The involvement of protein phosphorylation in stimulus–secretion coupling in the mouse exocrine pancreas

Michael L. ROBERTS* and Fred R. BUTCHER†

Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506, U.S.A.

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Secretagogue-induced protein phosphorylation was studied in the mouse pancreas *in vitro*, by using polyacrylamide-gel electrophoresis to separate the labelled proteins. Muscarinic cholinergic agonists increased the phosphorylation of a single band, which corresponded to M_r 32000, when the tissue was incubated with Ca^{2+} present in the extracellular medium, but not in Ca^{2+} -free Krebs solution. In the presence of Ca^{2+} , ionophore A23187 stimulated phosphorylation of the same band. The dose–response curve for carbachol-induced phosphorylation was biphasic, with maximum response at $1.0\text{ }\mu\text{M}$ -carbachol, and lesser responses when greater concentrations were used. This resembles the dose–response curve for carbachol-induced amylase secretion. The data suggest that the muscarinic-agonist-induced protein phosphorylation is stimulated secondarily to elevation of cytosol $[\text{Ca}^{2+}]$ and do not support the idea that diacylglycerol formed from hydrolysis of phosphatidylinositol is the activator of the protein kinase. Derivatives of cyclic AMP stimulated phosphorylation of bands corresponding to M_r 95500, 32000 and 20000. The effects of dibutyryl cyclic AMP and bethanechol on the protein of M_r 32000 were not additive, suggesting that the two agents produced phosphorylation of the same site(s) on this protein. Since derivatives of cyclic AMP, which are not very effective secretagogues in the exocrine pancreas, stimulate phosphorylation of the protein of M_r 32000, it is difficult to argue that phosphorylation of this particular protein leads to protein secretion.

Ca^{2+} is the second messenger that produces protein secretion from mouse pancreatic acinar cells when the muscarinic cholinergic receptors are activated (Williams & Chandler, 1975; Petersen & Ueda, 1976; Williams, 1980). Details of the mechanisms that produce this elevation of cytosol $[\text{Ca}^{2+}]$, and of the means by which this second messenger initiates secretion, remain unknown. Two phenomena that seem to be important in the regulation of cells that use Ca^{2+} as a second messenger are stimulus-induced phosphatidylinositol hydrolysis (Michell, 1975) and protein phosphorylation (DeLorenzo, 1976; Krueger *et al.*, 1977). Although both of these events have been shown to occur in exocrine-gland cells, the role that

either of them plays in stimulus–secretion coupling has not been defined. Some published results suggest the possibility that these two events may be closely linked. Hydrolysis of phosphatidylinositol leads to the formation of diacylglycerol (Michell, 1975), and, since a diacylglycerol-controlled protein kinase (protein kinase C) has been described (Kishimoto *et al.*, 1980), it has been suggested that stimulus-induced phosphatidylinositol hydrolysis may lead to phosphorylation of regulatory proteins (Kawahara *et al.*, 1980).

An alternative suggestion for a link between these two events arises from the finding that depletion of cellular ATP inhibits secretagogue-induced phosphatidylinositol hydrolysis in the mouse pancreas (Hokin, 1974). These results raise the possibility that a phosphorylation step is interposed between occupation of the receptors and initiation of phosphatidylinositol hydrolysis.

The major aim of the present study was to examine the relationship of agonist-induced changes in protein phosphorylation to agonist-induced changes in amylase release from mouse pancreas.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

* Permanent address: Department of Physiology, University of Adelaide, Adelaide, South Australia 5001, Australia.

† To whom reprint requests should be addressed.

Materials and methods

Materials

Carbachol (carbamoylcholine chloride), bethanechol (carbamoyl- β -methylcholine chloride), atropine sulphate, dibutyryl cyclic AMP, 8-bromo cyclic GMP and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 8-Bromo cyclic AMP was from ICN Nucleic Acid Research Institute, Irvine, CA, U.S.A. Acrylamide, *NN'*-methylenebisacrylamide and protein molecular-weight standards for polyacrylamide-gel electrophoresis were from Bio-Rad, Richmond, CA, U.S.A. [32 P]P_i was from New England Nuclear, Boston, MA, U.S.A., ionophore A23187 from Calbiochem, San Diego, CA, U.S.A., and ACS scintillant from Amersham, Arlington Heights, IL, U.S.A. All other chemicals were of reagent grade.

