PANCREOZYMIN AND CAERULEIN STIMULATE IN VITRO PROTEIN PHOSPHORYLATION IN THE RAT PANCREAS.

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SUMMARY. Pancreozymin 1.10^{-8} M and caerulein 1.10^{-9} M provoked a 30-40 % increase in ^{32}P orthophosphate incorporation into proteins preexisting in rat pancreas fragments incubated for 20-60 min. This effect was significant in all subcellular fractions but was most evident in the membrane proteins of zymogen granules. These results suggest that hormonal stimulus-secretion coupling in the rat exocrine pancreas involves the activation of a protein phosphotransferase and the subsequent phosphorylation of proteins directly implicated in exocytosis.

Acinar cells represent at least 90 % of the pancreas. Their secretion of hydrolases is stimulated by pancreozymin and caerulein, a related decapeptide. The role of cyclic AMP as mediator of ecbolic stimulation is still subject to controversy. Dibutyryl cAMP partially reproduces the effects of pancreozymin on enzymatic secretion but it neither induces a phospholipidic effect nor does it inhibit amino acid incorporation into proteins (1). The hormone concentration provoking a half-maximal stimulation of adenyl cyclase in pancreas homogenates ($1.5\ 10^{-6}$ M) is 200 times higher than that producing maximal in vitro secretion (2,1). Moreover, contradictory results have been published on the effects of pancreozymin on cAMP levels in the pancreas (3,4). The role of cAMP as mediator being not yet firmly established, we decided to investigate the effects of pancreozymin and caerulein on the in vitro phosphorylation of proteins in the rat exocrine pancreas. Cyclic AMP and possibly also cyclic GMP indeed activate protein phosphotransferases in the target organs of a number of hormones, and the resulting phosphorylation of some proteins modifies their biological activity and finally expresses the physiological effect of the hormones under consideration.

MATERIALS AND METHODS.

Pancreozymin was obtained from the GIH Research Unit of the Karolinska Institute (Stockholm, Sweden), synthetic caerulein from Farmitalia (Milano, Italy) and Trasylol from Bayer (Leverkusen , Germany). Cycloheximide and beef heart cytochrome C were purchased from Sigma, ^{32}P orthophosphate from the Institut National des Radioéléments (Mol, Belgium) and L [1-^{14}C]-leucine from the Radiochemical Centre (Amersham, England).

Male albino rats weighing 150-200 g and fed ad libitum were killed by decapitation. Randomly distributed pancreas fragments of approximately 20 mg were preincubated in order to eliminate wash-out effects at 37° in Krebs-Ringer bicarbonate buffer (pH 7.4) enriched with 10 mM glucose and Trasylol (500 UIK/ml). The fragments were transferred after 20 min to a fresh medium with the same general composition but containing also ^{32}P orthophosphate (30 to 60 μCi/ml) or 0.8 mM [1-^{14}C]-leucine (0.3 μCi/ml), and where specified , pancreozymin, caerulein and cycloheximide.

After incubation, the pancreas fragments were quickly washed, homogenized in ice-cold water (5 % w/v) and immediately treated with CCl_3COOH (final concentration 10 % w/v). In the supernatant, inorganic phosphate Pi was assayed spectrophotometrically after extraction at 0° with organic solvants (5) and the specific activity of free L [1-^{14}C]-leucine was determined (6), whenever indicated. To remove lipids, the acid-insoluble proteins were washed successively with 10 % CCl_3COOH, ether/ethanol (4:1, v/v), and ether. The nucleic acids were eliminated by a 20 min hydrolysis at 90° in 10 % CCl_3COOH. The precipitate was washed once more with 10 % CCl_3COOH and dissolved in N NaOH. Proteins were determined by the Folin assay and radioactivity was measured in a liquid scintillation spectrometer. Differential centrifugation and the isolation of the membrane proteins of zymogen granules are described in the legends of Table I and Fig. 2.

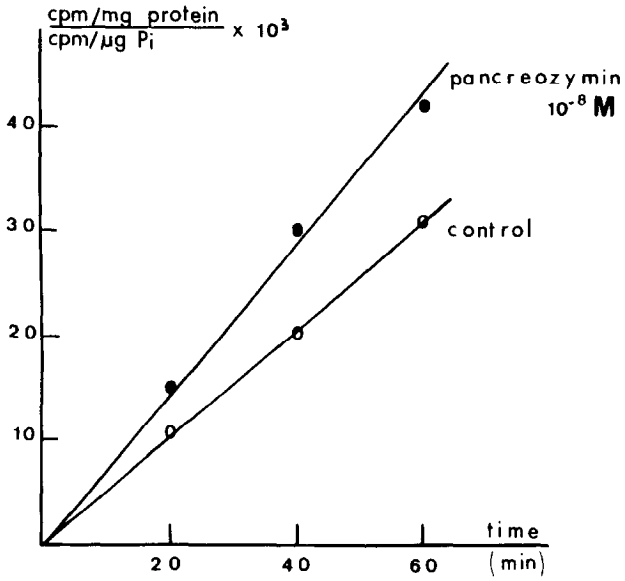

FIGURE 1. Effects of 10^{-8} M pancreozymin on the in vitro phosphorylation of total proteins. Rat pancreas fragments were randomly distributed into 6 vials and incubated during 20, 40, or 60 min. Each point represents the mean of 3 separate experiments.

