Biochimica et Biophysica Acta, 370 (1974) 573–584

BBA 67371

RAT PANCREAS ADENYLATE CYCLASE
II INACTIVATION AND PROTECTION OF ITS HORMONE RECEPTOR SITES*

H J M KEMPEN, J J H H M DE PONT and S L BONTING

Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen (The Netherlands)

(Received June 26th, 1974)

SUMMARY

1 The reason for the 100-fold lower sensitivity to pancreozymin than to secretin of rat pancreas adenylate cyclase (EC 4 6 1 1) has been investigated

2 Preincubation of the enzyme preparation in the cold decreases the activation of the enzyme by 3 10^{-7} M pancreozymin-C-octapeptide to a greater extent than that by 10^{-7} secretin, while the basal or fluoride stimulated activity is not affected

3 Addition of phosphatidylserine (1 mg/ml) to the assay medium causes a marked increase in the stimulation by 3 10^{-7} M pancreozymin-C-octapeptide, little increase in the stimulation by 10^{-7} M secretin and no effect on the basal and fluoride stimulated activities

4 This phospholipid effect decreases in the order phosphatidylcholine > phosphatidylserine > phosphatidylethanolamine > phosphatidylinositol, the last substance having little or no effect

5 Addition of phosphatidylserine lowers the half-maximally stimulating concentration of pancreozymin-C-octapeptide without changing the maximal enzyme activity obtained with this hormone

6 In the presence of phosphatidylserine (1 mg/ml) cyclic AMP production by the pancreozymin-C-octapeptide stimulated adenylate cyclase remains linear for 10 min, whereas in its absence production is linear for at most 2 min

7 The effects of addition of lysophosphatidylcholine, fatty acids and bovine serum albumin on the basal and pancreozymin-C-octapeptide stimulated adenylate cyclase activities indicate that the reduction in hormone sensitivity is not caused by phospholipase breakdown products, but rather by direct enzymatic removal of phospholipids from the hormone receptor area

8 In the presence of phosphatidylserine (1 mg/ml), like in its absence, there is no additivity of the effects of pancreozymin-C-octapeptide and secretin, confirming the previous conclusion that both hormones act on a single adenylate cyclase, which may have two hormone receptors

* Presented in preliminary form at the VIIth Alfred Benzon Symposium on "Secretory mechanisms of exocrine glands", Copenhagen, 9–12 September 1973, and in an abstract [25]

INTRODUCTION

Evidence for a role of cyclic AMP in the stimulation of pancreatic enzyme secretion by pancreozymin has been reported by Ridderstap and Bonting [1], working with the isolated rabbit pancreas in vitro They observe a stimulation of the enzyme secretion by theophylline and by exogenous cyclic AMP, and also a potentiation of the pancreozymin effect by theophylline These effects cannot be due to a wash-out effect through stimulation of the fluid secretion, as is shown by the subsequent observation of Rutten et al [2] that papaverine induces an enhanced protein secretion, while the fluid secretion is not changed or slightly decreased Kulka and Sternlicht [3] have obtained a stimulation of enzyme release from mouse pancreas in vitro with exogenous cyclic AMP, while Bauduin et al [4] have reported similar findings with dibutyryl cyclic AMP for rat pancreas slices On the other hand, a role for cyclic AMP has also been claimed for the stimulation by secretin of the fluid secretion from the isolated cat pancreas [5, 6]

In view of these findings, the enzymes concerned with the metabolism of cyclic AMP in the pancreas have been studied by Rutten et al [7] They have established the presence and properties of adenylate cyclase (EC 4 6 1 1, ATP pyrophosphate-lyase) in rat pancreas, and have demonstrated that it can be stimulated considerably by both secretin and pancreozymin Surprisingly, the half maximally stimulating concentration of the latter hormone was about 100 times higher than that of the former By selective peroxidation it could be shown that the pancreozymin effect was not due to contamination with secretin of the pancreozymin preparation used in these experiments The effects of these two hormones, when added together in maximally stimulating concentrations, are not additive This suggests that both hormones act on the same adenylate cyclase