Animals

Adult male CD1 mice from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. were used. They were starved overnight before the experiments, but allowed water *ad libitum*.

Solutions

In the early experiments, when the pattern of phosphorylation in the stimulated pancreas was being examined, bethanechol was used as a secretagogue and the Krebs solution used (adjusted to pH 7.4 at 37°C) had the composition (mM): NaCl, 126; KCl, 4.7; MgCl₂, 1.13; CaCl₂, 2.5; sodium fumarate, 2.7; sodium glutamate, 4.9; sodium pyruvate, 4.9; Hepes, 6; glucose, 2.8.

To compare the results from phosphorylation studies with those from measurements of phosphatidylinositol hydrolysis made previously (Tennes & Roberts, 1981), carbachol was used as the secretagogue and the Hepes was replaced by Tris (3 mM). This substitution had no obvious effect on the phosphorylation pattern obtained (results not shown).

For experiments in Ca²⁺-free conditions, CaCl₂ and MgCl₂ were omitted from the Krebs solution and replaced by an iso-osmotic amount of NaCl together with 0.1 mM-EGTA.

Protein-phosphorylation assays

All washes and incubations of the tissue were performed in Krebs solution, which was continuously bubbled with humidified O₂ and kept at 37°C. Pancreases were removed from mice and chopped with a McIlwain tissue chopper into pieces of 0.5 mm³. The minced gland was washed twice for 10 min, then taken up with a Pasteur pipette and dispersed into vials containing 2 ml of Krebs solution, each vial receiving about 30 mg wet wt. of tissue. [32 P]P_i (100 μ Ci in 50 μ l of Krebs solution) was added to each vial and the tissues were pre-incubated for 60 min. Secretagogues and/or antagonists were then added and the incubation was

allowed to proceed for 30 min. In experiments with ionophore A23187, the ionophore was added in 10 μ l of dimethyl sulphoxide to give a final A23187 concentration of 2 μ M, after the first 30 min of the pre-incubation. At the end of the 60 min pre-incubation period, CaCl₂ was added (final concn. 2.5 mM) and the incubation continued for 30 min. Control tissues for these latter experiments were treated with 10 μ l of dimethyl sulphoxide after 30 min of pre-incubation with [32 P]P_i, but apart from this they were handled identically with those treated with A23187. The incubation times indicated above were chosen on the basis of other experiments that established that the level of protein phosphorylation had reached a steady state. It should also be noted that agonist addition did not change the overall steady-state level of protein phosphorylation.

Incubations were terminated by adding 1 ml of a solution (adjusted to pH 6.8) containing (mM): NaF, 150; KH₂PO₄, 30; EDTA, 15; EGTA, 15; Tris, 150; ATP, 0.025. The tissues were immediately homogenized with a Polytron (Brinkman Instruments), and a portion (200 μ l) of the homogenate was solubilized by adding it to an equal volume of a solution containing 0.1 M-Tris, 4% (w/v) SDS, 20% (v/v) glycerol and that had been adjusted to pH 6.8, and then placing the mixture in a water bath at 100°C for 6 min. When the samples had cooled to room temperature, 4 μ l of 2-mercaptoethanol was added to each, and they were left at room temperature overnight. Another sample of each homogenate was precipitated with trichloroacetic acid and used to assay the protein content of the homogenate by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Solubilized samples were subjected to polyacrylamide-slab-gel electrophoresis in the presence of SDS in the discontinuous buffer system of Laemmli (1970), essentially as described by Ames (1974). The slabs were 1.5 mm thick and were either 12% acrylamide (for estimation of *M_r*) or linear gradients of 6–15% acrylamide. Molecular weights of the phosphoproteins were estimated by using the following proteins, with the indicated molecular weights, as standards: myosin, 200 000; β -galactosidase, 116 250; phosphorylase *b*, 92 500; bovine serum albumin, 66 200; ovalbumin, 45 000; and trypsin inhibitor, 20 000. The same amount of protein, usually 60 μ g, was applied to each lane of a gel. Gels were stained with Coomassie Blue R-250, destained, dried and used for autoradiography with Kodak NS-5T X-ray film.

