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Thiophosphorylation of hog gastric ($H^+ + K^+$)-ATPase membranes by endogenous protein kinases

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($H^+ + K^+$)-ATPase-enriched membranes from hog stomachs were tested for their capacity to autophosphorylate using [γ-^{32}P]ATP or [γ-^{35}S]ATP[S] as phosphate donors. The radioactive polypeptides were characterized by SDS-PAGE. In the presence of Mg^{2+} and 5 μM [γ-^{32}P]ATP, rapid and transient incorporation of ^{32}P occurred at 0°C. Radioactivity was essentially found in the major polypeptide of the material, the 95 kDa subunit of ($H^+ + K^+$)-ATPase. Under the same experimental conditions, thiophosphorylation was slower and reached a plateau within 1 h. Incorporation levels were higher with manganese than with magnesium. After one hour at 0°C, and in the presence of 10 mM manganese and 5 μM ATP[S], 0.58 ± 0.06 nmoles of thiophosphate were incorporated per mg of protein. Twenty seven percent of the thiophosphorylated amino acids were acylphosphates i.e. likely to be the ATPase thiophosphointermediate. The remaining thiophosphorylated amino acids (73%) were thought to be produced by protein kinases. This was supported by the autoradiographies of membrane SDS-PAGE which indicated that, in addition to the 95 kDa ATPase subunit, other polypeptides were thiophosphorylated especially at 108, 58, 47, 45 and 36–40 kDa. A previous study had provided strong evidence that chloride transport in gastric microsomes, is modulated by a protein kinase-dependent phosphorylation (Soumarmon, A., Abastado, M., Bonfils, S. and Lewin M.J.M. (1980) J. Biol. Chem. 255, 11682–11687). In the present work, we demonstrate that the peptidic inhibitor of cAMP-dependent protein kinases decreased thiophosphorylation of a 45 kDa polypeptide. We suggest that this polypeptide could be regarded as a candidate for the role of chloride transporter or chloride transport regulator.

Introduction

Gastric HCl secretion is thought to involve three main ionic transporters of the apical membrane of parietal cell: the ($H^+ + K^+$)-ATPase which actively secretes H^+ [1], a Cl^- and a K^+ conductive channel. The latter should be responsible for coupling between H^+ and Cl^- secretions in vivo [2,3]. Considerable information on ($H^+ + K^+$)-ATPase structure and function are available but little is known about the two ionic channels. We have provided evidence for the existence of a protein kinase-regulated electrogenic Cl^- transporter in isolated gastric membranes [4]. This transporter could be a good candidate as the apical chloride transporter of parietal cell.

Regulation of acid secretion by histamine or

Abbreviation: ATP[S], adenosine 5′-[γ-thio]triphosphate.

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somatostatin, is likely to require phosphorylation-dephosphorylation cascades of cytoplasmic enzymes [5–7]. Such events could take place at the secretory membrane level and act in the regulation of transporters. One of the putative targets could be the chloride conductance.

In the present work, we have tested phosphorylation capacities of gastric ($H^+ + K^+$)-ATPase-enriched membranes, using ATP[S] as a phosphorylating analog of ATP [8].

Materials and Methods

Materials

[γ-^{32}P]ATP (3 Ci/mmol), [γ-^{35}S]ATP[S] (600 Ci/mmol) and fluorographic solution (Amplify) were purchased from Amersham International (U.K.). Hepes, cAMP-dependent protein kinase inhibitor and ATP were obtained from Sigma (St Louis, MO, U.S.A.), Tris from Prolabo (Paris, France). *n*-Octyl glucoside, pyruvate kinase and ATP[S] were purchased from Boehringer (Mannheim, F.R.G.).

Monoclonal antibody against gastric ($H^+ + K^+$)-ATPase was a gift from Dr. Adam Smolka (Cure Institute, Los Angeles, CA, U.S.A.) [9].

Methods

Preparation of gastric membranes. Membranes were prepared as previously described [10]. Fresh hog stomachs were obtained from the slaughterhouse (Domont Abattoirs, Pontoise). The fundic mucosa was homogenized and differential centrifugations were run to obtain the microsomal fraction [4]. This fraction was purified on a discontinuous sucrose gradient and the 8.5–30% sucrose interface was used as the source of purified gastric membranes [10]. Specific activity of the K^+-dependent ATPase activity of the preparation ranged in between 63 and 132 μmol per mg of protein and per hour.

