ml secretin	≈15–40	16.3 ± 1.6	35.4 ± 2.4	$P<0.001$

DISCUSSION

The basal activity of rat pancreatic adenylate cyclase is lower than that reported for most other tissues. Addition of fluoride raises the activity to a level which is more commonly found in other tissues. The kinetic properties of the NaF-stimulated enzyme are rather similar to those observed for several other tissues, *e.g.* the pH optimum of 7.4, a K_m value for ATP of 0.3 mM and the Mg^{2+} activation and Ca^{2+} inhibition characteristics. The substrate inhibition (Fig. 2) is not uncommon for the fluoride-stimulated enzyme. Pohl *et al.*[11] reported a similar substrate inhibition in rat liver plasma membranes, when the adenylate cyclase was stimulated by NaF, but with glucagon as the activating agent there was no substrate inhibition. Although these authors do not calculate the K_m value for ATP, their v–$[S]$ curves suggest that it is the same in the presence of either activating agent.

The most interesting effects are, of course, those of different hormones on the enzyme activity. First, the synthetic secretin preparation used in our experiments has a strongly activating effect. This observation supports the suggestion of Case *et al.*[2] that secretin would exert its action on water and electrolyte secretion *via* cyclic AMP, and agrees with their observation[12] that cyclic AMP levels in the isolated cat pancreas rise after perfusion of secretin. To our knowledge, a stimulating effect of secretin on adenylate cyclase has so far been reported only for one other tissue, namely fat cells of the rat[8].

Pancreatic adenylate cyclase is not stimulated by glucagon although there is

a great similarity in structure between glucagon and secretin, which are thought to be derived from the same parent structure in evolution[14]. Rat liver adenylate cyclase, on the other hand, responds to glucagon, but not to secretin[8]. This supports once again the theory that adenylate cyclase in different tissues can have highly specific hormone receptor sites. The receptor site in fat cells appears to be less specific, since their adenylate cyclase is stimulated by secretin as well as by glucagon, although the maximal activation by the latter hormone is lower than that by the former[8]. Trypsin treatment of the fat cells, moreover, completely abolishes the activation by glucagon, but only partly that by secretin, suggesting partly or wholly differing receptor sites.

One further comment about the absence of pancreatic adenylate cyclase stimulation by glucagon is in order. The hormone is thought to exert its insulin releasing action in pancreatic β-cells through cyclic AMP[15] and thus would be expected to stimulate the adenylate cyclase present in these cells. Since however, the islets of Langerhans form only a few percent of the total amount of pancreatic tissue, they should make only a minor contribution to total adenylate cyclase activity. Hence, a stimulation of this small fraction of enzyme activity by glucagon might well go unnoticed in our experiments.

The effects on pancreatic adenylate cyclase of compounds affecting enzyme secretion have also been investigated in some detail. Pancreozymin causes nearly the same maximal stimulation of rat pancreatic adenylate cyclase as secretin, though at a 100-fold molar concentration. The relative insensitivity for pancreozymin could mean that this effect be due to contamination of the pancreozymin preparation with secretin. However, the results of the experiments, in which the hormones were subjected to prior oxidative treatment, appear to rule out this possibility. The non-additivity of the effects of maximally stimulating concentrations of pancreozymin and secretin suggests that both hormones exert their action on the same adenylate cyclase. The question arises which adenylate cyclase this would be: the adenylate cyclase of the enzyme secreting cells, or that of the fluid secreting cells. The possibility that both types of cells would have the same adenylate cyclase is highly improbable, because of the clearly different physiological actions of secretin and pancreozymin. A more likely possibility is that the adenylate cyclase system in the fluid secreting cells responds to both hormones. This hypothesis is supported by the observation of Heatley[10] that pure secretin in the pancreas has no effect on enzyme secretion, while pure pancreozymin stimulates both enzyme secretion and fluid secretion. The fact that in his experiments pancreozymin on a molar basis is less active than secretin on fluid secretion may explain, at least in part, why in our experiments much more pancreozymin than secretin is needed for the same effect on adenylate cyclase. Pentagastrin, which resembles pancreozymin and gastrin in having an identical C-terminal tetrapeptide, and gastrin stimulate pancreatic enzyme secretion *in vivo*[16–18] as well as *in vitro*[4]. However, pentagastrin has no stimulating effect on rat pancreatic adenylate cyclase. These observations lead us to the conclusion that the adenylate cyclase activity in our preparation represents mainly that of the fluid secreting cells. This could also explain why cholinergic agents, which cause enhanced enzyme secretion *in vitro* as well as *in vivo*[19] do not stimulate the pancreatic adenylate cyclase activity. However, the absence of enzyme stimulation by cholinergic agents may also be due to the inability of these substances to stimulate the enzyme[20].

Although our experiments do not contribute additional conclusive evidence

for a role of cyclic AMP in the mechanism of enzyme secretion, considerable support for such a role is found in the previously cited literature. An additional consideration is, that pancreozymin will not pass the cell membrane, because of its large molecular size which makes a messenger function for cyclic AMP or possibly another cyclic nucleotide likely.

Adrenergic compounds do not stimulate pancreatic adenylate cyclase which agrees with the observation that these compounds do not elicit any stimulatory response in the pancreas[19]. The fact that epinephrine does not inhibit secretin stimulated adenylate cyclase is of interest, because in the isolated rabbit pancreas α-adrenergic stimulation has been observed to inhibit fluid secretion[21].

The large range in both basal and fluoride- or secretin-stimulated adenylate cyclase activities, which we observed in normal rats, requires a comment. Since we felt that this large range might be due to differences in food uptake immediately before sacrifice of the animals, we compared the activities in enzyme preparations from starved animals and from animals extensively stimulated by free access to food and double injections of secretin and pancreozymin. Preparations from the starved animals give a specific activity coinciding with the lower end of the range, while preparations from the stimulated animals have an activity equal to the upper end. This suggests that the large range of activities in the normal rats is indeed due to differences in food uptake.

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