PHOSPHORYLATION OF A ZYMOGEN GRANULE MEMBRANE POLYPEPTIDE FROM RAT PANCREAS

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1. Introduction

Secretion of digestive enzymes and proenzymes by the exocrine pancreas is stimulated by the polypeptide hormone, pancreozymin, and by acetylcholine [1,2]. Adenosine 3′:5′ cyclic monophosphate (cyclic AMP), butyryl derivatives of cyclic AMP and theophylline stimulated pancreatic secretion in vitro, suggesting that cyclic AMP is an intracellular mediator of these hormones [3]. The role of cyclic AMP in the pancreas is still not clear [4,5]. A possible function is the regulation of protein kinases. A soluble, cyclic-AMP dependent protein kinase was partially purified from beef pancreas [6]. Furthermore Lambert et al. [7] observed that peptide hormone-stimulated secretion in pancreatic tissue slices was coupled to increased phosphorylation of zymogen granules. We now report that purified zymogen granule membranes from rat pancreas phosphorylated a granule membrane polypeptide, distinct from the major polypeptide of this membrane [8,9]. This phosphorylation, apparently due to an endogenous protein kinase, was not stimulated by cyclic AMP. There was minimal stimulation by guanosine 3′:5′ cyclic monophosphate (cyclic GMP).

2. Materials and methods

2.1. *Preparation of zymogen granule membranes*

Zymogen granules were prepared from unstarved, adult Sprague–Dawley rats as described earlier [10]. Granules were lysed at 0°C in 0.2 M $NaHCO_3$, pH 8.2, containing 0.05 mg soybean trypsin inhibitor (Sigma) per ml. Membranes were separated from granule contents by centrifugation, then purified by centrifugation in a discontinuous sucrose gradient to remove mitochondria [11], followed by extraction with 0.25 M NaBr [8,9]. By preparing zymogen granule membranes from mixtures containing granule contents labeled with [^{14}C] leucine in vitro, we established that adsorbed zymogens accounted for $< 3\%$ of the total particulate protein. Cytochrome *c* oxidase activity was absent, and mitochondrial membrane polypeptides were not detected by polyacrylamide gel electrophoresis [9].

2.2. *Phosphorylation of zymogen granule membranes*

[γ-^{32}P] ATP was prepared by the procedure of Richardson [12]. Phosphorylation of zymogen granule membranes was performed in 0.1 ml reaction mixtures containing 2.0 mg/ml membrane protein, and 0.150 mM [γ-^{32}P] ATP, 1.6×10^3 cpm/pmole, 1.0 mM $MgCl_2$, and 20 mM imidazole–HCl, pH 7.1. In some experiments 2.5 μM cyclic AMP or cyclic GMP (Sigma), or 1.5 mM UTP or ADP (P.L. Biochemicals) was added. Reactions were initiated by the addition of [γ-^{32}P] ATP. After incubation at 23°C, 0.3 ml of cold 0.3 N $HClO_4$ containing 5 mM H_3PO_4 and 2.5 mM ATP was rapidly added and reaction mixtures were centrifuged 10 min at 50 000 *g*. The pellets were washed 4 times by resuspension in the $HClO_4$ solution and recentrifugation, then washed once in distilled H_2O.

2.3. *Polyacrylamide gel electrophoresis of phosphorylated membranes*

Washed protein was dissolved in 1% sodium dodecyl sulfate (SDS) containing 2% (v/v) β-mercaptoethanol and 0.25 M sucrose. Sodium phosphate (pH 2.4) was then added to a final concentration of 50 mM, and the solution was applied to 0.6 X 11 cm gels containing 9% acrylamide. Electrophoresis in 1% SDS and 50 mM $NaPO_4$ pH 2.4 was performed according to the procedure of Avruch and Fairbanks [13]. Gels were cut into 2 mm slices and counted in a scintillation counter [14]. In some cases replicate gels were stained either by Coomassie blue for protein [15] or by periodate–Shiff reagent for carbolhydrate [16], then scanned on a spectrophotometer equipped with a Gilford linear transport.

3. Results and discussion

The polypeptide profile of zymogen granule membranes is shown by fig. 1A. Comparison with identical gels stained for carbohydrate suggested that the predominant band detected by Coomassie blue was also the major glycopolypeptide. These profiles closely resemble those previously obtained for rat zymogen granule membranes at pH 7.4 [8,9], indicating that there was no alteration in the relative mobilities of SDS-solubilized granule membrane polypeptides after precipitation with $HClO_4$. Fig. 1A also demonstrates that more than 70% of ^{32}P was incorporated into a single component, approximate molecular weight 130 000 [9], and not the major granule membrane polypeptide. This procedure would not detect acid-labile phosphate bonds, such as imidazole phosphate. When the membranes were completely dissolved prior to electrophoresis, radioactivity at the origin was negligible. There was little radioactivity in orthophosphate or phospholipid, which migrated with the tracking dye, or in the ATP region, immediately following the tracking dye [13]. Fig. 1B demonstrates that digestion of phosphorylated membranes with pepsin converted most of the radioactivity to lower molecular weight species, suggesting that the ^{32}P was protein bound. The phosphorylated granule membrane component was not hydrolyzed at room temperature at pH 2.4, 10, or at pH 5.4 in the presence of hydroxylamine (table 1). These results