Proteins. Proteins were measured by the Coomassie blue staining method according to Bradford [11] using bovine serum albumin fraction V as standard.

Phosphorylations. [^{32}P]ATP. Phosphorylation assays were carried out according to Wallmark et al. [12]. Incubation volume was 110 μl. The medium contained [γ-^{32}P]ATP (100000 cpm), 5 μM ATP, 2 mM $MgCl_2$ and 50 mM Hepes (pH 7.25) with Tris powder. The reaction was started by the addition of 50–130 μg of protein and run at 0°C for 10 s. It was stopped by the addition of 2 ml of either 10% $HClO_4$ containing 40 mM inorganic phosphate or 1 ml of 10% trichloroacetic acid containing 10 mM inorganic phosphate at 0°C. Precipitated samples were filtered on Millipore filters (HAWP 0.45 μm) which were then dissolved in 1 ml acetone and counted in a scintillation counter.

[^{35}S]ATP[S]. Thiophosphorylation assays were carried out using 6–50 nM [γ-^{35}S]ATP[S] ($7 \cdot 10^5$–$6 \cdot 10^6$ cpm per assay). The incubation medium (110 μl) contained radioactive ATP[S], 10 mM $MnCl_2$ and 50 mM Hepes (pH 7.25) with Tris powder. Reaction was started by the addition of 10–200 μg of membrane protein. Gastric membranes were prepared either in the presence or in the absence of dithiothreitol.

Thiophosphorylation of both preparations was run for 1 h at either 0 or 30°C. It was stopped as described above for the ^{32}P phosphorylation reactions.

Stability in hot acid and hydroxylamine was tested as described by Kirchenberger et al. [13].

($H^+ + K^+$)-ATPase activity and Cl^--transport were measured as previously reported [4,10].

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Laemmli's technique with minor modifications was used [10,14]. The separating gel and stacking gel were 10% and 5% polyacrylamide, respectively. Radiolabelled membranes were precipitated in acid and pelleted by centrifugation. Pellets were suspended at room temperature in 50 mM Tris-HCl (pH 6.8), 2 mM EDTA, 2% SDS, 0.01% mercaptoethanol, 10% glycerol and 0.25% Bromophenol blue. The suspension stood for 60 min before loading 100 μl samples over the stacking gel. Electrophoresis was started at 15–20 mA. When the blue dye reached the separating gel, power was increased up to 35–40 mA. The migration was run overnight at 0–4°C. The gel slabs were fixed and stained in 10% acetic acid, 10% trichloroacetic acid, 30% methanol and 0.15% Coomassie blue R250 for 3 h at room temperature. They were destained in 5% acetic acid, 10% ethanol for 4–6 h followed by 8% acetic acid, 25% ethanol overnight at room temperature.

Autoradiography. Stained gels for ^{32}P radiography were dried and then exposed to Kodak X-AR Omat 5 films. Gels for ^{35}S radiography were treated by fluorography using Amplify (Amersham), and dried and then exposed to Kodak X-AR OMAT 5 films at −70°C. Gels containing 100000 cpm samples were exposed to the film for 2 days.

Results

I. [^{32}P]Phosphorylation

In the presence of 5 μM ATP, [γ-^{32}P]ATP tracer and 2 mM Mg^{2+}, gastric membranes rapidly incorporated ^{32}P [10,12]. Maximal incorporation was reached after 10 s at 0°C and followed by a rapid decrease. Phosphorylated intermediates were stabilized by acid precipitation at 0–4°C. The acid-precipitated radioactivity was liberated (93–97%) at 90°C or after hydroxylamine treatment. This suggested that phosphate groups were incorporated as acyl phosphate and were related to the phosphointermediate of $(H^+ + K^+)$-ATPase (Table I). This finding was consistent with the SDS-PAGE autoradiography indicating that radioactivity was mainly incorporated in the 95 kDa band referred to as the $(H^+ + K^+)$-ATPase subunit. Furthermore, little ^{32}P incorporation remained when phosphorylation was run in the presence of K^+ which is known to stimulate the rate of ATPase dephosphorylation (Fig. 1) [12].

