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THE INFLUENCE OF DETERGENTS AND TRYPSIN ON THE STIMULATION OF AMYLASE SECRETION BY EITHER PANCREOZYMIN OR SODIUM FLUORIDE IN THE PERFUSED RAT PANCREAS

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Abstract—(1) Rat pancreas fragments were perfused for 2 hr with Krebs–Ringer bicarbonate buffer enriched with 10 mM glucose and Trasylol (500 UIK/ml). Amylase output was estimated at 5-min intervals on successive samples of the effluent. (2) Pancreozymin at a concentration of 7.10^{-9} M doubled amylase output when introduced after 1 hr of preincubation. Administration of 10 mM NaF promoted a biphasic effect. The initial and transient hypersecretory peak was followed by a second and more prolonged period of hypersecretion. It is assumed that the primary component of the biphasic response was due to hyperosmolarity, and it is tentatively suggested that the secondary response to NaF was the result of variations in the phosphorylation of membrane proteins. (3) Paired tissue fragments were pre-exposed for 30 min to digitonin, sodium dodecylsulfate, Triton X-100, or bovine trypsin (the proteolytic enzyme in the absence of Trasylol). The basal output of amylase rose with increasing detergent concentrations (from 100 to 500 μg/ml) but not after trypsin pretreatment. The four agents were equally effective in reducing the sensitivity to pancreozymin. They did not impair the initial osmotic response to NaF, but did curtail the prolonged second NaF hypersecretory effect. Digitonin and dodecylsulfate were less effective in this respect than either Triton X-100 or trypsin though being equally detrimental to pancreozymin action. (4) These observations suggest that the regulation *in vitro* of secretion by pancreozymin and NaF in intact acinar cells of the rat pancreas involves two distinct loci of a membrane–lipoprotein complex.

The mode of action of pancreozymin upon the pancreatic acinar cell is a much debated question. Three hypotheses may account for stimulus–secretion coupling: (1) the stimulation of an adenylate cyclase of the plasma membrane; (2) the translocation of calcium and (3) the activation of guanylate cyclase. None of these possibilities have been clearly demonstrated up to the present time. However, since a hormone with a mol. wt of approx 4000 cannot readily penetrate the plasma membrane, the binding of pancreozymin to appropriate plasma membrane receptors must represent an essential part of the transmission of the message.

Unfortunately, it is still difficult to undertake a direct study of pancreozymin binding. The purification of plasma membrane is a delicate operation in the case of a tissue rich in digestive hydrolases. Furthermore, the preparation of radioactive pancreozymin of high specific activity presents difficulties with respect to the lack of free phenol function available for iodination. These considerations warrant an indirect approach to the problem, involving the manipulation of intact cells. We have investigated the influence of 30-min pretreatment with detergent or trypsin (EC 3.4.4.4) on the time course *in vitro* of basal amylase output. We have also compared the action of these agents, known to alter membrane function, on the stimulation of secretion by pancreozymin and sodium fluoride when the secretagogues are added at the end of 1 hr of perifusion.

METHODS

Pancreatic tissue was obtained from batches of four male Wistar strain rats (175–225 g). The fragments were prepared and the complete experimental set-up organized as described previously [1]. In each experiment, pancreatic fragments from a combined pool were randomly distributed among six 1·8-ml perfusion cells. Each cell received ten fragments weighing approx 300 mg altogether. The fragments were perfused (superfused) at 37° with constant delivery (20 ml/hr) of Krebs–Ringer bicarbonate buffer (pH 7·4), enriched with 10 mM glucose and saturated with a gas mixture of 95% O_2 + 5% CO_2. Trasylol–Bayer (the Kunitz inhibitor of proteolytic enzymes) was also present at a concentration of 500 kallikrein inhibitor units (UIK)/ml, except as indicated. Five-min successive samples of the effluent were directed to a fraction collector maintained at 2°. A proportioning pump (Model I, Technicon Instruments Co., Tarrytown, U.S.A.) and three-way stopcocks allowed the smooth switch over to other solutions.

