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PHOSPHORYLATION OF MEMBRANES FROM THE RAT GASTRIC MUCOSA *

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Summary

Gastric mucosal membranes derived primarily from parietal cells were found to contain endogenous protein kinase systems as well as several phosphate-accepting substrates. One specific membrane protein with a molecular weight of 88 000 was phosphorylated only in the presence of calcium, while the degree of phosphorylation of three other membrane proteins was similarly increased. The activity of the calcium-dependent protein kinase was found to be totally inhibited in the presence of trifluoperazine, a phenothiazine known to specifically inactivate calmodulin. These results suggest that a calmodulin- and calcium-dependent phosphorylation system may be a component of the parietal cell membrane.

Phosphorylation of the membrane proteins was not affected by either cyclic AMP or cyclic GMP. The heat-stable inhibitor protein of cyclic AMP-dependent protein kinase did not inhibit the endogenous protein kinase activity suggesting that the membrane enzyme is not similar to the cytosolic protein kinase. However, the catalytic subunit of the soluble enzyme was capable of phosphorylating a number of membrane proteins indicating that after maximal autophosphorylation of the gastric membranes, phosphate-acceptor sites are still available to the cytosolic cyclic AMP-dependent protein kinase.

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Abbreviations: GppNHp, 5′-guanylylimidodiphosphate; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Introduction

Gastric acid secretion is regulated by neurocrine, endocrine and paracrine effectors [1], but the precise nature of the interaction of these stimuli as well as the mechanisms underlying their actions on the parietal cell is still unknown. The effects of histamine appear to be mediated by cyclic AMP. In studies utilizing isolated parietal cells, histamine has been shown to stimulate cyclic AMP formation as well as the acid secretory mechanism as indicated by an increase in oxygen consumption and aminopyrine accumulation; phosphodiesterase inhibitors, such as isobutylmethylxanthine, potentiated histamine's stimulation of these processes [2,3]. In addition, dibutyryl cyclic AMP has been shown to mimic the effect of histamine in increasing aminopyrine uptake [4].

Similar studies with the acetylcholine analog, carbachol, and gastrin indicate that the intracellular mediators of these agonists are distinct from cyclic AMP [2]. Cholinergic stimulation of acid secretion appears to involve alterations in the fluxes of calcium across the plasma membrane of the parietal cell [4]. The mechanism by which the effects of gastrin are mediated still remain to be elucidated.

Since cyclic AMP and calcium are involved in modulating the acid secretory mechanism in the parietal cell, it would seem likely that their effects may involve the regulation of the activity of protein kinases and that the phosphorylation of unique membrane proteins could provide a mechanism for regulating the morphological structure of the apical and tubulovesicular membranes and the transport systems within these membranes. However, very little is presently known about either the gastric mucosal protein kinases or their substrates. The rabbit gastric mucosa has been shown to contain a soluble cyclic AMP-dependent protein kinase [5] as well as a membrane-associated enzyme that is stimulated by cyclic AMP [6]. In the guinea pig gastric mucosa, a histamine-induced increase in tissue cyclic AMP levels was shown to be correlated with an in situ activation of the soluble protein kinase [7]. To further elucidate the role of protein kinases in the mediation of gastric acid secretion, the present study was undertaken to characterize the factors regulating the processes of membrane protein phosphorylation in the rat gastric mucosa and to compare the role of cytosolic and membrane-associated protein kinases in catalyzing the phosphorylation of specific membrane proteins.

Materials and Methods

Materials. All radioactive compounds and reagents for liquid scintillation counting were obtained from New England Nuclear. Bovine brain calmodulin was a gift from Dr. Charles Brostrom. Trifluoperazine was provided by Smith, Kline and French Pharmaceuticals. Molecular weight protein standards and the reagents for polyacrylamide gels were purchased from Bio-Rad. ATP, GTP, cyclic AMP and cyclic GMP were supplied by Boehringer Mannheim. All other chemicals were of reagent grade.