The effects of both hormones on rat pancreatic adenylate cyclase have now been investigated in more detail First we have considered whether the low pancreozymin sensitivity was perhaps due to an artifactual inactivation of the cyclase before or during its assay Evidence has been obtained for a phospholipolytic inactivation of the sensitivity to pancreozymin-C-octapeptide, which can be averted by the addition of phospholipids to the assay medium With this modification in the assay procedure, the effects of both hormones have been reevaluated

MATERIALS AND METHODS

Materials

The synthetic C-terminal octapeptide of pancreozymin (SQ 19 844, gift of Dr M Ondetti, The Squibb Institute for Medical Research, New Brunswick, N J , U S A), in this paper denoted as pancreozymin-C-octapeptide, is used instead of pancreozymin Synthetic secretin has been kindly donated by Dr M Wunsch (Max Planck Institut fur Biochemie, Munich, GFR)

Phosphatidylcholine is prepared from egg yolk Phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol are isolated from bovine brain white matter by column-chromatography on DEAE-cellulose, as described by Rouser et al [8] For phosphatidylserine, the modification of Sanders [9] is used After elution from the column, phosphatidylinositol is twice re-chromatographed by preparative thin-

layer chromatography on silica gel The phospholipids are stored in benzene–ethanol (4 1, by vol) at −20 °C Phosphatidylinositol, purified from pig liver, is obtained from Serdary Research, London, Ontario, Canada Lysophosphatidylcholine has been prepared by Dr Daemen of this laboratory from egg yolk phosphatidylcholine by treatment with snake venom, followed by extraction in hexane–ethanol until chromatographically pure, and is stored in 95% ethanol at −20 °C Palmitic, oleic and linoleic acid are from Fluka (Buchs, Switzerland) Bovine serum albumin (Fraction V) is obtained from Sigma Chemical Co (St Louis, Mo , U S A), fatty acids are removed according to the procedure of Chen [10] The substrate [α-^{32}P]ATP is purchased from The Radiochemical Centre (Amersham, England), and is routinely purified by ion-exchange chromatography

Methods

Tissue preparation and adenylate cylcase assays are performed as previously described [7]

Phospholipids, lysophosphatidylcholine and fatty acids, after evaporation of solvents in a stream of N_2, are suspended in a solution, containing 10 mM Tris–HCl buffer, 2 5 mM $MgCl_2$, 2 5 mM Na_2EDTA (final pH 7 4), by means of ultrasonic treatment at 0 °C under N_2 (Branson sonifier, Type B 12, equipped with microtip, power delivery 50 W) Sonication is performed in 10-s periods until no further clarification is observed The suspensions are centrifuged at 7000 × *g* for 1 min in order to remove metal particles Bovine serum albumin is dissolved in the Tris buffer described above, and the pH is adjusted to 7 4 by addition of 2 M NaOH Lipids, serum albumin, hormones and fluoride are all added to the assay medium in 5-μl volumes The resulting solution is thoroughly stirred before the start of the assay In the absence of added substances, 5 μl double distilled water is added, so as to keep the final assay volume at 50 μl All concentrations are given as final concentrations in the assay medium The concentration of lipid is expressed in mg/ml, derived from dry weight determination Phospholipase A assay is performed according to Figarella and Ribeiro [11] at 37 °C in a modified substrate medium, containing egg yolk lecithin, 0 5 mM Tris-HCl buffer, 0 6 mM sodium desoxycholate, 2 5 mM $MgCl_2$, 2 5 mM EDTA, and 0 2 mg/ml soybean trypsin inhibitor (pH 7 4)

RESULTS

Stability of the adenylate cyclase activity

In preliminary experiments it had been noticed, that brief pre-incubation of the enzyme preparation at 37 °C yields decreased basal and fluoride-stimulated adenylate cyclase activities, sensitive only to unusually high hormone concentrations This suggests, that inactivation of the cyclase can take place in the normal assay procedure In order to determine, at which stage of the procedure inactivation takes place (preparation of the 4000 × *g* pellet at 0 °C or enzyme assay at 37 °C), the effects of pre-incubation at 0 and 37 °C on the basal adenylate cyclase activity and the hormone- and fluoride-stimulated activities have been investigated