By using the autoradiographs as a guide, bands of interest were cut from the dried gels, and radioactivity was measured by liquid-scintillation counting. The results for these experiments are expressed as a ratio between the radioactivity (c.p.m.) in an affected band and that in each of two reference

bands, M_r 64 500 and 53 700, in the same lane. The phosphorylation state of these reference bands did not appear, from the autoradiographs, to change on stimulation of the pancreas. This procedure of expressing the results as a ratio was used to facilitate pooling of results from separate experiments.

Statistics

Results are presented as means ($\pm$ S.E.M.), with the numbers of samples in the group in parentheses. The significance of differences between group means was determined by using Student's *t* test.

Results

Pancreatic cells incorporated ^{32}P into a number of proteins, which could be resolved by SDS/polyacrylamide-gel electrophoresis and autoradiography. Addition of bethanechol ($10\mu\text{M}$) to the incubations increased the degree of labelling of a single protein band, which corresponded to M_r 32 000 (Fig. 1). This effect of bethanechol was blocked by atropine (results not shown). Dibutyl cyclic AMP (1mM) produced increased labelling of proteins with M_r 95 500, 32 000 and 20 000 (Fig. 1). The results obtained with dibutyl cyclic AMP were confirmed by using another cyclic AMP derivative, 8-bromo cyclic AMP (0.2mM), which stimulated labelling of the same bands (detailed results not shown). Incubation of the tissue with bethanechol and dibutyl cyclic AMP gave increases in phosphorylation of the M_r 20 000 and M_r 32 000 bands similar to those produced by dibutyl cyclic AMP alone, but appeared to have less effect on the M_r 95 500 band.

The autoradiographic findings were confirmed by measuring the ^{32}P content of the bands apparently affected by incubation of the tissue with bethanechol and/or dibutyl cyclic AMP (Table 1).

Fig. 1. Protein phosphorylation in mouse pancreas slices stimulated by bethanechol or dibutyl cyclic AMP. Mouse pancreas slices were incubated as described in the Materials and methods section. The incubations were stopped 30 min after addition of either bethanechol ($10\mu\text{M}$) and/or dibutyl cyclic AMP (dbcAMP) (1mM), and the tissue was homogenized, processed as described in the text and subjected to electrophoresis on a 6–15% linear gradient polyacrylamide slab gel. The autoradiograph made from such a gel is shown here. The molecular weights for proteins that showed changes in degree of phosphorylation when the slices were stimulated are shown on the right-hand margin (95.5 K represents M_r 95 500 etc.). The treatment for any sample is shown beneath the lane in which the sample was run.

For both unstimulated tissues and tissues stimulated with bethanechol or dibutyl cyclic AMP, virtually all of the ^{32}P that had been incorporated

Table 1. Effect of bethanechol and dibutyl cyclic AMP on protein phosphorylation in the mouse pancreas

Gels were prepared for autoradiography as described in the legend to Fig. 1. The areas corresponding to the location of proteins of M_r 95 500, 32 000 and 20 000 were cut from the gels as were two reference bands, M_r 64 500 and 53 700. The ^{32}P content of these areas was determined by liquid-scintillation counting. The results are presented as means $\pm$ S.E.M. of the ratio of radioactivity (c.p.m.) in the band divided by the sum of the radioactivity (c.p.m.) in reference bands. For each treatment, *n* is the number of samples. Significance of the differences from controls: *, $P < 0.05$; **, $P < 0.001$. Unmarked values were not significantly different from controls.

Treatment	^{32}P incorporation into bands corresponding to:		
	M_r 95 500	M_r 32 000	M_r 20 000
Control ($n = 9$)	0.64 ± 0.04	0.50 ± 0.02	0.24 ± 0.01
Bethanechol ($10\mu\text{M}$) ($n = 9$)	0.61 ± 0.03	$0.68 \pm 0.03^{**}$	0.27 ± 0.01
Dibutyl cyclic AMP (1mM) ($n = 7$)	$0.77 \pm 0.05^*$	$0.72 \pm 0.04^{**}$	$0.39 \pm 0.03^{**}$
Dibutyl cyclic AMP (1mM) + bethanechol ($10\mu\text{M}$) ($n = 7$)	0.70 ± 0.04	$0.73 \pm 0.08^*$	$0.39 \pm 0.02^{**}$