Isolation of gastric membranes. Stomachs were obtained from five non-fasting male Sprague-Dawley rats, weighing 200—250 g. The fundic area was flooded with 1 M NaCl and then wiped to remove the surface cells [8]. The

mucosa was then minced in a 0.25 M sucrose/0.5 mM EDTA/10 mM Tris-HCl buffer, pH 7.5 (10 ml/g tissue). Homogenization was carried out in a Dounce homogenizer with 30 strokes of a loose-fitting pestle. The homogenate was filtered through cheesecloth, diluted with an equal volume of homogenizing buffer and then further homogenized with 15 strokes of a tight-fitting pestle.

The homogenate was centrifuged at 2600 × *g* for 15 min in a Sorvall RC2-B centrifuge and the resulting supernatant was then centrifuged at 10 000 × *g* for 20 min. The white outer portion of the pellet was resuspended in the supernatant and recentrifuged at 10 000 × *g* for 20 min. The outer pellet was resuspended in the supernatant and centrifuged at 20 000 × *g* for 20 min. The outer pellet was then resuspended in 75 ml homogenizing buffer and centrifuged at 20 000 × *g* for 20 min.

The final 20 000 × *g* pellet was resuspended in 13.5 ml 50% sucrose/0.5 mM EDTA/10 mM Tris-HCl (pH 7.5), divided into three tubes and overlaid with the following discontinuous gradient: 3.5 ml 40% sucrose, 2.5 ml 30% sucrose and 2.7 ml of 20% sucrose (w/v), each buffered with 0.5 mM EDTA/5 mM Tris-HCl (pH 7.5). Centrifugation was carried out in a Beckman L5-65 ultracentrifuge with a SW 41 rotor at 150 000 × *g* for 90 min. The membranes which collect at the 30/40% interface were removed with a Pasteur pipet, diluted with additional homogenizing buffer, and pelleted by centrifugation at 200 000 × *g* for 30 min. All studies utilized freshly prepared gastric membranes.

Protein phosphorylation. The incorporation of ^{32}P into membrane proteins from [γ-^{32}P]ATP was determined by a modification of the method of Schulman and Greengard [9]. The standard assay mixture in a final volume of 1.5 ml contained: 50 mM 1,4-piperazinediethanesulfonic acid (Pipes) buffer (pH 7.0), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.2 mM EGTA, 5 μM [γ-^{32}P]ATP (10 μCi), 40—60 μg membrane protein, and where indicated 0.5 mM $CaCl_2$. Incubation was carried out for 1 min at 37°C unless otherwise indicated. The membranes were pelleted by centrifugation at 10 000 × *g* for 1 min in an Eppendorf microfuge and dissolved in 125 μl 1% SDS containing 1% mercaptoethanol, 10% sucrose and 0.01% bromophenol blue. The samples were boiled for 10 min prior to being subjected to electrophoresis.

Samples terminated by centrifugation to remove the remaining [^{32}P]ATP were compared to samples of smaller volume terminated directly by the addition of SDS; no significant differences in the pattern or degree of phosphorylation of the membrane proteins were observed. In addition, to establish whether the ATPases present in the preparation were interfering with the assay, phosphorylation of the membranes was carried out in the presence of 20 mM NaF. No significant differences in the incorporation of $^{32}P_i$ were observed.

Polyacrylamide gel electrophoresis. Membrane proteins were resolved by electrophoresis carried out for 4 h at 18 mA/gel in a Hoefer Scientific model 500 slab gel apparatus using a 7.5% separating gel and a 3% stacking gel as described by Laemmli [10]. The gels were stained with 0.2% Coomassie brilliant blue in 12.5% trichloroacetic acid and destained with 7% acetic acid. The gels were then vacuum-dried on filter paper for autoradiography on Kodak SB panoramic dental X-ray film. Autoradiographs were scanned at 550 nm in a Gilford model 240 recording spectrophotometer equipped with a gel-scanning accessory as described by Garrison [11].

Molecular weights of the membrane proteins were estimated from standard log molecular weight vs. mobility curves [12] using proteins of known molecular weight.

Protein purifications. The catalytic subunit of cyclic AMP-dependent protein kinase (1000 histone units/μg) was isolated from hog gastric mucosa as described previously [13,14].

The heat-stable protein kinase inhibitor was prepared from rabbit skeletal muscle by the method of Schlender and Reimann [15].