Fig 1 shows that after 30 min pre-incubation at 0 °C the stimulation by 3 10^{-7} M pancreozymin-C-octapeptide, and to a smaller extent that by 3 10^{-7} M secretin, are reduced with respect to the controls, but that the basal and fluoride-stimulated

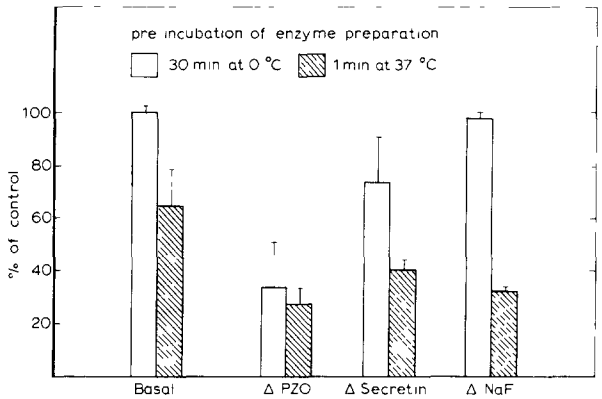

Fig 1 Effects of preincubation of the enzyme preparation on the basal and stimulated rat pancreas adenylate cyclase activities, expressed as percentage of activity without preincubation in each case △PZO, △Secretin and △NaF refer to activation due to the presence of 3 10^{-7} M pancreozymin-C-octapeptide, 10^{-7} M secretin and 10^{-2} M NaF, respectively The length of the vertical lines represents the standard error for three experiments with different enzyme preparations

activities are not affected On the other hand, pre-incubation at 37 °C for 1 min considerably decreases both the basal activity, as well as the stimulation by hormones and fluoride This suggests that the hormone sensitivity, particularly to pancreozymin-C-octapeptide, can already decrease during the preparation of the 4000 × *g* pellet at 0 °C

Further experiments have been aimed at determining whether phospholipase A activity, normally present in pancreas, may be responsible for the observed decrease in hormone sensitivity It is known, that phospholipase A can selectively decrease or abolish the hormone sensitivity of liver or thyroid adenylate cyclase without affecting the basal and fluoride-stimulated activities [12–14] When assayed in a medium, similar in ionic composition to the homogenization medium, the washed

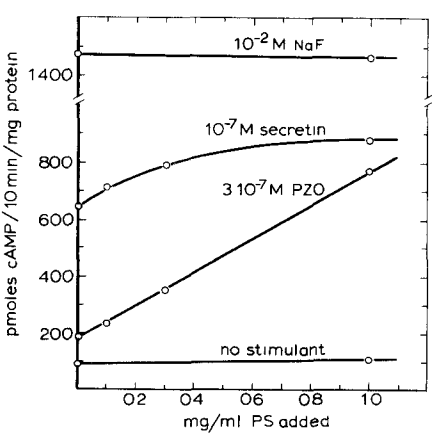

Fig 2 Effects of addition to the assay medium of increasing concentrations of phosphatidylserine (PS) on basal and stimulated rat pancreas adenylate cyclase activities

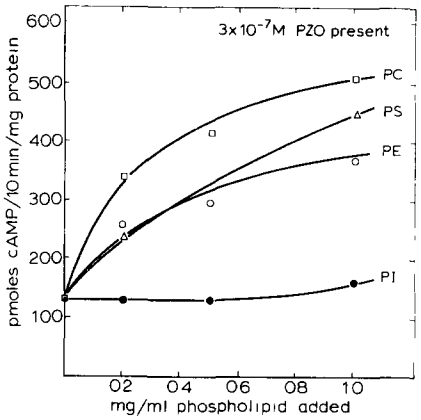

Fig 3 Effect of addition to the assay medium of increasing concentrations of various phospholipids on the pancreozymin-C-octapeptide stimulated rat pancreas adenylate cyclase activity □—□ phosphatidylcholine, △—△ phosphatidylserine, ○—○ phosphatidylethanolamine, ●—● phosphatidylinositol

4000 × *g* pellet used as enzyme preparation appears to contain 38% (S E 3%, $n = 3$) of the phospholipase activity initially present in the fresh total homogenate