The same experimental protocol was followed in all trials. The first three perfusion cells served as controls and the pancreatic fragments were unexposed

to detergent or trypsin. In a second set of three cells, the noxious agent was tested at a single concentration: the fragments were exposed for 30 min to 0·010, 0·025 or 0·050% concentrations (w/w) of either digitonin, sodium dodecylsulfate, Triton X-100 or bovine trypsin. Normal Krebs–Ringer–glucose medium was perfused during the following 30 min to eliminate detergent or trypsin. Pancreozymin (320 Crick, Harper and Raper units/ml) or 10 mM NaF was added to the perfusion medium of two of the three cells after the second 30 min. Trasylol was present at all times except for 30 min in those experiments when bovine trypsin was present. Each experimental curve illustrated in Figs. 1–4 can be directly compared to the corresponding curve in Figs. 5–8 obtained after perfusion of paired fragments.

α-Amylase in the perfusion medium and in the tissue was estimated by the saccharogenic method of Noelting and Bernfeld [2] as automated by Vandermeers *et al.* [3]. One unit of amylase is defined as the amount of enzyme which liberates a reducing power equivalent to 1 μmole of maltose per min at 25°. Hydrolase secretion was expressed as per cent of total initial content secreted in 5 min, the total amylase content being estimated by adding the amounts secreted to the residual enzyme in the fragments.

Digitonin and NaF were purchased from Merck (Darmstadt, Germany). Sodium dodecylsulfate (SDS) was obtained from BDH Chemicals Ltd. (Poole, England); Triton X-100 from Rohm and Haas Co. (Philadelphia, U.S.A.); bovine trypsin (2× crystallized salt-free, lyophilized) from Worthington Biochemical Corp. (Freehold, U.S.A.) and pancreozymin from the GIH Research Unit of the Karolinska Institute (Stockholm, Sweden).

RESULTS

Control values of amylase secretion. In the absence of any of the four agents under study, the basal output of amylase decreased rapidly during the first 10 min of perfusion. This period corresponded to the clearing up of cell debris accumulated during the preliminary manipulations of the pancreases and to the washing-out of hydrolases stagnating in ductules. Thereafter, the secretion remained relatively stable, the average output of amylase being approx 0·4% per 5 min of the initial tissue content.

Effects of pancreozymin with or without preliminary treatment with detergents of trypsin. Figures 1–4 illustrate the effects of increasing concentrations of digitonin, sodium dodecylsulfate, Triton X-100 and bovine trypsin on amylase output. It is clear that this basal output increased under all circumstances except following trypsin treatment or low concentrations of Triton X-100.

The continuous infusion of pancreozymin, added in supra-maximal concentration (7.10^{-9} M, [1]) after 1 hr of perfusion without any of the four agents,