A minor band of radioactivity was also found at 220 kDa. This was attributed to incompletely dissociated ATPase because of its sensitivity of K^+ dephosphorylation and because of the positive reaction of this polypeptide with $(H^+ + K^+)$-ATPase monoclonal antibody [9]. No significant amount of other phosphorylated intermediates was detected even with increasing ATP concentrations.

TABLE I

CHEMICAL STABILITY OF PHOSPHORYLATED AND THIOPHOSPHORYLATED PROTEINS FROM HOG GASTRIC MEMBRANES

Gastric membranes prepared in dithiothreitol were either phosphorylated 10 s at 0–4°C using 5 μM ATP, [γ-^{32}P]ATP (100000 cpm) and 2 mM $MgCl_2$ or thiophosphorylated 45 min at 0–4°C in the presence of 5 μM ATP[S], [γ-^{35}S]ATP[S] (130000 cpm). Thiophosphorylation assays contained either 2 mM $MgCl_2$ or 10 mM $MnCl_2$. Reactions were stopped by 1 ml of trichloroacetic acid 10% (TCA) and samples were centrifuged. Pellets were treated either for 15 min at 90°C by 0.2 ml of 10% trichloroacetic acid plus 0.1 mM Na_2HPO_4 or for 15 min at 30°C by 0.5 ml of 0.8 M hydroxylamine in 50 mM acetate (pH 5.8). Controls remained in 0.2 ml trichloroacetic acid on ice. Samples were then cooled and precipitated with 1 ml of 20% trichloroacetic acid containing 10 mM NaH_2PO_4 and centrifuged. Radioactivity in the pellets was counted. Results are means of independent triplicate assays, and are expressed as pmoles of phosphate or thiophosphate per mg of protein. Treatment with hot trichloroacetic acid hydrolyzes acyl phosphate, histidyl phosphate and lysyl phosphate. Treatment with hydroxylamine hydrolyzes acyl phosphate.

Conditions	^{32}P radioactivity	^{35}S radioactivity	
	Mg^{2+}	Mg^{2+}	Mn^{2+}
Control, 4°C	705	287	615
TCA 10%, 90°C	19	101	412
Hydroxylamine 0.8 M	48	114	452

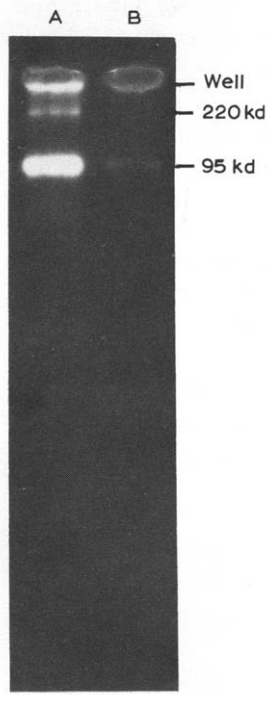

Fig. 1. Autoradiography of SDS-PAGE of [^{32}P]phosphorylated hog gastric membranes. Gastric membranes, prepared in the presence of dithiothreitol, were phosphorylated 10 s at 0°C using 5 μM [γ-^{32}P]ATP and 2 mM $MgCl_2$ with (lane B) and without (lane A) 20 mM KCl. Reaction was stopped by 1 ml 10% trichloroacetic acid and samples were centrifuged. Pellets were dissolved and immediately loaded onto a 10% polyacrylamide gel.

II. [^{35}S]Thiophosphorylation

a. ATP[S] and ATP interactions with ($H^+ + K^+$)-ATPase. K^+-stimulated ATPase activity was decreased by addition of ATP[S] (Fig. 2). ATP[S] (1 mM) inhibited 50% of ATPase activity when measured at 37°C in the presence of 2 mM ATP. However, significant inhibition of [^{32}P]ATP phosphorylation at 0°C required preincubation of ATP[S] with the membranes prior to ATP addition.