Effects of addition of phospholipids to the assay medium for adenylate cyclase

Fig 2 shows that the stimulation of the adenylate cyclase by 3 10^{-7} M pancreozymin-C-octapeptide is greatly increased by addition of phosphatidylserine in increasing concentrations The stimulation by 10^{-7} M secretin, which is much higher than that by pancreozymin-C-octapeptide to begin with, is only slightly increased by the addition of phosphatidylserine The basal and fluoride-stimulated activities are not significantly affected by the phospholipid

Various natural phospholipids have been tested for their effect on the pancreo-

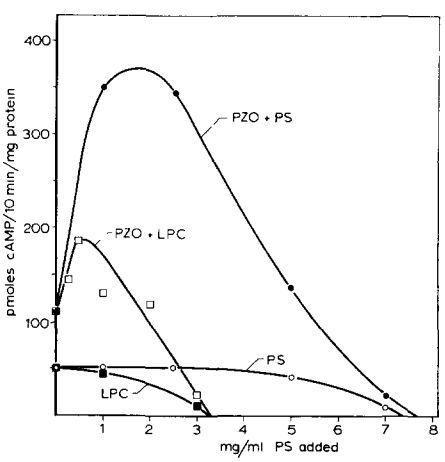

Fig 4 Effect of addition to the assay medium of increasing concentrations of phosphatidylserine and lysophosphatidylcholine on the basal and pancreozymin-C-octapeptide (3 10^{-7} m) stimulated rat pancreas adenylate cyclase activities

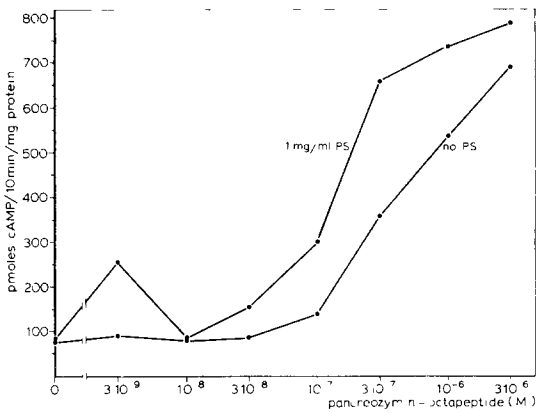

Fig 5 Stimulation curves for the effect of pancreozymin-C-octapeptide on rat pancreas adenylate cyclase activity in the presence or absence of phosphatidylserine (1 mg/ml)

zymin-C-octapeptide-stimulated adenylate cyclase activity (Fig 3) In a concentration of 1 mg/ml, the effect decreases in the following order phosphatidylcholine > phosphatidylserine > phosphatidylethanolamine (all from bovine brain) > phosphatidylinositol (from pig liver) Phosphatidylinositol from bovine brain is not effective at all None of these phospholipids has an appreciable effect on the basal activity in the concentrations tested The optimal effect of phosphatidylserine is found between 1 0 and 2 5 mg/ml (Fig 4), addition of 5 mg/ml has little effect, while higher concentrations are inhibitory to the basal as well as the hormone-stimulated activity

The stimulation curve for pancreozymin-C-octapeptide is markedly shifted to lower hormone concentrations in the presence of 1 mg/ml phosphatidylserine (Fig 5) The half-maximal stimulating concentration of the hormone is lowered from 7 0 10^{-7} to 1 5 10^{-7} M, while the maximally stimulated enzyme activity is not significantly of the altered

In the absence of added phospholipid the pancreozymin-C-octapeptide-stimulated rate of cyclic AMP production decreases rapidly during the first minute of incubation at 37 °C, whereas in the presence of 1 mg/ml phosphatidylserine it remains constant during the first 10 min and decreases only slightly thereafter (Fig 6) On the other hand, the added phospholipid has no effect on the basal and fluoride-stimulated production rates Apparently, the added phospholipid acts by preventing a reduction hormonal stimulation during the incubation

Mechanism of the phospholipid effect

Addition of phospholipid during preparation or pre-incubation of the enzyme preparation in the cold is not more effective than addition at the start of the assay (not shown) This suggests that the added phospholipid cannot protect the adenylate cyclase against inactivation occurring during the preparation of the enzyme in the cold