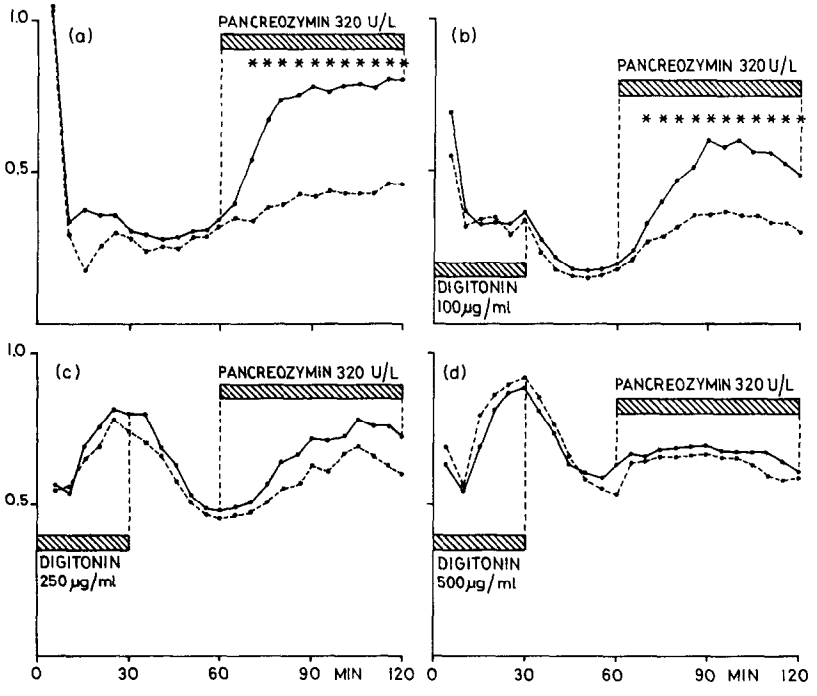

Fig. 1. Effect of digitonin pretreatment on amylase secretion by rat pancreas fragments perifused in the presence (●——●) and in the absence (●- - -●) of pancreozymin. When added, pancreozymin (320 CHR U/l.) was introduced (■) after 60 min in a hormone-free medium. (a) shows the control secretion profile obtained with a Krebs–Ringer bicarbonate buffer, enriched with 10 mM glucose and Trasylol (500 UIK/ml). (b–d) illustrate the effects of digitonin on amylase output from paired fragments. The drug was administered in increasing concentrations (100, 250, and 500 μg/ml) and withdrawn at time 30 min. Each curve is the mean of three experiments. Asterisks show in this and subsequent figures where differences are significant ($P < 0.05$), when values obtained with the test substance are compared with paired control data.

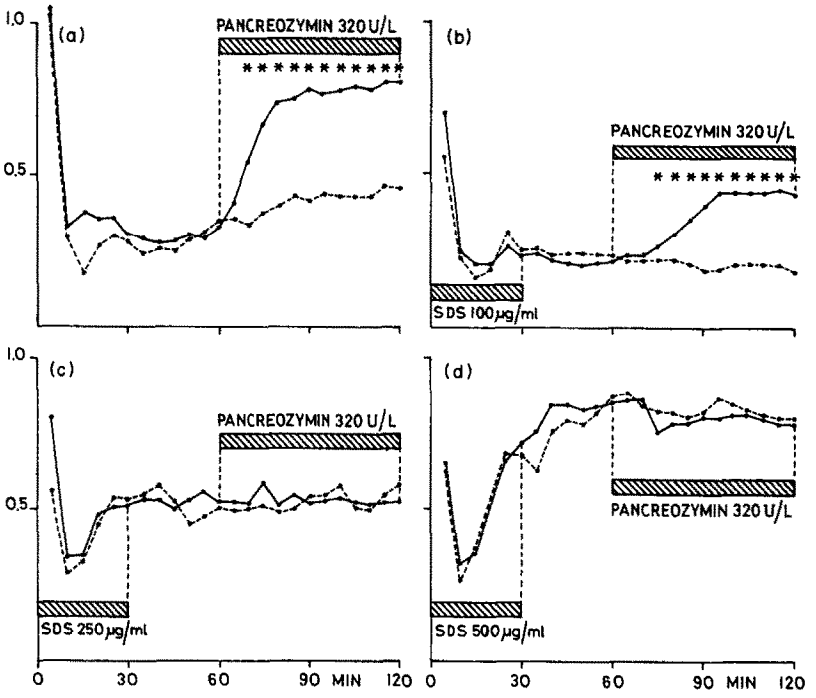

Fig. 2. Effect of sodium dodecylsulfate (SDS) pretreatment on amylase secretion in the presence (●——●) and in the absence (●- - -●) of pancreozymin. SDS was administered for 30 min in increasing concentration (0·01–0·05%). Same representation of data as in Fig. 1. Means of three experiments.

rapidly stimulated amylase output, so that a plateau corresponding to a 2-fold increase in basal secretion was attained within 20 min of hormone administration.

This stimulation decreased progressively and finally disappeared after pretreatment with increasing concentrations of the three detergents, or of trypsin.

Effects of NaF with or without preliminary treatment with detergents or trypsin. The introduction of 10 mM NaF in the medium resulted in biphasic stimulation