b. Thiophosphorylation. Rate of thiophosphorylation was slower than rate of phosphorylation and, at 0°C, maximal level of thiophosphorylation was reached after 1 h. Thiophosphointermediates were more stable than phosphointermediates and the kinetic reaction remained as a plateau for at least 1 h (Fig. 3). In the presence of 5 μM ATP[S] and manganese at 0°C, maximal incorporation varied from 0.3 to 0.8 nmol/mg of protein with a mean incorporation of 0.58 ± 0.06 nmol ($n = 7$). Magnesium and manganese were activators but magnesium was not as efficient as manganese. Maximal stimulation was obtained using 10 mM manganese and under these conditions, ^{35}S incorporation was 2–3-fold higher than in the presence of 2 mM magnesium. Moreover, the qualitative

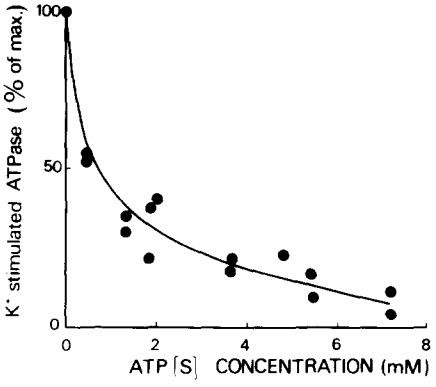

Fig. 2. Inhibition of ($H^+ + K^+$)-ATPase by ATP[S]. Hog gastric membranes were incubated 10 min at 37°C in the presence of 2 mM MgATP, 4 mM phospho*enol*pyruvate, 1 μl/ml pyruvate kinase, 40 mM Hepes-Tris (pH 7) and increasing concentrations of ATP[S]. Three experiments were performed using different preparations of membranes. For each experiment, K^+-stimulated activity was calculated as the difference between the activity in the presence of Mg^{2+} and K^+ minus the activity in the presence of Mg^{2+} alone. 100% was 69 ± 5 μmol P_i per mg of protein per hour.

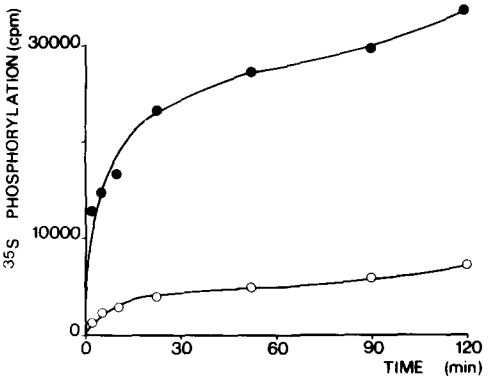

Fig. 3. Kinetics of thiophosphorylation of hog gastric membranes at 0°C. Membranes (10 μg protein) were incubated in 110 μl of 40 mM Hepes-Tris (pH 7) containing [γ-^{35}S]ATP-[S] (750000 cpm), 10 mM $MnCl_2$, 0.2 M sucrose with (○——○) or without (●——●) 5 μM unlabelled ATP[S]. Reaction was stopped by the addition of 1 ml 10% trichloroacetic acid containing 10 mM NaH_2PO_4. Precipitates were collected by filtration on Millipore filters (0.45 μm) which were then dissolved in 1 ml acetone. At 120 min in the presence of 5 μM ATP[S], 0.45 nmol thiophosphate were incorporated per mg of protein.

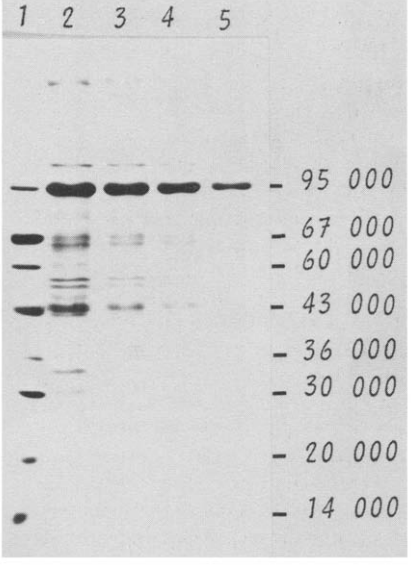

Fig. 4. Coomassie blue staining of SDS-PAGE of hog gastric membranes. Polyacrylamide gel had a 5–20% gradient. Electrophoresis was run and stained using 20, 15, 10 and 5 μg of protein in the wells 2–5, respectively. The left lane (1), was loaded with molecular weight markers: phosphorylase *b* (95000), albumin (67000), catalase subunit (60000), ovalbumin (43000), lactatate dehydrogenase subunit (36000) and carbonic anhydrase (30000).