Fig 6 shows that upon addition of phosphatidylserine (1 mg/ml) 2 min after the start of the incubation (circles) the activity in the presence of pancreozymin-C-octapeptide is not higher than that observed without addition of the phospholipid

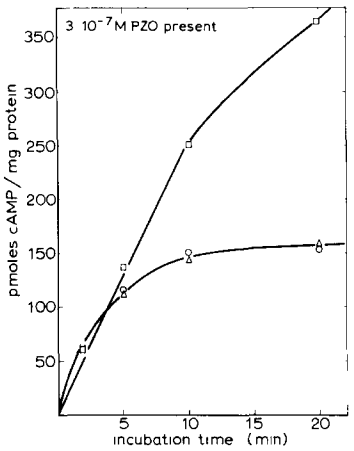

Fig 6 Time course for the pancreozymin-C-octapeptide-stimulated formation of cyclic AMP by rat pancreas adenylate cyclase phosphatidylserine (1 mg/ml) is absent (△—△), present from $t = 0$ (□—□), or added after 2 min (○—○)

(triangles) This indicates that the major inactivation of the pancreozymin receptor occurs during the first 2 min of incubation at 37 °C

In view of the likelihood that the hormone receptor inactivation is due to the action of phospholipase A present in the 4000 × g pellet, we have investigated the effects of the products of phospholipase action lysophospholipids and free fatty acids Addition of lysophosphatidylcholine in concentrations below 2 mg/ml in the presence of the octapeptide results in a small enhancement of the hormone effect, while at higher concentrations the activity is greatly inhibited (Fig 4) The basal activity is decreased by all concentrations of lysophosphatidylcholine The enhancement of the pancreozymin-C-octapeptide-stimulated activity observed with low concentrations precludes the possibility that endogenously formed lysophospholipids could be responsible for the loss of the hormone receptor activity

On the other hand, palmitic, oleic and linoleic acid inhibit the pancreozymin-C-octapeptide-stimulated adenylate cyclase activity, the saturated palmitic acid being less inhibitory than the unsaturated oleic and linoleic acid In a concentration of 0 5 mg/ml palmitic acid causes a decrease of 23% (Fig 7), oleic acid of 42% (Fig 7) and linoleic acid of 79% (57% at 0 2 mg/ml)

The fatty acid effect has been further investigated by testing whether bovine serum albumin, which is known to bind fatty acids, could elevate the hormone response The addition of 10 mg/ml bovine serum albumin to the assay medium indeed results in a greatly increased stimulation by pancreozymin-C-octapeptide, while the basal activity is again not affected (Fig 7) However, when oleic acid and serum albumin are added together, nearly the same amount of inhibition is found as with oleic acid alone (Fig 7) Furthermore, a fatty acid depleted preparation of serum albumin gives the same increase in pancreozymin-C-octapeptide-stimulated activity as does untreated serum albumin (Fig 7) Finally, oleic acid gives the same degree of inhibition in the presence of 1 mg/ml phosphatidylserine as in its absence (Fig 7) Simultaneous addition of serum albumin (10 mg/ml) and phosphatidylserine (1 mg/ml) does not give more stimulation than albumin alone (Fig 7)