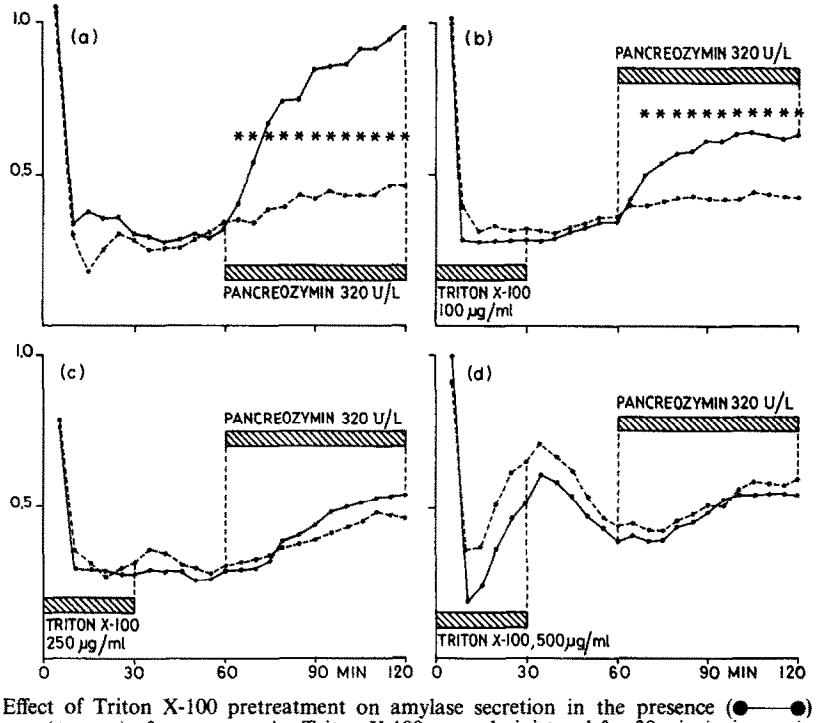

Fig. 3. Effect of Triton X-100 pretreatment on amylase secretion in the presence (●——●) and in the absence (●- - -●) of pancreozymin. Triton X-100 was administered for 30 min in increasing concn (0·01–0·05%). Same representation of data as in Fig. 1. Means of three experiments.

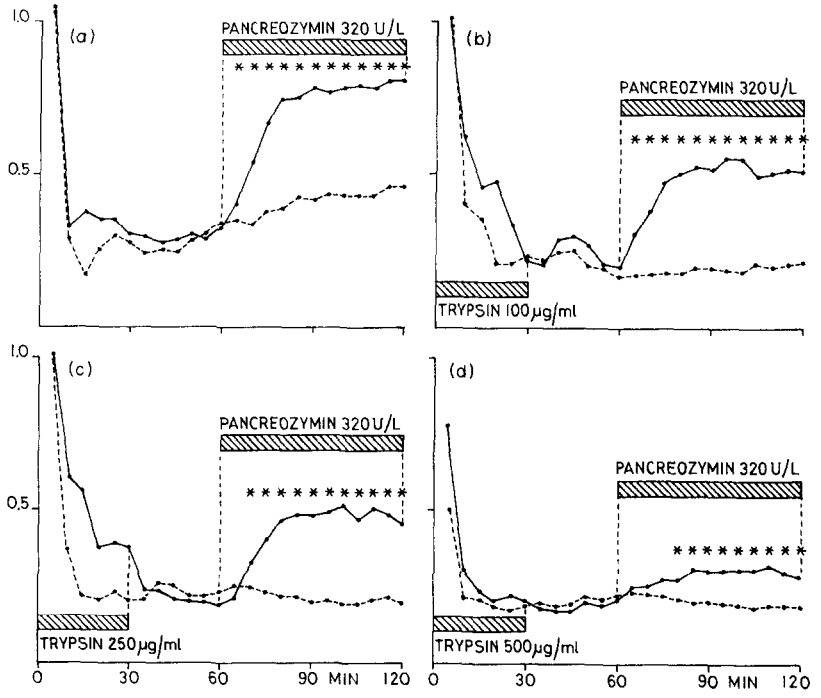

Fig. 4. Effect of bovine trypsin pretreatment on amylase secretion in the presence (●——●) and in the absence (●- - -●) of pancreozymin. Same methodology as in Fig. 1 except for the absence of Trasylol during the 30-min exposure to increasing concentrations of trypsin (0·01–0·05%). Means of three experiments.

of amylase secretion (Figs. 5–8). The rapid initial peak was unspecific and due only to the rise in osmolarity resulting from the addition of NaF. The second and prolonged wave of hypersecretion was due to NaF itself as will be discussed later on.

When the same experiments were repeated after exposure to detergents or to bovine trypsin, the immediate and unspecific response to the NaF load was not reduced, no matter how great the concentration of the agent being tested (Figs. 5–8). In fact, in the