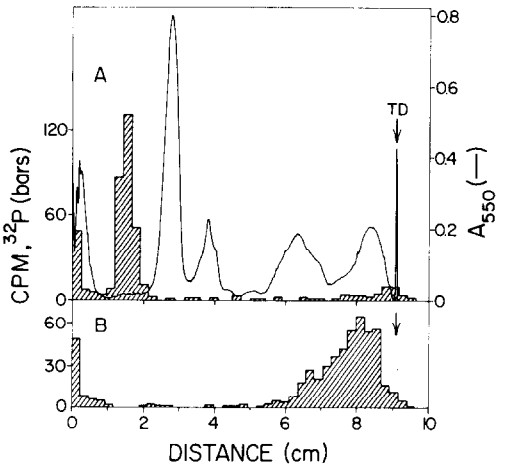

Fig. 1. Distribution of ^{32}P and protein stain in gels of phosphorylated zymogen granule membranes. Zymogen granule membranes (200 μg protein) were incubated with [γ-^{32}P] ATP for 5 sec and membranes were precipitated and washed as described in Materials and methods. Samples (100 to 200 μg protein) were subjected to polyacrylamide gel electrophoresis at pH 2.4. After 3.5 hr at 10 v/cm, the gels were either fractionated and counted, or stained with Coomassie blue. Fig. 1A: Comparison of the protein stain pattern and radioactivity in gel fractions. TD denotes the position of the tracking dye, pyronin B. Fig. 1B: Phosphorylated granule membranes (200 μg protein) were incubated in 0.01 N HCl and pepsin (1 mg/ml) at 23°C for 10 min, then subjected to electrophoresis at pH 2.4. The radioactivity profile of membranes incubated in 0.01 N HCl minus pepsin was similar to fig. 1A.

suggested that acyl [^{32}P] phosphate did not account for a significant portion of the product. The product was hydrolyzed in dilute alkali at 37°C, indicative of O-phosphate esters of serine and/or threonine [17,18].

Although [γ-^{32}P] ATP was degraded by an endogenous ATPase, ATP was not rate limiting in these kinase assays. The average specific activity for 6 different preparations was 7.0 pmoles of [^{32}P] phosphate transferred per mg protein per min. When 3 mM ATP and 3 mM $MgCl_2$ were added to reaction mixtures at subsequent intervals, no loss of ^{32}P from the labeled membrane was observed. This suggested that the label did not turnover. Other characteristics of the kinase are summarized in table 2. The activity was stimulated by $MgCl_2$ and somewhat inhibited by $CaCl_2$. Phosphorylation did not diminish in the

Table 1
Stability of the phosphorylated granule membrane component

Treatment	^{32}P Incorporation
Experiment 1 (10 min, 23°C)	cpm
none (control)	324
pH 2.4, 0.1 M sodium phosphate	319
pH 5.4, 0.2 M sodium acetate plus 0.8 M hydroxylamine	365
pH 10, 0.1 M sodium borate	256
Experiment 2 (30 min, 37°C)	
none (control)	213
1 N NaOH	5

Washed, phosphorylated zymogen granule membranes were prepared as described in fig. 1, then were resuspended under conditions listed below. After the second incubation, membranes were collected by reprecipitation with 0.3 N $HClO_4$, 5 mM H_3PO_4 and 2.5 mM ATP, washed once with water, and analyzed by SDS-polyacrylamide gel electrophoresis at pH 2.4. The radioactivity in the polypeptide region was determined as described in fig. 1. The standard error was 2.5%.

presence of excess ADP, suggesting that membrane bound [^{32}P] phosphate was not transferred to ADP and that phosphorylation was not inhibited by ADP. On the other hand, UTP inhibited membrane phosphorylation in the presence of excess $MgCl_2$. These properties are similar to those described for a protein kinase in erythrocyte ghosts [19]. However, cyclic AMP did not stimulate activity, and there was minimal activation by cyclic GMP. Possibly the granule membrane protein kinase is not activated by cyclic nucleotides. Alternatively, the kinase may have been modified during membrane isolation.

Table 2
Partial characterization of granule membrane protein kinase activity

Addition	% Incorporation
none	(100)
2.5 mM $CaCl_2$	86
2.5 mM EDTA, minus $MgCl_2$	40
1.5 mM ADP plus 3 mM $MgCl_2$	117
1.5 mM UTP plus 3 mM $MgCl_2$	21
2.5 μM cyclic AMP + 2.5 mM theophylline	93
2.5 μM cyclic GMP	113

Assays of [^{32}P] phosphate in incorporation into zymogen granule membranes were carried out as described in fig. 1. Reaction mixtures were supplemented as described above.

The importance in secretion of zymogen granule phosphorylation by an endogenous kinase is not clear. However, there are several similarities with other storage granules. Phosphorylation of adenohypophyseal storage granule membranes by an endogenous protein kinase was not stimulated by cyclic AMP [18]. Apparently a protein kinase and ATPase are also associated with chromaffin granule membranes [20]. Hence the phosphorylation of storage granule membranes may be of general significance. Exocytosis involves the fusion of granule membranes with the plasma membrane. The phosphorylation of specific granule membrane polypeptides could profoundly alter the properties of the storage granule and thus promote fusion or facilitate another step in secretion. The mode of regulating membrane protein kinases and the function of such phosphorylated membrane components pose intriguing questions for future investigations.

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