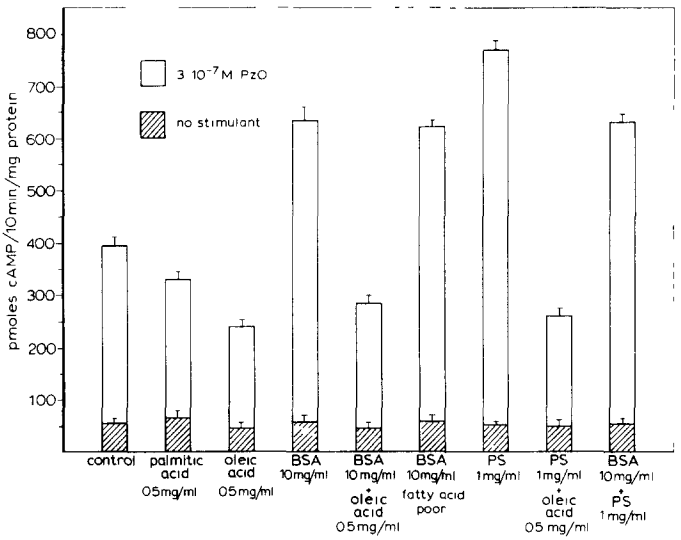

Fig 7 Effects on the basal and pancreozymin-C-octapeptide stimulated rat pancreas adenylate cyclase activities of addition to the assay medium of 0 5 mg/ml palmitic acid, 0 5 mg/ml oleic acid, 10 mg/ml bovine serum albumin, 10 mg/ml bovine serum albumin + 0 5 mg/ml oleic acid, 10 mg/ml fatty-acid-poor bovine serum albumin, 1 mg/ml phosphatidylserine, 1 mg/ml phosphatidylserine + 0 5 mg/ml oleic acid, and 10 mg/ml bovine serum albumin + 1 mg/ml phosphatidylserine The length of the vertical lines indicates the range of duplicate determinations, all performed with the same enzyme preparation

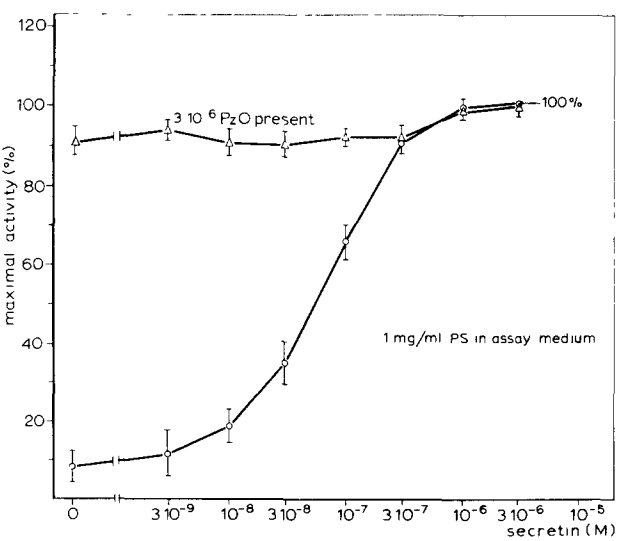

Fig 8 Stimulation curves for the effect of secretin on rat pancreas adenylate cyclase in the presence or absence of 3 10^{-6} M pancreozymin-C-octapeptide Phosphatidylserine is present in a concentration of 1 mg/ml The length of the vertical bars indicates the standard error for three experiments performed with different enzyme preparations Activities are expressed as percentage on the activity obtained with 3 10^{-6} M secretin

Lack of additivity of the hormone effects

Having established that in the presence of 1 mg/ml phosphatidylserine the pancreozymin-C-octapeptide-stimulated cyclase activity is linear in time for up to 10 min, we have determined the stimulation curves for the two hormones, separately and in combination, in the presence of the phospholipid

The half maximally stimulating concentration of secretin is 5 10^{-8} M, while maximal enzyme activity is obtained at 10^{-6} M (Fig 8) The stimulation curve for pancreozymin-C-octapeptide displays an unexpected biphasic appearance Maximal activity is obtained invariably at 10^{-6} M, while in 6 out of 8 experiments a secondary peak of stimulation at low hormone concentrations (10^{-9}–3 10^{-9} M) is observed The half maximally stimulating concentration of pancreozymin-C-octapeptide in the 10^{-8} M–10^{-6} M range of the stimulation curve is estimated at 5 10^{-8} M (Fig 9), the same as that for secretin The maximal level of stimulation, reached with pancreozymin-C-octapeptide, is about 90% of that obtained with secretin (Figs 8 and 9)

When the stimulation curve of secretin is determined in the presence of a maximally stimulating concentration of pancreozymin-C-octapeptide (3 10^{-6} M), the same maximal level of stimulation is observed and at the same concentration of secretin as without pancreozymin-C-octapeptide (Fig 8) In the reverse experiment similar results are obtained the enzyme activity, once fully stimulated by secretin, is not further increased (nor decreased) by adding increasing amounts of pancreozymin-C-octapeptide (Fig 9) This means, that the effects of the two hormones on the adenylate cyclase activity are neither additive nor competitive, when measured in the presence of 1 mg/ml added phospholipid