Fig. 2. Protein phosphorylation in mouse pancreas slices treated with 8-bromo cyclic GMP

The procedure was the same as described for Fig. 1, except that the samples were stimulated with (a) nothing, (b) betanecol ($10\mu\text{M}$) or (c) 8-bromo cyclic GMP (0.5 mM).

into proteins could be released by treating the gel with 0.5 M-NaOH at 90°C for 15 min before neutralization of pH, staining and autoradiography (detailed results not shown). Treatment with NaOH did not leach significant amounts of protein from the gel, as judged by subsequent staining. The $[^{32}\text{P}]$ -phosphoproteins were stable to treatment with 10% (w/v) trichloroacetic acid at 90°C for 40 min.

No changes in the phosphorylation state of the proteins was observed after the addition of 8-bromo cyclic GMP (0.5 mM) to the incubations (Fig. 2).

The dose-response relationship for phosphorylation of the M_r -32000 band incubated by carbachol appeared biphasic, with the maximum response at

Fig. 3. Dose-response curve for the increase in phosphorylation of the M_r -32000 protein band in mouse pancreas slices stimulated with carbachol

The procedure was as described in Fig. 1, except that carbachol (0 – $10\mu\text{M}$) was used as a stimulus. The band corresponding to M_r 32000 was cut from the dried gel, along with two reference bands of M_r 64500 and 53700, and counted for radioactivity by liquid scintillation. The results are plotted as means $\pm$ S.E.M. of the ratio radioactivity (c.p.m.) in M_r -32000 band divided by the sum of the radioactivity of the two reference bands. Each point is the mean for either seven or nine samples.

$1\mu\text{M}$ -carbachol and a tendency toward decreased responses at higher concentrations of the secretagogue (Fig. 3).

Addition of Ca^{2+} to tissue preincubated with the ionophore A23187 produced an increase in phosphorylation of the protein band of M_r 32000 (Fig. 4). The impression gained from the autoradiographs was confirmed when the ^{32}P content of the bands from the gel was measured by liquid-scintillation counting. The radioactivity (c.p.m.) in the M_r -32000 band divided by that in the reference bands was 0.81 ± 0.03 ($n = 6$) for A23187-treated tissues and 0.59 ± 0.02 ($n = 6$) for the control tissues, a significant difference ($P < 0.001$). When pieces of pancreas were washed and incubated in Ca^{2+} -free Krebs medium, carbachol produced no increase in ^{32}P incorporation in any of the bands, as judged from the autoradiographs (Fig. 4). The radioactivity in the M_r -32000 band divided by that in the reference bands was 0.53 ± 0.03 ($n = 6$) for control samples in Ca^{2+} -free Krebs solution and 0.58 ± 0.04 ($n = 6$) for carbachol ($10\mu\text{M}$)-stimulated samples in this medium, a difference that was not significant.

Fig. 4. Roles of Ca^{2+} in phosphorylation of the M_r -32 000 protein band in mouse pancreas slices

(a) Mouse pancreas slices were preincubated for 30 min in Ca^{2+} -free Krebs solution with $[^{32}\text{P}]\text{P}_i$ as described in the text, and then treated with either $10\ \mu\text{l}$ of dimethyl sulfoxide (lanes 1 and 3) or ionophore A23187 (final concn. $2\ \mu\text{M}$) in $10\ \mu\text{l}$ of dimethyl sulfoxide (lanes 2 and 4). Then 30 min later, CaCl_2 was added (final concn. $2.5\ \text{mM}$) and the incubation was continued for 30 min. When the incubations were stopped, the tissue was homogenized and processed as described in the text and subjected to electrophoresis on a 6–15% linear gradient polyacrylamide-gel slab. The autoradiogram derived from the gel is shown here. (b) Mouse pancreas slices were treated as described for Fig. 1, except that Ca^{2+} -free Krebs solution was used and carbachol ($10\ \mu\text{M}$) was the stimulus. The resulting autoradiogram is shown here. Lanes 5 and 7 are unstimulated controls and lanes 6 and 8 are from carbachol-treated tissues.