RESULTS AND DISCUSSION.

Incorporation of ^{32}P orthophosphate into total proteins proceeded linearly for at least 60 min and was stimulated by 10^{-8} M pancreozymin (Fig. 1). Similarly, the exposure to 10^{-9} M caerulein induced a 39 % increase in total protein phosphorylation after 40 min incubation (Table I) and promoted a 3.5 fold increase in amylase secretion.

The average Pi content of pancreas after 40 min incubation was 7.1 µmoles/g initial weight, while the Pi content of the medium was 1.16 µmoles/ml. Both Pi content and specific radioactivity of Pi were not significantly affected by pancreozymin and caerulein (data not shown). The increased incorporation into proteins could not therefore be the result of facilitated uptake of Pi. However, to take into account any slight variability in these parameters, we always expressed the labelling of proteins with ^{32}P in cpm/mg proteins : cpm/µg Pi. The specific radioactivity of tissue Pi can be taken as specific

TABLE I. Distribution of enzyme activities and phosphorylated proteins in the pancreas after 40 min incubation with or without 1.10^{-9} M caerulein. Enzymatic activities are in % of total homogenates. The labelling of proteins with ^{32}P is expressed in (cpm/mg proteins : cpm/µg Pi) x 10^3. Means of 5 experiments ± SEM. Student's test between paired data. In this subcellular fractionation study, approx. 1 g of pooled fragments were homogenized in 10 mM Tris-maleate buffer (pH 6.0) - 0.3 M sucrose (5 % w/v) with a Teflon-glass homogenizer having a clearance of 0.11 mm. After filtration through medical gauze, differential centrifugation was conducted at 0-4° as indicated underneath.The 180 x g fraction contained cell debris, nuclei and plasma membranes. The 1000 x g pellet was made of a bottom white layer containing mainly zymogen granules and a brownish top layer. The latter was removed and joined to the 1000 x g supernatant. The 4000 x g pellet contained the bulk of mitochondria and some zymogen granules. Heavy and light microsomes were distributed among the 15,000 and 100,000 x g sediments. Each centrifugation pellet was resuspended in 2.5 ml cold water. Aliquots were kept for α-amylase (10), cytochrome C oxidase (11) and Folin assays and the remainder of the fraction was treated with CCl_3COOH for extraction of proteins as described under material and methods for total proteins.

Fraction	Proteins	Amylase		Cyt. C oxidase		^{32}P incorporation into proteins			
	%/ fraction	U/ fraction	U/mg protein	U/ fraction	U/mg protein	no caerulein	10^{-9}M caerulein added	stimulation %	P
TOTAL HOMOGENATE	100	100	100	100	100	24 ± 8	34 ± 10	+ 39	< 0.005
180 x g x 10 min sed.	7	5	60	6	90	39 ± 15	53 ± 22	+ 35	< 0.050
1 000 x g x 10 min sed.	12	26	208	14	106	6 ± 3	10 ± 6	+ 63	< 0.050
4 000 x g x 10 min sed.	23	28	120	78	302	12 ± 4	18 ± 4	+ 46	< 0.010
15 000 x g x 15 min sed.	11	7	59	9	64	18 ± 6	28 ± 6	+ 55	< 0.001
100 000 x g x 60 min sed.	12	4	32	5	47	25 ± 7	30 ± 7	+ 21	< 0.025
100 000 x g x 60 min sup.	27	20	75	0	0	23 ± 7	28 ± 7	+ 24	< 0.025
RECOVERY (in %)	92	91	-	113	-				

TABLE II. Influence of cycloheximide and/or pancreozymin 1.10^{-8}M on total protein synthesis, protein phosphorylation and amylase secretion. Results are in % of controls, after 40 min incubation.

	Cycloheximide (µg/ml)	Incorporation into proteins of ^{14}C leucine*	^{32}P **	Amylase secretion
Control	0	100	100	100
	25	46	85	94
	100	17	90	110
Pancreozymin 1.10^{-8} M	0	61	144	253
	25	22	140	305
	100	11	137	262

* ^{14}C leucine incorporation is estimated as specific radioactivity of proteins divided by specific radioactivity of L [1-^{14}C]-leucine in the acido-soluble extract of the pancreas.

** The radioactivity of ^{32}P labelled proteins is expressed as in Table I.

radioactivity of [γ-^{32}P] ATP, the immediate precursor of phosphoproteins, considering the reported rapid exchange of the γ-phosphate of ATP with Pi (7).