Carbamylcholine (10^{-6} M), which like its parent compound acetylcholine,

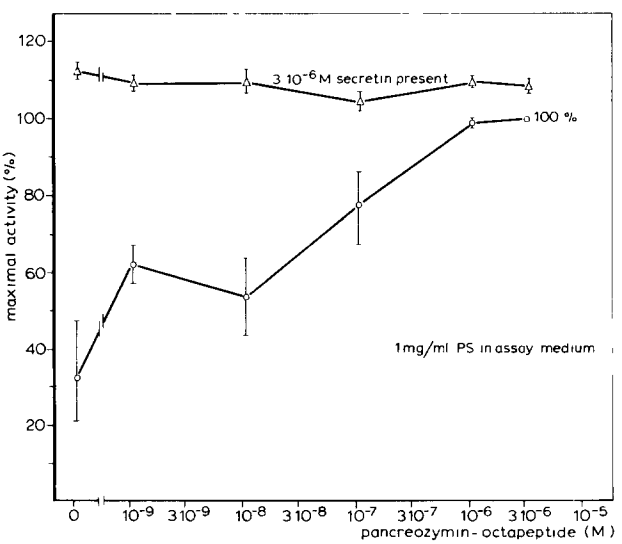

Fig 9 Stimulation curves for the effect of pancreozymin-C-octapeptide on rat pancreas adenylate cyclase in the presence or absence of 3 10^{-6} M secretin Phosphatidylserine is present in a concentration of 1 mg/ml The vertical bars represent the standard error for eight experiments performed with different enzyme preparations Activities are expressed as percentage of the activity obtained with 3 10^{-6} M pancreozymin-C-octapeptide

stimulates pancreatic enzyme secretion, has no effect, either when tested alone or in combination with secretin (10^{-7} M) or pancreozymin-C-octapeptide (3 10^{-7} M), on the pancreatic adenylate cyclase activity in the presence of added phospholipids

DISCUSSION

The reason for undertaking these studies was the 100 times lower sensitivity of rat pancreas adenylate cyclase activity towards pancreozymin than to secretin, observed previously [7] The non-additivity of the effects of the two hormones, which was noticed in the same study, suggests that the activity represents a single enzyme The available evidence for a role of cyclic AMP in the stimulation of pancreatic enzyme secretion [1–4] and the fact that 96% of rat pancreas volume consists of acinar tissue [15] favor the conclusion that this enzyme would be located in the acinar cells In this light the low sensitivity of the enzyme to pancreozymin was surprising

The possibility of an artifactual loss of hormone receptor activity during preparation and/or assay of the enzyme has been considered Evidence for such a loss is obtained from the experiments, in which the 4000 × *g* pellet serving as enzyme preparation is pre-incubated at 0 °C Pre-incubation for 30 min at 0 °C decreases the pancreozymin-C-octapeptide stimulation strongly, but has little effect on the basal, secretin-stimulated and fluoride-stimulated activities Since preparation of the 4000 × *g* pellet requires exposure for 30 min at 0 °C, it seems likely that especially the pancreozymin receptor is damaged during this procedure Observation of the kinetics of cyclic AMP production in the presence of pancreozymin-C-octapeptide during assay at 37 °C indicates that it is linear for at most 1 min and that after 8 min all activity is lost Hence, there seems to be a further loss of pancreozymin receptor activity during the assay

The recent reports that treatment with phospholipase A or C causes a loss of hormone sensitivity of adenylate cyclase in other tissues, without [12–14] or with [16, 17] loss of basal and fluoride-stimulated activity, suggest an explanation Phospholipase activity is present in our adenylate cyclase preparation, even after repeated washings of the 4000 × *g* pellet with hypotonic salt solution to remove secretory enzymes [18] Since in our experiments trypsin inhibitor and EDTA are present throughout, phospholipase A_2 and protease activity are unlikely to occur [19], but phospholipase A_1 [20] and/or lipase [21] activity can be present

In studies where hormone sensitivity is lowered by intentional phospholipase A treatment, partial recovery is observed upon addition of phospholipids [12, 13, 17] The addition of 1 mg/ml phosphatidylserine to our enzyme preparation during assay gives a clear activation of the pancreozymin stimulation, while the other activities are little or not at all affected Phosphatidylcholine and phosphatidylethanolamine have a slightly larger and smaller effect respectively, while phosphatidylinositol has virtually no effect This strengthens the suggestion that the previously observed low pancreozymin-stimulated activity was due to (phospho)lipolytic attack on the hormone receptor during preparation and/or assay of the enzyme