effect of both cations was different. In the presence of magnesium and at 0°C, 0.29 nmol of thiophosphointermediates could be synthetized which represented 0.18 nmol of acylphosphate plus 0.11 nmol of other ester phosphates. In the presence of manganese and at the same temperature, the same amount of acyl phosphate was found but more other ester phosphates were synthetized (0.43 nmol) (Table I). Thus manganese improved thiophosphorylation of serine, threonine or tyrosine i.e. the activity of protein kinase. More radioactivity (factor 2.7 ± 0.6 ($n = 5$)) was incorporated at 30°C than at 0°C.

c. Autoradiography of thiophosphorylated-membrane PAGE. The protein patterns of hog gastric membrane-SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were not different whether membranes were phosphorylated or not. The major band was 95 kDa and as shown in Fig. 4, increasing protein load revealed minor bands, especially at 108, 67, 52, 49, 43, 32 and 30 kDa.

SDS-PAGE autoradiographies of [^{35}S]thiophosphorylated membranes displayed a whole pattern of radioactive polypeptides (control lanes of Fig. 5), in sharp contrast with the only two bands obtained with [γ-^{32}P]ATP (Fig. 1). The 95 kDa band and a 47 kDa one were labelled. At 30°C, the 47 kDa band was less labelled than at 0°C and it was underlined by a more intense 45 kDa