Assays. K^+-ATPase activity, defined as the difference between activity in the presence and absence of K^+, was measured in a medium containing 2 mM ATP, 2 mM $MgCl_2$, 40 mM Tris-HCl buffer (pH 7.4), and 5—10 μg membrane protein, with and without 20 mM KCl, in a final volume of 1 ml. Incubation was carried out for 15 min at 37°C and the reaction terminated by the addition of 0.1 ml cold 25% trichloroacetic acid. The samples were centrifuged and 0.9 ml supernatant removed for phosphate determination as described by Chen et al. [16].

K^+-stimulated *p*-nitrophenyl phosphatase was assayed in a medium containing 5 mM *p*-nitrophenyl phosphate, 6 mM $MgCl_2$, 1 mM EDTA, 40 mM Tris-HCl buffer (pH 7.5) and 15 μg membrane protein in the presence and absence of 20 mM KCl. The *p*-nitrophenol released was measured spectrophotometrically at 410 nm [17].

5′-Nucleotidase was determined in a medium containing 2 mM AMP, 2 mM $MgCl_2$, 40 mM Tris-HCl buffer (pH 7.4) and 30 μg membrane protein and the P_i released was measured as described by Chen et al. [16].

Adenylate cyclase activity was determined by a modification of the method of Salomon et al. [18]. The incubation medium contained 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 10 mM theophylline, 10 mM phospho*enol*pyruvate, 12.5 μg pyruvate kinase, 1.2 mM [α-^{32}P]ATP (0.5 μCi), and 15—20 μg membrane protein, in a final volume of 50 μl.

Guanylate cyclase activity was measured by a modification of the method of Krishna and Krishnan [19]. The incubation medium in a final volume of 0.1 ml contained 40 mM Tris-HCl buffer (pH 7.4) 3.3 mM $MnCl_2$, 1 mM cyclic GMP, 2 mM theophylline, 1 mM [α-^{32}P]GTP (0.5 μCi) and 30—40 μg membrane protein.

Succinate dehydrogenase was assayed spectrophotometrically by recording the amount of succinate oxidized in the presence of ferricyanide at 420 nm according to the method of King [20]. NADPH cytochrome *c* reductase was measured by the method of Masters et al. [21] and glucose-6-phosphatase was determined by the method of Solyom and Trams [22]. Protein concentration was determined by the method of Lowry et al. [23] using bovine serum albumin as standard.

Results

Characterization of gastric membranes

Using K^+-ATPase as a marker for the parietal cell apical membrane [24], membranes which were enriched in this enzyme were isolated from the fundic region of the rat gastric mucosa. As shown in Table I, the membranes with a

TABLE I

COMPARISON OF ENZYME ACTIVITIES IN GASTRIC MUCOSAL HOMOGENATES AND IN PURIFIED MEMBRANES

Activities are expressed in μmol/h per mg protein and represent the mean value ± S.E. of four experiments. pNPPase, *p*-nitrophenyl phosphatase.

Fraction	Mg^{2+}-ATPase	K^+-ATPase	K^+-pNPPase	5′-Nucleotidase
Homogenate	8.9 ± 0.8	1.4 ± 0.4	1.06 ± 0.6	0.71 ± 0.04
Membrane	44.6 ± 5.4	26.5 ± 0.3	18.3 ± 1.8	2.3 ± 0.3

density of 1.13 exhibited an 18-fold increase in K^+-stimulated ATPase and K^+-stimulated ATPase and K^+-stimulated *p*-nitrophenyl phosphatase activities with respect to the homogenate. Mg^{2+}-ATPase and 5′-nucleotidase were also enriched in this membrane fraction but not to the same degree as K^+-ATPase. This pattern of enzyme activities is similar to that reported for microsomes isolated from frog oxyntic cells [25,26] and the gastric mucosa of dog [27,28] and hog [29,31]. It should be noted, however, that unlike other species, K^+-ATPase activity in the rat was not found in the particulate material obtained from the post 20 000 × *g* supernatant but rather in the 20 000 × *g* outer pellet. This difference may result from the techniques used in homogenization.