The earlier finding that treatment of the rat with a combined injection of secretin and pancreozymin [7] raises the hormone stimulated enzyme activity may then be ascribed to a depletion of (phospho)lipase from the pancreas before the animal is sacrificed The approx 5-fold lowering of the half-maximally stimulating concentra-

tion of pancreozymin-C-octapeptide observed in the present study suggests that phospholipid addition increases the hormone affinity of the receptor The maximal activity of the enzyme upon saturation of the hormone receptor is, however, not significantly increased by the phospholipid

Assuming that the hormone receptor is inactivated by the action of endogenous phospholipase A, for which we have cited some evidence, then the question arises as to how this process takes place One possibility is that the products, fatty acids and lysophospholipids, would inactivate the receptor However, lysophosphatidylcholine in concentrations below 2 mg/ml does not inhibit but slightly stimulates the pancreozymin-C-octapeptide-stimulated activity, hence it is unlikely that formation of lysophospholipids plays a role The inhibitory effect of high lysophosphatidylcholine concentrations (above 2 mg/ml) may be a detergent effect, which could also explain the inhibitory effects of high phospholipid doses (above 5 mg/ml) Fatty acids, particularly unsaturated ones, in low concentrations (0 2 and 0 5 mg/ml) are inhibitory However, the effect is not reversed by addition of phosphatidylserine Neither does addition of bovine serum albumin, which is known for its ability to bind fatty acids, reverse the effect of added fatty acids Hence, the inactivation of the hormone receptor does not seem to be caused by the products of phospholipase action, but rather by enzymatic removal of phospholipids from the hormone receptor area

Next is the question how phospholipid addition increases the lowered hormone receptor activity The major effect of phospholipid addition occurs during the first 2 min at 37 °C after the start of the assay, since neither addition during the preparation of the 4000 $\times$ *g* pellet, nor addition more than 2 min after the start of the assay has any effect There could be three different explanations of the phospholipid effect (1) the added phospholipid could saturate the phospholipase and thus decrease its effect on the phospholipids around the hormone receptor site, (2) it could substitute lost phospholipids, (3) it could protect the conformation of the hormone receptor The first two explanations are unlikely in view of the similarity and non-additivity of the effects of phospholipids and bovine serum albumin A "conformation-protective effect" also agrees with our observations that the addition of phosphatidy serine does not increase the initial rate of the enzyme, but keeps it constant for 10 min, whereas without addition of phospholipid the rate becomes zero within 10 min

When pancreozymin-C-octapeptide is present in fully stimulating concentration, addition of secretin does not give an additive effect in the presence of 1 mg/ml phosphatidylserine The reverse, addition of pancreozymin-C-octapeptide to a fully stimulating concentration of secretin in the presence of the phospholipid, shows no additivity either This is clear proof that we are dealing with a single enzyme Since the two hormone sensitivities behave differently towards pre-incubation and towards added phospholipids, the enzyme apparently has two hormone receptors, one for pancreozymin and one for secretin, as indeed would be expected from the very differrent structures of these peptide hormones The presence of two independent receptors is also suggested, moreover, by the observation that pancreozymin-C-octapeptide does not shift the maximally stimulating concentration of secretin, indicating that there is no competition The presence of several independent hormone receptors has also been claimed for the adenylate cyclase of fat cells [22]

The location of this enzyme is still uncertain, since in the rat pancreas pancreozymin and secretin each stimulate both enzyme and fluid secretion [23], while secre-

tin potentiates the effect of pancreozymin on enzyme secretion [24] The enzyme could thus be located in the acinar as well as in the ductular/centro-acinar cells Studies aimed at resolving this uncertainty are now in progress

ACKNOWLEDGEMENTS

The excellent technical assistance of Miss A M M Jakobs is gratefully acknowledged

This investigation was supported in part by the Netherlands Organization for the Advancement of Basic Research (ZWO), through the Foundation of Biophysics

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