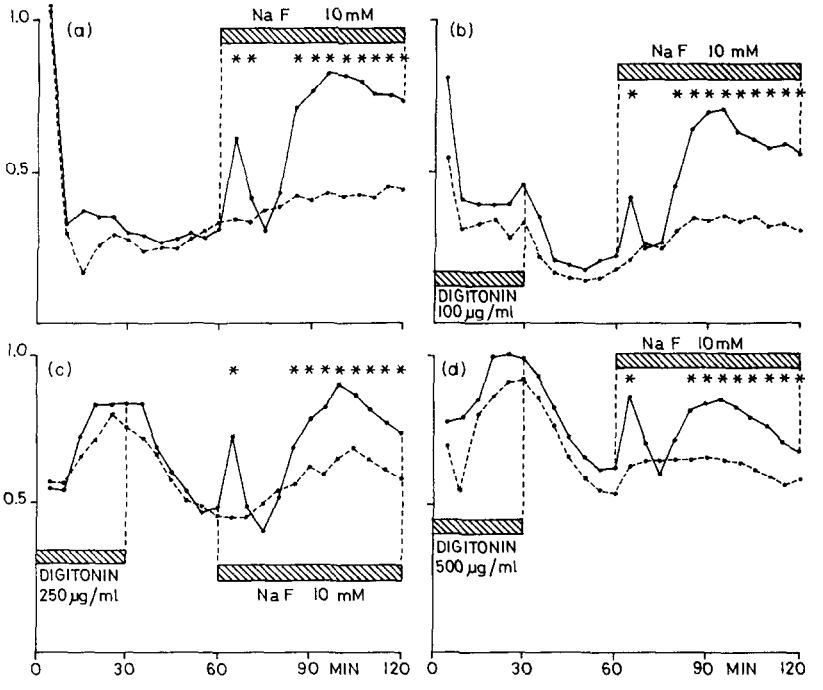

Fig. 5. Effect of digitonin pretreatment on amylase output by rat pancreatic fragments perfused in the presence (●——●) and in the absence (●- - -●) of 10 mM NaF. Same representation of data as in Fig. 1. Means of three experiments.

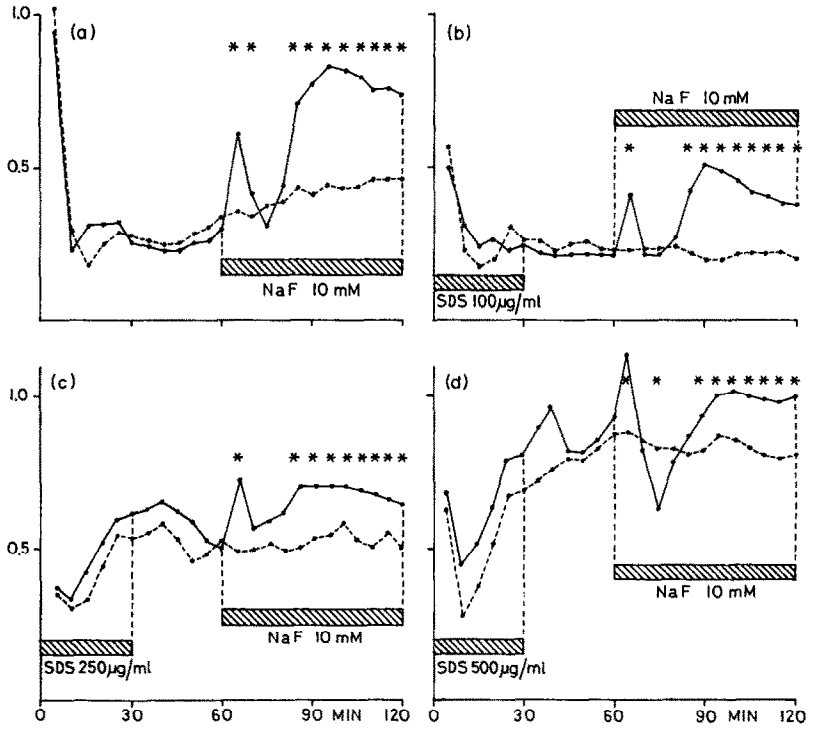

Fig. 6. Effect of sodium dodecylsulfate (SDS) pretreatment on amylase secretion in the presence (●——●) and in the absence (●- - -●) of 10 mM NaF. Same representation of data as in Fig. 1. Means of three experiments.

presence of Triton X-100 an increase in the initial secretory peak was observed. On the other hand, the second NaF peak decreased after pretreatment with increasing concentrations of the three detergents and of trypsin. Although diminished, this hypersecretion persisted at concentrations of digitonin and sodium dodecylsulfate higher than those which had produced a complete insensitivity to pancreozymin (Figs. 5 and 6). In other words, sodium dodecylsulfate and digitonin concentrations which abolished the secretory