The gastric membranes had no appreciable ($Na^+ + K^+$)-ATPase activity and the K^+-ATPase present was Na^+ independent and ouabain insensitive (Table II). The basal Mg^{2+}-ATPase was stimulated by only 10% by HCO_3^- and neither the basal nor K^+-stimulated activities were affected by SCN^-, suggesting that the enzymes were not of mitochondrial origin [32]. In addition, lack of contamination by mitochondria was demonstrated by insignificant succinate dehydrogenase activity and insignificant NADPH cytochrome *c* reductase and glucose-6-phosphatase activities indicated the absence of endoplasmic reticulum.

These membranes also contained adenylate cyclase and guanylate cyclase activities as shown in Table III. The 3.6-fold enrichment of adenylate cyclase in the membranes was similar to the degree of purification for 5′-nucleotidase. The adenylate cyclase was stimulated by both NaF and the GTP analog 5′-guanylylimidodiphosphate (GppNHp). However, neither histamine nor acetylcho-

TABLE II

EFFECTS OF VARIOUS AGENTS ON Mg^{2+}-ATPase AND K^+-STIMULATED ATPase ACTIVITIES IN RAT GASTRIC MEMBRANES

Activities are expressed in μmol/h per mg protein and represent the mean value ± S.E. of three determinations.

	Basal Mg^{2+}	$Mg^{2+} + K^+$	ΔK^+
Control	45.3 ± 2.3	63.6 ± 3.4	18.3
120 mM NaCl	47.7 ± 1.9	58.5 ± 5.0	10.8
1 mM ouabain	44.8 ± 3.4	62.4 ± 4.1	17.6
20 mM $NaHCO_3$	50.2 ± 0.8	67.5 ± 1.7	17.3
10 mM NaSCN	46.2 ± 4.2	65.1 ± 2.2	18.9
10 mM NaF	38.8 ± 1.2	42.6 ± 0.9	3.8

TABLE III

COMPARISON OF ADENYLATE CYCLASE AND GUANYLATE CYCLASE ACTIVITIES IN SUBFRACTIONS OF RAT GASTRIC MUCOSA

Activities are expressed in pmol/min per mg protein and represent the mean value ± S.E. of three determinations. n.d., not determined.

Fraction	Adenylate cyclase			Guanylate cyclase	
	Basal	10 mM NaF	50 μM GppNHp	Basal	5 mM $CaCl_2$
Homogenate	7.6 ± 1.2	39.3 ± 3.3	n.d.	25.9 ± 2.3	30.1 ± 4.8
Membrane	27.9 ± 7.7	376.0 ± 25.8	181.9 ± 9.2	10.7 ± 2.0	78.3 ± 10.3
Cytosol	—	—	—	25.5 ± 1.9	26.8 ± 3.1

line was found to activate the enzyme, and GppNHp was without effect in enhancing enzyme responsiveness to either hormone.

As previously shown in the guinea pig [33], guanylate cyclase in the gastric mucosa was associated with both the cytosolic and plasma membrane fractions. Although the major portion of guanylate cyclase activity was found in the cytosol, the enzyme form which is stimulated by Ca^{2+} was completely localized in the membrane fraction.

The complex polypeptide pattern of the purified gastric membranes is shown in Fig. 1. There were eleven major bands with the most prominent peaks represeting 105 000 and 50 000 dalton polypeptides. In addition, gels run with higher percentages of acrylamide indicated that smaller peptides having molecular weights of approx. 20 000, 18 000 and 16 000 were also components of the gastric membranes.