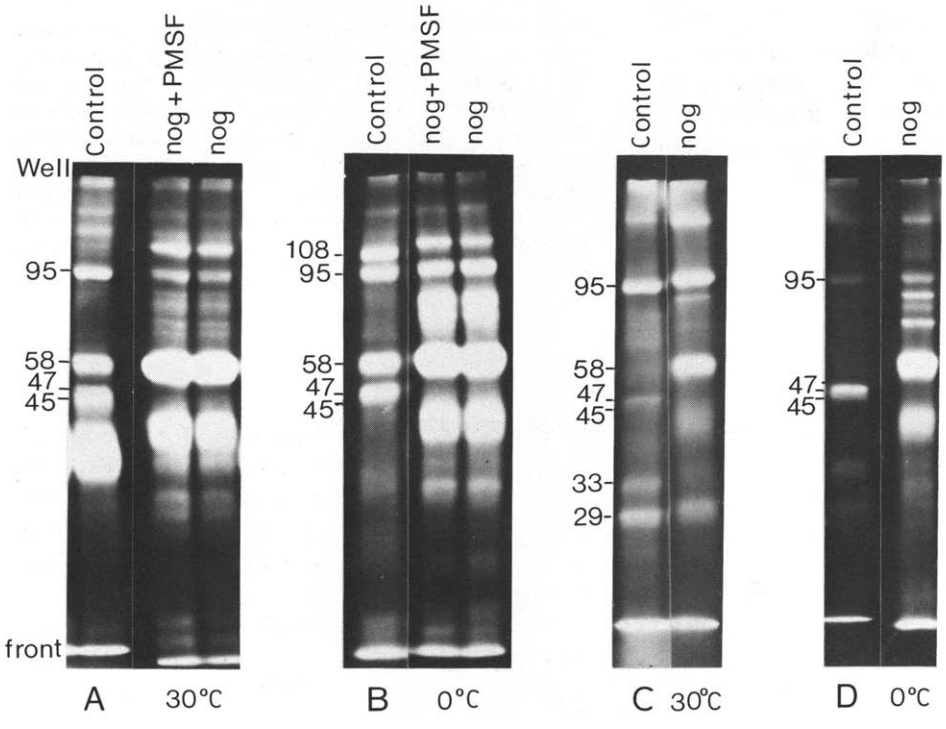

Fig. 5. Autoradiography of SDS-PAGE of thiophosphorylated hog gastric membranes. Thiophosphorylation of hog gastric membranes, prepared either in the presence (series A and B) or in the absence (series C and D) of dithiothreitol, was carried out for 1 h using 20 μg protein in 110 μl of a medium containing [γ-^{35}S]ATP[S] ($5 \cdot 10^6$ cpm) and 10 mM $MnCl_2$. Temperature of incubation (0° or 30°C) are specified under the figure. Whether membranes were solubilized with *n*-octyl glucoside (nog) prior to the assay or not (control) is specified over the figure. Solubilization by *n*-octyl glucoside was carried out using 1.5% detergent and 0.4 mg protein/ml i.e. a 1:40 (protein-to-detergent) ratio, with and without phenylmethylsulfonyl fluoride (PMSF). After 1 h of thiophosphorylation, samples were precipitated in acid and pellets were dissolved in SDS. Polyacrylamide gel electrophoreses were run, gels were stained, dried and exposed for autoradiography. As specified in the text, intensity of labelling was always much greater using *n*-octyl glucoside. Time of exposure for photographic printing had to be varied because of difference in negative intensity due to variation in the activity in the presence and in the absence of the detergent. This explains why the 95 kDa band which contained more label in the presence of the detergent seems weaker on the photographic print. Molecular weights are given in kDa.

band. Labelling of the 45 kDa polypeptide required the presence of dithiothreitol. It was only in dithiothreitol-prepared membranes that a 58 kDa polypeptide, a 108 kDa one and a wide zone in between 36 and 40 kDa could be labelled. The 108 kDa bands was more important at 0°C than at 30°C, whereas the large 36–40 kDa zone was only seen at 30°C. The differences between the autoradiographic patterns of assays at 0°C and 30°C suggested temperature-dependent proteolysis. However, if membranes were incubated at 30°C for 1 h prior to the thiophosphorylation assay at 0°C, no decrease of the 108 kDa polypeptide labelling and no phosphorylation of the 36–40 kDa zone were noted. Furthermore, protease inhibitors such as PMSF and bacitracin failed to improve labelling of the 108 kDa polypeptide at 30°C. PMSF decreased thiophosphorylation of the 36–40 kDa zone, perhaps by sulfonylation of seryl residues. Thus, increase of the assay temperature was not favoring proteolysis (of the 108 kDa polypeptide) but it was suggested to trigger different protein kinases.

Effect of n-octyl glucoside

Incubations in the presence of 1.5% *n*-octyl glucoside decreased the [^{32}P]phosphorylation capacity to almost zero while, by contrast, it stimulated 2–7-fold the thiophosphorylation capacity. *n*-Octyl glucoside is known to solubilize ($H^+ + K^+$)-ATPase. In the present conditions i.e. a detergent-to-protein ratio of 40 : 1, solubilization alters enzyme activity by decreasing the level of ATPase phosphointermediate [10,15]. The discrepancy between the stimulation of thiophosphorylation and the inhibition of phosphorylation suggested an activation of protein kinases (and protein phosphatases). Autoradiographies of SDS-PAGE showed that, in the presence of *n*-octyl glucoside, all bands were more labelled except the 47 and 45 kDa polypeptides (Fig. 5). Labelling of the 58 kDa polypeptide was markedly enhanced and seen both in dithiothreitol and in dithiothreitol-free membranes. Other bands of thiophosphopeptides were noted especially in between 58 and 95 kDa (Fig. 5).