Discussion

The proteins that showed increased incorporation of ^{32}P after stimulation with muscarinic agonists or cyclic AMP derivatives also showed considerable incorporation of ^{32}P in unstimulated gland tissue. This is not surprising, if these proteins are involved in secretory mechanisms, because the mouse pancreas shows fairly pronounced release of amylase even in the unstimulated state (Kulka & Sternlicht, 1968; Williams & Chandler, 1975; Williams, 1977). The acid-stability and alkali-lability of the bands incorporating ^{32}P into the pancreatic proteins are consistent with the presence of phosphoserine or phosphothreonine residues in these proteins (Krueger *et al.*, 1977).

Phosphorylation of the band of M_r 32 000, which was stimulated by either bethanechol or cyclic AMP, apparently involved the same site on the same protein, because adding both agents to the incubations produced no greater phosphorylation of this protein than did dibutyl cyclic AMP alone.

Pancreatic-islet β -cells, like the acinar cells, can be stimulated by cyclic AMP derivatives (Malaisse *et al.*, 1967; Sussman & Vaughan, 1967) and cholinergic agonists (Iversen, 1973; Loubatieres-Marianai *et al.*, 1973; Gagerman *et al.*, 1978), but it is unlikely that the changes observed here occurred in the β -cells. The amount of islet tissue present would be too small to account for changes of the magnitude that were measured in the present experiments. In addition, Ca^{2+} -dependent phosphorylation of intact insulinoma cells involves a protein of M_r 60 000, with no evidence of increased phosphorylation of proteins in the size range of the protein that showed Ca^{2+} -dependent phosphorylation in the present studies (Schubart *et al.*, 1980). Schubart *et al.* (1977) reported that insulinoma cells showed cyclic AMP-dependent phosphorylation of a protein of M_r 28 000, somewhere near the size of one of the proteins that we observed to undergo cyclic AMP-dependent phosphorylation. However, as noted above, the cyclic AMP- and Ca^{2+} -dependent phosphorylation observed in the present studies appeared to involve the same protein, and, as there is no evidence for Ca^{2+} -dependent phosphorylation of a β -cell protein of this M_r , it seems reasonable to conclude that the changes described here are taking place in the exocrine pancreas.

The biphasic dose-response curve for the phosphorylation stimulated by carbachol is very similar to that for amylase secretion stimulated by this secretagogue (Kulka & Sternlicht, 1968; Savion & Selinger, 1978). The decreased amylase secretion with high concentrations of agonists is associated with disorganization of the structure at the luminal end of the acinar cell (Savion & Selinger, 1978) and appears to result from the toxic effect of high cytosol $[\text{Ca}^{2+}]$ (Roberts & Woodland, 1982). Not all responses that can be measured in the pancreas show a biphasic dose-response curve. Phosphatidylinositol hydrolysis, which is thought to be an early event in stimulus-secretion coupling (Michell, 1975), increases with increasing concentrations of muscarinic agonists up to at least $1\ \text{mM}$ (Hokin, 1974; Tennes & Roberts, 1981). The close similarity of the dose-response curves for carbachol-induced phosphorylation and amylase secretion suggests that phosphorylation of the protein of M_r 32 000 may be linked to the final events in the stimulus-secretion pathway.

The association of this approach with secretion is supported by reports that a protein of similar size is phosphorylated in other secretory tissues, including

rat and mouse parotid glands (Jahn *et al.*, 1980; Kanamori & Hayakawa, 1980; Jahn & Soling, 1981a; Spearman *et al.*, 1981) and rat lacrimal gland (Jahn & Soling, 1981b). In the rat exocrine pancreas (Freedman & Jamieson, 1981) and hamster insulinoma cells (Schubert *et al.*, 1977), proteins of M_r approx. 28000–29000 are phosphorylated after stimulation of secretion. Upon cell fractionation, these proteins have been localized in the ribosomal fraction, suggesting that the role of these proteins may be in secretagogue-induced protein synthesis rather than in secretion. However, phosphorylation of the M_r -32000 protein seen in the present experiments is unlikely to be associated with secretagogue-induced protein synthesis, since mice starved overnight as in the present experiments do not show secretagogue-induced protein synthesis (Korc *et al.*, 1981).