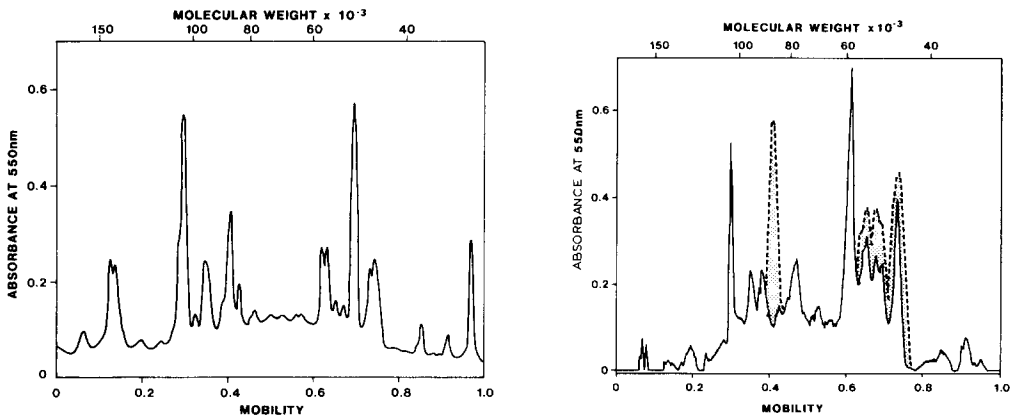

Fig. 1. SDS-polyacrylamide gel electrophoretic pattern of purified gastric membranes. The amount of membrane protein applied was 40 μg. Membrane proteins were stained with Coomassie blue prior to absorbance scanning.

Fig. 2. Endogenous phosphorylation of gastric membrane proteins in the absence and presence of calcium. Membranes incubated with [γ-^{32}P]ATP were resolved by SDS slab gel electrophoresis and the resultant autoradiographs scanned at 550 nm to determine the absorbance. Mobility of the bands is relative to the tracking dye, bromophenol blue. The areas enclosed by the dashed lines represent the increase in phosphorylation observed in the presence of 0.5 mM calcium.

Endogenous phosphorylation of gastric membranes

Gastric membranes were phosphorylated by an intrinsic protein kinase when incubated with [γ-^{32}P]ATP. The tracing of Fig. 2 represents the basic pattern of [^{32}P]phosphate incorporation into membrane proteins as resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. While several proteins were capable of serving as phosphate acceptors for the endogenous protein kinase, substantial amounts of radioactivity were incorporated into eight major bands. The highest level of incorporation was into a protein having a molecular weight of approx. 59 000 (R_F = 0.61).

The incorporation of [^{32}P]phosphate into the protein with a molecular weight of approx. 105 000 (R_F = 0.3) probably represents the phosphorylation of K^+-ATPase [28]. The degree of phosphorylation of this band was reduced in the presence of K^+ and the phosphate bond was labile when the membranes were treated with 0.5 N NaOH at 0°C prior to electrophoresis indicating that the phosphate attachment to this protein was via an acyl-phosphate bond [34]. All of the other phosphorylated membrane proteins were unaffected by such treatment indicating that the [^{32}P]phosphate was covalently attached to the proteins through a phosphate-ester linkage.

Calcium-dependent phosphorylation of gastric membranes

Calcium caused a marked stimulation of endogenous phosphorylation of a protein having a molecular weight of approx. 88 000 (R_F = 0.4) as shown in Fig. 2. The phosphorylation of this protein appears to be catalyzed by a specific calcium-dependent protein kinase since no incorporation of phosphate occurred in the presence of EGTA. In addition, the degree of phosphorylation of three other membrane proteins having apparent molecular weights of 54 000, 52 000 and 47 000 was increased in the presence of calcium. The phosphorylation of these proteins depended specifically on calcium since neither Co^{2+}, Sr^{2+}, nor Mn^{2+} were capable of effectively substituting for the cation. In addition, this calcium-dependent protein kinase appeared not to use GTP as a phosphoryl donor since the presence of GTP at a concentration ten times that of [^{32}P]ATP in the incubation medium did not decrease the level of membrane phosphorylation stimulated by calcium.

Under standard assay conditions, the endogenous, calcium-dependent phosphorylation of the 88 000 molecular weight protein reached a maximal level within 2.5 min (Fig. 3). Afterwards the level of phosphorylation decreased indicating that the effects of a phosphoprotein phosphatase began to predominate.