Thiophosphorylation and Cl^- transport

As previously found on similar preparations,

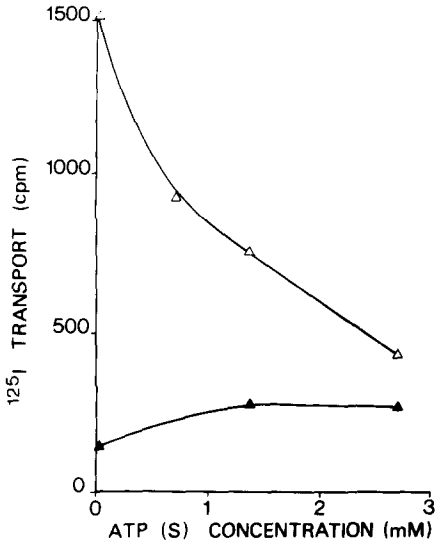

Fig. 6. Effect of ATP[S] on ^{125}I uptake by hog gastric microsomes. Gastric microsomes (50 μl at 13.7 mg protein/ml) were diluted in 300 μl of 0.25 M sucrose, 40 mM Hepes-Tris (pH 7.2) containing 1 μl pyruvate kinase, 5 mM phospho*enol*pyruvate in the presence (△———△) or in the absence (▲———▲) of 2.5 mM MgATP and of increasing concentrations of ATP[S]. Anion uptake was initiated by the addition of radioactive iodide (275000 cpm/100 μl) and stopped after 2 min at 30°C by filtration on Dowex columns as previously detailed [4]. Eluted radioactivity was counted.

ATP stimulated 10-fold the rate of iodide uptake by fresh hog gastric microsomes [4] (Fig. 6). ATP[S] mimicked this effect but maximal level of stimulation was only a factor two. In the presence of ATP, ATP[S] acted as an inhibitor of ATP stimulation (Fig. 6).

Stimulation by ATP of Cl^- transport had been related to the presence of a cAMP-dependent protein kinase [4]. ATP[S] could act as a thiophosphorylating agent of the chloride transporter. In the presence of 5 units of protein kinase inhibitor and 56 μg of membrane protein, thiophosphorylation of the 45 kDa polypeptide was specifically decreased (Fig. 7). In our conditions of assay, addition of cAMP failed to improve the labelling of the 45 kDa peptide as it failed to improve the rate of anion uptake by the vesicles. It suggested that only the catalytic subunit of the kinase was involved.

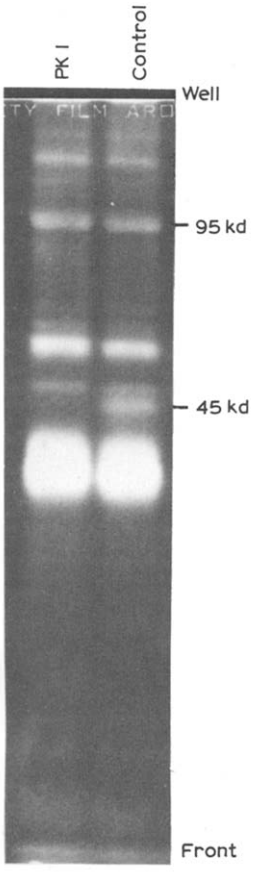

Fig. 7. Effect of protein kinase inhibitor on hog gastric membranes thiophosphorylation. Membranes (56 μg protein) were thiophosphorylated at 30°C using [γ-^{35}S]ATP[S] ($3 \cdot 10^6$ cpm), 10 mM $MnCl_2$, 40 mM Hepes (pH 7.2) and 2 mM dithiothreitol. Effect of protein kinase inhibitor (PK I, 5 units) was tested by reference to a control with no inhibitor as specified over thiophosphorylation. Membranes (56 μg protein) were thiophosphorylated at 30°C using [γ-^{35}S]ATP[S] ($3 \cdot 10^6$ cpm), 10 mM $MnCl_2$, 40 mM Hepes (pH 7.2) and 2 mM dithiothreitol. Effect of protein kinase inhibitor (PK I, 5 units) was tested by reference to a control with no inhibitor as specified over the figure.

Discussion

Previous studies have shown that hog gastric ($H^+ + K^+$)-ATPase can autophosphorylate to synthesize an acylphosphate and no other membrane protein was phosphorylated [10,12,16]. The present paper shows that ATPase can also be thiophosphorylated. It also provides evidence for thiophosphorylation of other membrane proteins due to the presence of membrane protein kinases.

Rat gastric membranes have been reported to contain a Ca^{2+}-dependent protein kinase which phosphorylates a 88 kDa polypeptide and increases the phosphorylation of 47, 52 and 59 kDa polypeptides [17]. The 88 kDa polypeptide was not seen in hog gastric membranes, but phosphorylation of 47 and 58 kDa polypeptides was observed. Although rat stomach is secreting acid, it is known that vesicular H^+ transport activity, as well as membrane K^+-ATPase activity, are more difficult to measure in rat than in hog [18,19]. The role of phosphorylation in regulation of ion transport should be more easily approached using hog membranes.