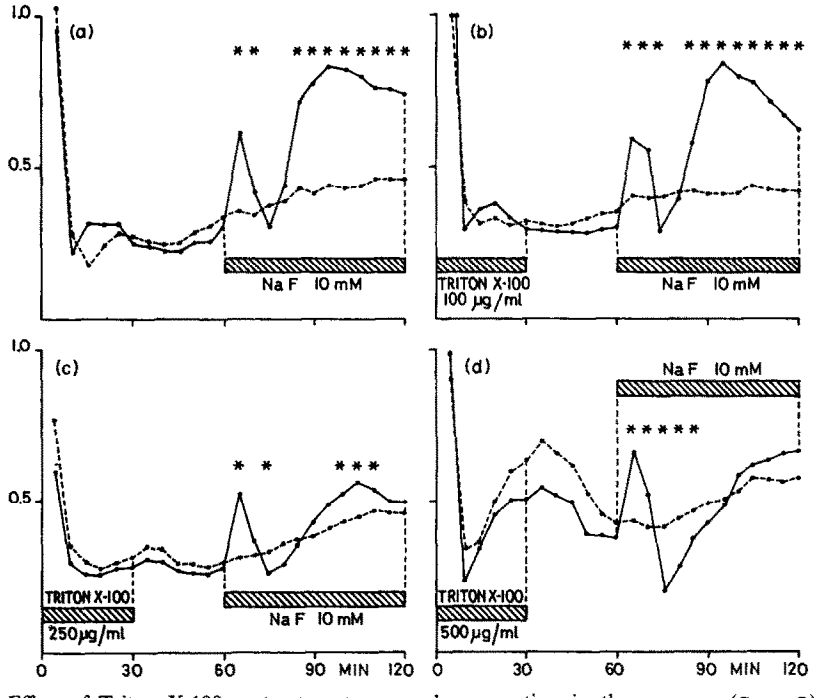

Fig. 7. Effect of Triton X-100 pretreatment on amylase secretion in the presence (●——●) and in the absence (●- - -●) of 10 mM NaF. Same representation of data as in Fig. 1. Means of three experiments.

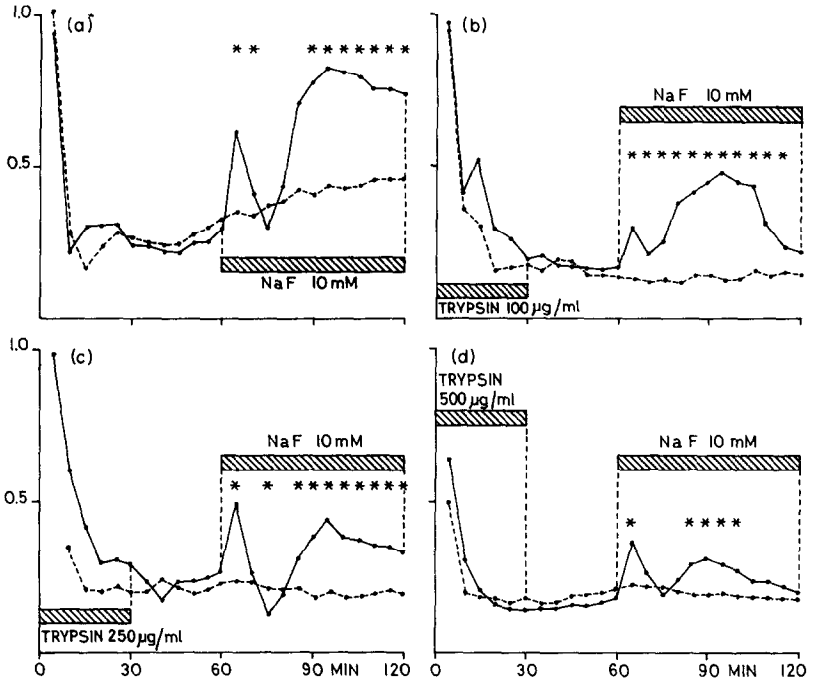

Fig. 8. Effect of bovine trypsin pretreatment on amylase secretion in the presence (●——●) and in the absence (●——●) of 10 mM NaF. Same methodology as in Fig. 1 except for the absence of Trasylol during the 30-min exposure to increasing concentrations of trypsin (0·01–0·05%). Means of three experiments.

effect of pancreozymin merely inhibited the second secretory effect due to NaF. Concentrations of Triton X-100 and trypsin able to abolish the pancreozymin effect also abolished the secretory effect of NaF.