The effect of varying the concentration of calcium on the endogenous phosphorylation of this protein is shown in Fig. 4. In the absence of added calmodulin, maximal phosphorylation was observed at a concentration of 0.3 mM calcium. Although this was a rather high level of calcium, it has been previously shown that for the cyclic nucleotide phosphodiesterase activated by calcium, the effectiveness of calcium in stimulating enzyme activity was directly dependent upon the level of the protein activator, calmodulin, present [35,36]. In addition, studies of the calcium- and calmodulin-dependent phosphorylation of synaptosomal membranes have shown that maximal phosphorylation occurred at a calcium concentration of approx. 10^{-3} M [37], suggesting that in studies involving membrane preparations, significant amounts of the added calcium

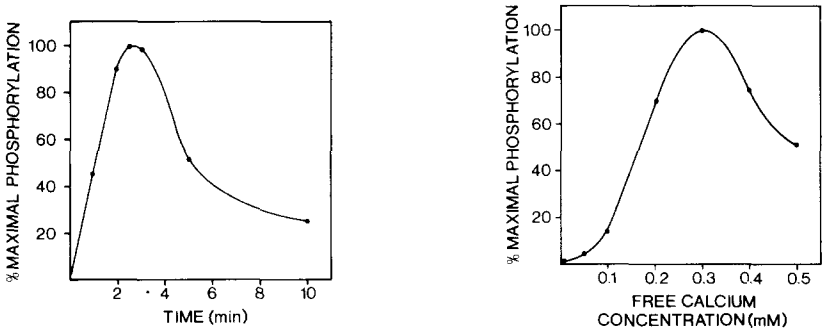

Fig. 3. Time course of the calcium-dependent phosphorylation of rat gastric membranes. The relative amounts of [^{32}P]phosphate incorporated into the membrane protein (M_r = 88 000) in the presence of calcium were calculated by integrating the area under the peak obtained in the absorbance scans of the autoradiographs.

Fig. 4. Effect of varying the concentration of calcium on the endogenous phosphorylation of the M_r 88 000 membrane protein. Free Ca^{2+} concentration was varied by use of a Ca^{2+}/EGTA buffer containing 0.2 mM EGTA.

may be unavailable for activation due to binding to various membrane proteins. The decrease in phosphorylation observed with high levels of calcium may indicate that calcium-stimulated phosphatases are associated with this membrane system or that high concentrations of calcium inhibit the kinase as has been observed for other protein kinases [38].

Mediation of the calcium-dependent phosphorylation by calmodulin

Since many tissues have been shown to contain a membrane-associated, calcium-dependent phosphorylation system which requires the cytosolic calcium-dependent regulatory protein, calmodulin [39], studies were initiated to determine whether the calcium-dependent phosphorylation of the gastric membranes was similarly mediated by a calmodulin mechanism. To remove any cytosolic calmodulin adhering to the gastric membranes, various washing procedures utilizing calcium-chelating agents were employed [39,40]. However, as shown in Fig. 5, such procedures did not alter the pattern of calcium-dependent phosphorylation. The addition of exogenous calmodulin resulted in a 2-fold increase in the level of phosphorylation of the 88 000 molecular weight protein, while only a small increment in the phosphorylation of the three other membrane proteins affected by calcium was observed. No additional proteins were phosphorylated in the presence of exogenous calmodulin.

Furthermore, the calcium-dependent phosphorylation of the gastric membranes was found to be totally inhibited in the presence of trifluoperazine, a phenothiazine known to specifically bind and inactivate calmodulin [41]. This was not a nonspecific effect of the drug since levels as low as a 20 μM were effective in totally inhibiting the membrane phosphorylation stimulated by calcium. These results suggest that the calcium-dependent protein kinase in the gastric membranes requires calmodulin for activity.