Because the same degree of phosphorylation of the M_r -32000 protein is produced by both dibutyryl cyclic AMP and bethanechol, and because cyclic AMP derivatives are less effective as stimuli for pancreatic protein secretion than are muscarinic agonists (Kulka & Sternlicht, 1968), it would seem that phosphorylation of this protein alone does not lead directly to, or result from, exocytosis. Ca^{2+} may be required within the cell for another role which facilitates exocytosis, and this would explain the greater efficacy of muscarinic agonists, when compared with dibutyryl cyclic AMP, to act as secretagogues.

In a number of species, cyclic AMP derivatives potentiate the effect of Ca^{2+} on pancreatic protein secretion. These include the rat (Heisler *et al.*, 1972; Sighn, 1979), guinea-pig (Gardner & Jackson, 1977) and cat (Schulz, 1975). Similar studies do not appear to have been performed on mice, but if dibutyryl cyclic AMP does prove to potentiate cholinergically induced pancreatic protein secretion, as might be expected, the effect would not be a result of their common site of action on the protein of M_r 32000, since bethanechol and dibutyryl cyclic AMP did not have an additive effect with regard to phosphorylation of this protein. In the rat parotid gland, where cyclic AMP appears to be the second messenger for β -adrenoceptor-induced amylase secretion (Butcher & Putney, 1980), phosphorylation of a protein of M_r 20000 occurs when the gland is stimulated to secrete (Spearman *et al.*, 1981), and it is possible that the pancreatic protein of M_r 20000, which was found to be phosphorylated in the presence of cyclic AMP derivatives, could be responsible for potentiation of secretion in the pancreas.

The results presented here do not support the proposition that the phosphorylation induced by cholinergic secretagogues in the mouse pancreas is due to activation of a protein kinase by diacyl-

glycerol, liberated from phosphatidylinositol. The ionophore A23187, with Ca^{2+} , induced an increase in phosphorylation of the M_r -32000 protein, and yet it does not produce hydrolysis of phosphatidylinositol in the mouse pancreas (K. A. Tennes & M. L. Roberts, unpublished work). Secretagogues such as carbachol induce an increase in cytosol $[\text{Ca}^{2+}]$ in pancreatic acinar cells (Williams, 1980), and this appears to be a sufficient stimulus to increase phosphorylation.

Hokin (1974) reported that 2,4-dinitrophenol inhibited acetylcholine-induced hydrolysis of phosphatidylinositol in mouse pancreas, and although Michell (1975) could not conclusively confirm this, we have found that both 2,4-dinitrophenol and oligomycin completely inhibit carbachol-induced phosphatidylinositol hydrolysis in mouse pancreas (K. A. Tennes & M. L. Roberts, unpublished work). One reason for the inhibition of phosphatidylinositol hydrolysis by metabolic inhibitors could be that phosphatidylinositol hydrolysis, like many other events in cell metabolism, is activated by phosphorylation of one of the enzymes involved in the response and that this activation becomes impossible in the absence of ATP. The results of the present study do not support this idea. The only protein that showed increased phosphorylation with muscarinic agonists, which also activate phosphatidylinositol hydrolysis, was that of M_r 32000. Phosphorylation of this protein does not correlate with activation of phosphatidylinositol hydrolysis because: (1) in Ca^{2+} -free medium, carbachol has no effect on protein phosphorylation, but produces phosphatidylinositol hydrolysis (Tennes & Roberts, 1981); (2) A23187, with Ca^{2+} , induced phosphorylation of this protein, but did not produce phosphatidylinositol hydrolysis (K. A. Tennes & M. L. Roberts, unpublished work); (3) the dose-response curves for the two events show that, as the concentrations of carbachol is increased above $1\mu\text{M}$, phosphorylation decreases while phosphatidylinositol hydrolysis increases (Tennes & Roberts, 1981).

Finally it should be emphasized that observing an increased labelling of a particular protein does not necessarily indicate a mechanism involving activation of a protein kinase. Similar effects on the phosphoprotein labelling pattern could be effected through alteration of protein-phosphate turnover involving an action on phosphatase activity instead of an action on kinase activity. Further studies are required to discern which of these mechanisms pertain in the exocrine pancreas.

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