Cycloheximide, at doses which reduced total protein synthesis by 54-83 % (Table II), did not interfere with basal or stimulated amylase secretion and did not inhibit the increase of ^{32}P incorporation into proteins due to 1.10^{-8} M pancreozymin, indicating that phosphorylation was taking place on preexisting proteins and was not dependent on increased protein synthesis (Incidentally, we confirmed (1) that pancreozymin itself inhibited protein synthesis in the pancreas).

About 97 % of all ^{32}P transferred to the acid-stable phosphoproteins was alkali-labile, i.e. hydrolysed by 1 M NaOH treatment for 5 min at 100° . The product of a partial acid hydrolysis (in 12 N HCl for 24 h at 37°) of proteins

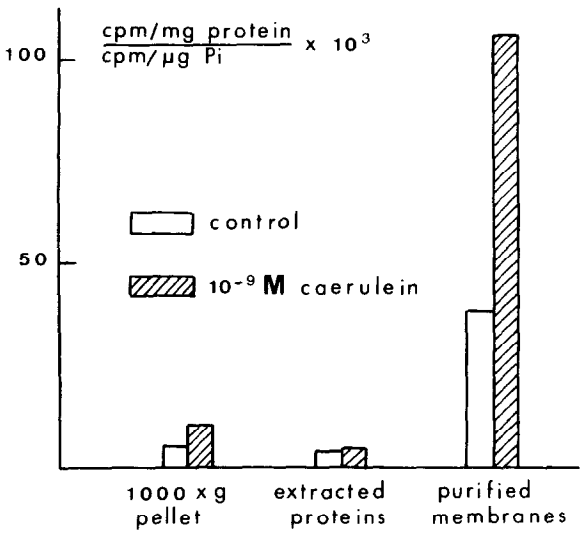

FIGURE 2. In vitro protein phosphorylation of zymogen granules in rat pancreatic fragments after 40 min incubation with or without 1.10^{-9} M caerulein. Means of 4 experiments.
The white bottom layer of the 1000 x g pellet, isolated as indicated in Table II was treated according to Meldolesi et al. (9). Secretory proteins were released by 0.2 M bicarbonate buffer (pH 7.8). Membranes were separated from contaminating mitochondria by flotation in a discontinuous sucrose density gradient : they were recovered at the interface between layers of 1.00 M and 0.30 M sucrose.

extracted from the pancreas after ^{32}P phosphate incorporation was submitted to descending paper chromatography (8). One radioactive spot remaining at the origin could correspond to phosphopeptides, two others co-chromatographed with phosphoserine and phosphothreonine standards. No radioactivity moved at the same Rf as Pi. These results indicate that labelled phosphate occurred in acid-stable phosphoproteins mostly as O-phosphoserine and O-phosphothreonine residues.

Increased phosphorylation of proteins under pancreozymin and caerulein could be related to the stimulation of secretion only if these peptides were regulating the phosphorylation of specific proteins directly involved in stimulus-secretion coupling.

As a first approach to this problem, ^{32}P incorporation data into the

proteins of six subcellular fractions were compared (Table I). Large variations were observed among fractions under basal conditions, with the specific activity in 180 x g pellet being 6.5 times higher than that of the 1000 g fraction rich in zymogen granules. Caerulein significantly enhanced protein phosphorylation in all fractions tested, but this stimulation was proportionately greater in fractions rich in zymogen granules (1000 x g), "heavy microsomes" (15,000 x g), mitochondria (4000 x g) and nuclei plus plasma membranes (180 x g). On the other hand, proteins in "light microsomes" and cytosol responded poorly to caerulein.

A marked differential stimulation by caerulein was observed in 3 components of the fraction rich in zymogen granules after lysis of the granules and separation of membranes from contaminating mitochondria and unextracted granules (Fig. 2). This procedure (9) allowed the release of 92 % amylase (10) and 84 % proteins from the granule fraction. The zymogen membranes were still contaminated with mitochondrial membranes, but the specific activity of cytochrome oxidase (11) was four times lower than that present in the bottom pellet made up of the contaminating intact mitochondria and submitochondrial particles (which represented 8 % of the proteins present in the initial 100 x g pellet).

Fig. 2 illustrates that soluble hydrolases were virtually unlabelled with ^{32}P. Their slight radioactivity was not significantly increased by caerulein. On the contrary, the highest basal phosphorylation was obtained in membrane proteins, and increased 2.7-fold under caerulein. The phenomenon observed in the proteins of zymogen granule membranes was probably underestimated, since the proteins extracted from whole mitochondria had a lower specific activity and since they were less responsive to caerulein (4000 x g pellet in Table I).

In conclusion, phosphorylation of serine and threonine residues in the membrane proteins of zymogen granules, mediated through protein kinase, could

change membrane properties and facilitate exocytosis. A rapid phosphorylation of the membrane proteins of secretory granules has been described in the adrenal medulla (12) and the anterior pituitary gland (13).

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