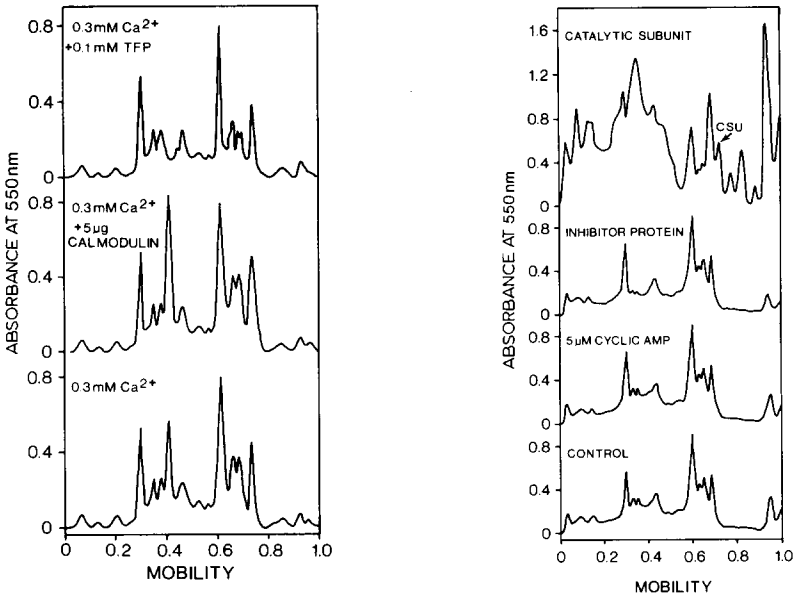

Fig. 5. Comparison of the effects of exogenous calmodulin and trifluoperazine on the calcium-dependent phosphorylation of the gastric membrane proteins. Prior to incubation, the membranes were repeatedly washed with 5 mM Tris-HCl, pH 7.0, 0.5 mM EDTA and 1 mM mercaptoethanol to remove any adhering cytosolic calmodulin.

Fig. 6. Phosphorylation of gastric membrane proteins by cyclic AMP-dependent protein kinases. The phosphorylation patterns in the presence of 5 μM cyclic AMP, the inhibitor protein of the cytosolic cyclic AMP-dependent protein kinase (amount which inhibits 1600 histone kinase units by 50%), or the catalytic subunit of the cytosolic cyclic AMP-dependent protein kinase (6000 units) were obtained following incubations carried out for 2 min. The band indicated by the arrow represents the autophosphorylation of the catalytic subunit.

Effects of cyclic nucleotides on membrane phosphorylation

The endogenous phosphorylation of the gastric membranes was not altered by the addition of cyclic AMP varying in concentration from 0.5 to 5 μM (Fig. 6). Similarly, cyclic GMP was without effect in regulating the degree of [^{32}P]-phosphate incorporation into membrane proteins. In addition, the heat-stable inhibitor protein of cyclic AMP-dependent protein kinase did not inhibit the endogenous protein kinase suggesting that the membrane enzyme is not similar to the soluble protein kinase.

Stimulation of membrane phosphorylation by cyclic AMP-dependent protein kinase

Since cyclic AMP does not regulate the phosphorylation of the gastric membranes directly, it was of interest to determine whether substrates for the soluble, cyclic AMP-dependent protein kinase of gastric mucosa were also present in these membranes. The results presented in Fig. 6 indicate that the gastric membranes do contain several protein substrates for the catalytic subunit of the soluble kinase. The incorporation of ^{32}P into several of the proteins phosphorylated by the endogenous protein kinase was further stimulated by

the catalytic subunit. In addition, a number of membrane proteins not previously phosphorylated did serve as substrates for this enzyme. The pattern shown represents an initial level of catalytic subunit-catalyzed phosphorylation; enhanced phosphorylation was observed with longer periods of incubation reaching a maximum at 10 min. It should be noted, however, that the substrate for the endogenous, calcium-dependent protein kinase (M_r = 88 000; R_F = 0.4) did not appear to be phosphorylated by the catalytic subunit. Since the catalytic subunit catalyzed a large incorporation of ^{32}P into a membrane protein with an R_F of 0.35, it is difficult to resolve this scan in the 0.4 region. However, comparing autoradiographs where less catalytic subunit was used to phosphorylate the membranes, it could be seen that the 88 000 molecular weight protein was not a substrate.

Discussion

Cyclic AMP and calcium have been suggested as mediators regulating gastric acid secretion in response to histamine and acetylcholine, respectively, and may act by modulating the activity of specific protein kinases. In the present study, gastric membranes derived primarily from parietal cells were shown to contain endogenous protein kinases which catalyze the phosphorylation of endogenous membrane-associated proteins. Similarly, the presence of intrinsic membrane protein kinases have been demonstrated in a wide variety of tissues [42,43], suggesting that the phosphorylation of specific membrane proteins may be a generalized mechanism by which membrane permeability and transport processes are regulated.