DISCUSSION

Nature of the immediate and secondary effects of NaF on amylase secretion. The acute secretory effect of 10 mM NaF was osmotic. Indeed, the immediate effects of 10 mM NaCl, KCl or sucrose were closely analogous. Furthermore, by withdrawing an equivalent amount of NaCl, the brief hypersecretory response occurring in the presence of 10 mM NaF disappears (data not shown).

The second and prolonged response to NaF was also obtained in the absence of hyperosmolarity, i.e. with isosmolarity maintained throughout the experimental period at the expense of NaCl (data not shown). This secondary effect of NaF is difficult to interpret. An inhibition of glycolysis appears not to be involved. Indeed, Bauduin [4] observed no amylase hypersecretion in response to sodium oxamate which is as efficient as NaF in blocking glycolysis in the exocrine pancreas. NaF is an excellent activator of a number of adenylate cyclases [5, 6] in acellular preparations but has never been described as stimulating this enzyme in intact cells [5] and the exocrine pancreas does not appear to be an exception to the rule [7, 8]. On the other hand, NaF inhibits hepatic phosphorylase phosphatase [9, 10] and this compound may exert a similar effect on phospho-protein phosphatase(s) in the exocrine pancreas. The hypersecretion of pancreatic hydrolases is indeed accompanied by increased protein phosphorylation and this effect is especially important for proteins present in the membrane of zymogen granules [11]. NaF may therefore intervene in stabilizing pancreatic phosphoproteins—we observed recently that NaF strongly inhibits the total protein phosphatase activity present in pancreas homogenates (unpublished data).

It is also perhaps worth noting that this drug can activate a protein phosphatase present in the plasma membrane of granulocytes and platelets [12]. This suggests a possible mechanism for the activation of adenylate cyclase in these formed elements of the blood. Finally, in intact thyroid cells (but not in liver) the levels of cyclic GMP, and the incorporation of tritiated guanine into cyclic GMP increase in response to NaF [13]. This suggests that in at least one intact tissue, NaF is likely to activate a guanylate cyclase.

At any rate, it appears that NaF acts distal to the pancreozymin receptor when it exerts its secondary effect.

Effects of detergents and trypsin on the stimulating action exerted by pancreozymin. Our data are consistent with others describing the fragility of a series of hormone receptors and suggesting their existence as lipoproteins entities in the plasma membrane. Digitonin, sodium dodecylsulfate and Triton X-100 inhibit at first, and abolish at higher concentrations, the stimulation of adenylate cyclase by glucagon in purified hepatic plasma membrane [14]. This inhibition parallels a marked reduction in the binding of the hormone to its receptor site [15] and can be partially restored with the addition of phosphatidylserine. Similarly, the incubation of fat cell membranes in the presence of digitonin alters the response of adenylate cyclase to glucagon, ACTH, secretin and epinephrine

[14]. Like responses are found in the case of trypsin [16].

The treatment of plasma membrane with some detergents may solubilize a membrane receptor. Cuatrecasas [17] was able to pull the insulin receptor off liver membranes with Triton X-100. Similar results were obtained by Levey [18] on myocardial adenylate cyclase.

Dissociation of the effects of digitonin and sodium dodecylsulfate on the stimulation of amylase secretion by NaF and pancreozymin. In our experiments with Triton X-100 and bovine trypsin, there existed a parallel between the disappearance of the stimulatory effect of pancreozymin and of the prolonged second hypersecretory effect of NaF.

On the other hand, after pretreatment with digitonin or sodium dodecylsulfate, the proper effect of NaF persisted whereas the response to pancreozymin totally disappeared. These results are in accord with those of Birnbaumer *et al.* [14]. These authors have found that digitonin and sodium dodecylsulfate inhibit the response of liver membrane adenylate cyclase to glucagon while increasing the response to NaF.

Taken together, our data suggest that pancreozymin and NaF act on two distinct loci of the plasma membrane of acinar cells and that the lipoprotein structure of this membrane was altered less extensively by digitonin (a glycoside) and sodium dodecylsulfate (a long chain anionic surfactant) than by equal weights of Triton X-100 (a nonionic detergent) and bovine trypsin.

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