ATP[S] is an analog of ATP in which an atom of sulfur replaces an oxygen [8]. using equivalent concentrations of both nucleotides (5 μM), levels of thiophosphorylation and phosphorylation were in the same range. This strongly suggests that thiophosphorylation is not a minor activity of the material. That ATP[S] may serve as a substrate for ($H^+ + K^+$)-ATPase is supported by ATP[S] inhibition of ATPase activity and of ATPase phosphorylation as well as by the labelling of the 95 kDa subunit by radioactive ATP[S] and the sensitivity of thiophosphointermediates to hydroxylamine. However, it is likely that rate of ATP[S] binding to the ATPase at 0°C was slower than rate of ATP binding because ATPase phosphorylation was poorly inhibited if no preincubation with the thionucleotide was run. Once bound to the ATPase, the ATP[S] (or thiophosphate) should stay longer than ATP. Only 0.5 mM of ATP[S] was required to inhibit 50% of the ATPase activity (in the presence of 2 mM ATP). These preliminary observations suggest that ATP[S] could be useful to study specific aspects of gastric ATPase catalytic cycle as has been recently shown with ($Na^+ + K^+$)-ATPase [20].

ATP[S] was also shown to be the substrate of protein kinase mediated phosphorylations [21]. In the present study, this property was emphasized by using manganese as the activating cation instead of magnesium. Evidence for the implication of protein kinases was derived from the phosphorylated amino acids. Sensitivity of the [^{32}P]phosphoamino acids to hydroxylamine treatment suggested that 93–97% of the phosphopep-

tides were the ATPase subunit. Sensitivity of the [^{35}S]thiophosphoamino acids to the same treatment suggested that only 26% were acyl residues (after an incubation at 0°C in presence of Mn^{2+}). This represented 0.16–0.18 nmol per mg of protein and suggested that only part of gastric ATPase was thiophosphorylated. ATPase phosphointermediates accumulate because the rate of dephosphorylation is slow as compared to the rate of phosphorylation. Low yield of ATPase thiophosphorylation should be due to the modification of one or both rates. Hydroxylamine-insensitive and hot acid-insensitive thiophosphoesters (67–73% of the compounds) were likely serine, threonine or tyrosine residues. However, precise identification of the thiophosphorylated amino acids will require a more sophisticated analysis [22].

Thiophosphoproteins were already described to be poor substrates of protein phosphatases [21]. In the present study, this was confirmed when the kinetics of thiophosphorylation remained as a plateau. Failure to phosphorylate proteins other than the ATPase with ATP, could be explained by the presence of potent membrane protein phosphatases.

Up to now, the role of thiophosphorylable proteins is unknown. The treatment of membranes with a high concentration of NaBr released a few extrinsic proteins and demonstrated that most protein kinases and most thiophosphorylable proteins, for instance the 45 kDa polypeptide, were intrinsic membrane constituents (unpublished data). Capacity for chloride transport at the apical membrane of gastric epithelial cell is increased by histamine. This effector stimulates cAMP synthesis and increases the activity of type I cAMP-dependent protein kinase [5,7]. It was recently proposed that chloride transporter is opened in the stimulated cells but closed in the resting ones [4]. Efforts to characterize the chloride transporter have shown that preparations of $(H^+ + K^+)$-ATPase membranes carry a quiescent chloride transporter. Chloride conductance was unmasked by ageing, and by trypsin or Cu^{2+}-phenanthroline treatment [23,24]. Chloride conductance was also unmasked by MgATP [4]. Effect of ATP appeared to require a protein kinase because it was inhibited by the peptide inhibitor of cAMP-dependent protein kinase. In the present study, we demonstrate that the kinase inhibitor decreased the thiophosphorylation of only one peptide, of 45 kDa. Thiophosphorylation of the 45 kDa polypeptide was improved by dithiothreitol which, as previously shown [25], maintains the ATP activation of the Cl^--transporter. We may then propose that chloride transporter of gastric apical membranes is regulated by a cAMP-dependent protein kinase and, in that proposal, the 45 kDa polypeptide can be regarded as a candidate for the role of chloride transporter or chloride-transport regulator.

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