The gastric membranes contain at least one protein kinase which appears to be insensitive to cyclic nucleotides; in the absence of any stimulatory agent, several membrane proteins were found to be phosphorylated to a significant degree. This independent protein kinase was not inhibited by the heat-stable inhibitor protein of the cyclic AMP-dependent protein kinase [44], thereby eliminating the possibility that the observed activity was due merely to the association of the catalytic subunit of the cytosolic enzyme with the membrane. The intrinsic protein kinases in synaptic membranes [45] and cardiac microsomes [46], as well as several other membrane preparations [42], were similarly found to be unaffected by the soluble cyclic AMP-dependent protein kinase inhibitor.

The membranes were also found to possess a calcium-dependent protein kinase which selectively phosphorylated an 88 000 molecular weight membrane protein and additionally stimulated the degree of phosphate incorporation into three other substrates phosphorylated by the independent protein kinase. Recent reports have shown that neuronal membranes contain a protein kinase which requires calcium [9] and that comparable calcium-dependent protein phosphorylating systems are present in membranes from a variety of tissues [39]. In each of these systems, activation of the membrane, calcium-stimulated kinase was dependent upon the presence of the cytoplasmic, calcium-dependent regulatory protein, calmodulin. In addition to membrane-associated kinases dependent upon calmodulin, other calcium-dependent protein kinases, including phosphorylase kinase [47] and the myosin light chain kinases from

smooth [48], and skeletal muscles [49] as well as human platelets [50], have been shown to be similarly activated by a calmodulin mechanism. A number of other enzymes have also been found to be regulated by calmodulin suggesting that this protein may be the intracellular receptor for calcium serving to mediate the physiological effects of calcium in coupling stimulation and response [51].

Unlike the previously described membrane-associated, calcium-dependent protein kinases, the enzyme present in the gastric membranes did not require the addition of exogenous calmodulin. However, the activity of this calcium-dependent protein kinase was totally inhibited in the presence of trifluoperazine, a phenothiazine known to specifically bind and inactivate calmodulin [41]. These results suggest that calmodulin may be a component of the gastric membrane. Similarly, calmodulin has been shown to be a component of the postsynaptic densities which are associated with the cytoplasmic surface of the postsynaptic membrane [52]. This subcellular fraction was also found to contain a calmodulin-dependent protein kinase which was activated in the absence of exogenous calmodulin and inhibited by either EGTA or the antipsychotic, chlorpromazine [53].

An alternative possibility is that the cytosolic calmodulin is very tightly adhered to the calcium-dependent kinase of the gastric membranes since extensive washings with calcium-chelating agents did not eliminate the calcium-stimulated phosphorylation of the membrane proteins. In comparison, calmodulin has been shown to be capable of binding to brain microsomes in a calcium-independent manner [54]. Similarly, the binding of calmodulin to phosphorylase kinase from skeletal muscle differs from the binding to other calcium-dependent enzymes in that calmodulin is present as a non-dissociable subunit [47]. It would appear that the affinity of calmodulin binding to its effector proteins may vary with physiological function.

In contrast to the rabbit gastric mucosa [6], gastric membranes from the rat fundic mucosa do not contain a cyclic AMP-dependent protein kinase. If cyclic AMP is to modulate membrane function in this tissue, the cytosolic protein kinase must be capable of phosphorylating membrane proteins. Indeed, the catalytic subunit of the soluble cyclic AMP-dependent protein kinase was found to catalyze the phosphorylation of several membrane proteins although it did not modify the protein specifically phosphorylated by the calcium- and calmodulin-dependent membrane protein kinase. Therefore, after maximal autophosphorylation, phosphate-acceptor sites are still available to the cytosolic enzyme.

The finding of an endogenous protein kinase regulated by calcium in the gastric membranes, as well as the ability of the cytosolic, cyclic AMP-dependent protein kinase to modify specific membrane substrates suggests that the phosphorylation of membrane proteins may be the mechanism which provides an interface for certain of the interactions of the cyclic nucleotides and calcium in regulating gastric acid secretion. It will be of interest to establish the functional significance of the membrane proteins specifically phosphorylated in response to either calcium or cyclic AMP.

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