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Phosphorylation of H,K-ATPase α-Subunit in Microsomes from Rabbit Gastric Mucosa by cAMP-Dependent Protein Kinase

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A 100-kDa protein that is a main component of the microsomal fraction from rabbit gastric mucosa is phosphorylated by cAMP-dependent protein kinase (PKA) in the presence of 0.2% Triton X-100. Microsomes from rabbit gastric mucosa possess activity of H,K-ATPase but not activity of Na,K-ATPase. Incubation of microsomes with 5 μM fluorescein 5′-isothiocyanate (FITC) results in both an inhibition of H,K-ATPase and labeling of a protein with an electrophoretic mobility corresponding to the mobility of the protein phosphorylated by PKA. The data suggest that the α-subunit of H,K-ATPase can be a potential target for PKA phosphorylation.

KEY WORDS: H,K-ATPase; H^+-secretion; cAMP-dependent protein kinase; phosphorylation; gastric mucosa.

INTRODUCTION

The gastric H,K-ATPase (proton pump), a close relative of Na,K-ATPase, is responsible for HCl secretion by the gastric parietal cells [1]. This ATPase catalyzes K-dependent ATP hydrolysis coupled to the active transport of H^+ and K^+ across the membrane. The enzyme consists of a large catalytic (M_r about 100 kDa) and a small glycosylated subunit (M_r about 55 kDa), designated α- and β-subunits, respectively [2]. In contrast to Na,K-ATPase, the H,K-ATPase has not, so far, been obtained as a highly purified enzyme. However, primary structures of both H,K-ATPase subunits have been deduced from the corresponding cDNAs [3, 4].

Acid secretion by the parietal cells is stimulated by histamine which interacts with H_2-receptors [5]. Stimulation of secretion is accompanied by a significant change in the morphology of parietal cells: the surface of the apical membrane is

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increased to form secretory canaliculi covered by microvilli. These changes are due to the fusion of the apical membrane with membrane fragments containing the H,K-ATPase that are stored in the cytoplasm [1].

Stimulation of acid secretion by histamine induces an increase in cAMP level in parietal cells that, in turn, leads to activation of PKA [5]. As a result, many intracellular proteins are phosphorylated by PKA [6]. However, only some have been identified to date. The goal of the current study was to determine whether H,K-ATPase was one such target of PKA phosphorylation.

METHODS

Microsomes enriched in H,K-ATPase were prepared from rabbit gastric mucosa as described previously [7]. The catalytic subunit of PKA (type II) from bovine brain (specific activity 0.5 unit/mg protein) was kindly provided by Professor E. S. Severin (Laboratory of Enzyme Chemistry, Moscow State University).

ATPase activity was measured by the coupled enzyme assay [8] at 37°C with 0.3 M sucrose, 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 3 mM ATP, 1.5 mM phosphoenolpyruvate, 0.2 mM NADH, 10 units/ml lactate dehydrogenase, and 15 units/ml pyruvate kinase. NADH oxidation was not observed in the absence of ATP. K-stimulated activity, representing H,K-ATPase, was estimated as the difference between the activity in the presence of 3 mM $MgCl_2$ + 20 mM KCl and basal activity in the presence of 3 mM $MgCl_2$ alone. To identify Na,K-ATPase activity, 100 mM NaCl was added to incubation medium. The reaction was initiated by the addition of protein (20 μg/ml) and was monitored continuously at 340 nm.

Phosphorylation of microsomes was performed at 30°C with 25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1 μM pepstatin, 10 μM leupeptin, 100 μg/ml soybean trypsin inhibitor, 10 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride, 2 μg PKA, and 8 μg of microsomal protein in a volume of 40 μl. When used, Triton X-100 was 0.2% (v/v). Reaction was started by the addition of 100 μM ATP containing [γ-^{32}P]ATP (1000–2000 cpm/pmol), proceeded for 30 min, and was stopped by the addition of 10 μl of SDS-containing buffer. Samples were subjected to SDS polyacrylamide gel electrophoresis according to Laemmli [9], the gels were stained with Coomassie Brilliant Blue. Phosphorylated proteins were visualized by autoradiography.

Labeling of microsomes with FITC was carried out at room temperature for 1 hr as described earlier [10]. The reaction was stopped by 100-fold dilution of the incubation medium with cold 50 mM Tris-HCl + 0.25 M sucrose. Microsomes were pelleted at 100,000 g for 1 hr, suspended in 50 mM Tris-HCl, and subjected to SDS polyacrylamide gel electrophoresis. FITC-labeled proteins were detected in unstained gels using a transilluminator UV light box.

Fluorescence measurements were made using a Hitachi-F3000 spectrofluorometer in a volume of 3 ml containing 25 μg/ml protein in 50 mM Tris-HCl (pH 7.4). Excitation and emission wavelengths were 495 nm and 520 nm, respectively.

Protein was assayed by the method of Lowry *et al.* [11].

Materials were obtained as follows: EDTA, Tris, ATP, PIPES, sucrose, NADH, phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase were from Sigma

(USA), SDS and ouabain were from Serva (Germany), dithiothreitol and inhibitors of proteases were from Boehringer Mannheim (Germany). [γ-^{32}P]ATP was purchased from Isotop (Obninsk, Russia). The sodium salt of ATP was converted into Tris salt by ion-exchange chromatography. All other chemicals were of reagent grade.

RESULTS AND DISCUSSION

Aliquots of the microsomal fraction from rabbit gastric mucosa were incubated with PKA and [γ-^{32}P]ATP under different conditions and then proteins were separated by SDS polyacrylamide gel electrophoresis. Figure 1A shows the results of Coomassie Blue staining of the gels whereas autoradiograms of the same gels are shown in Fig. 1B. Lane 3 (Fig. 1A) demonstrates the results of incubation of the

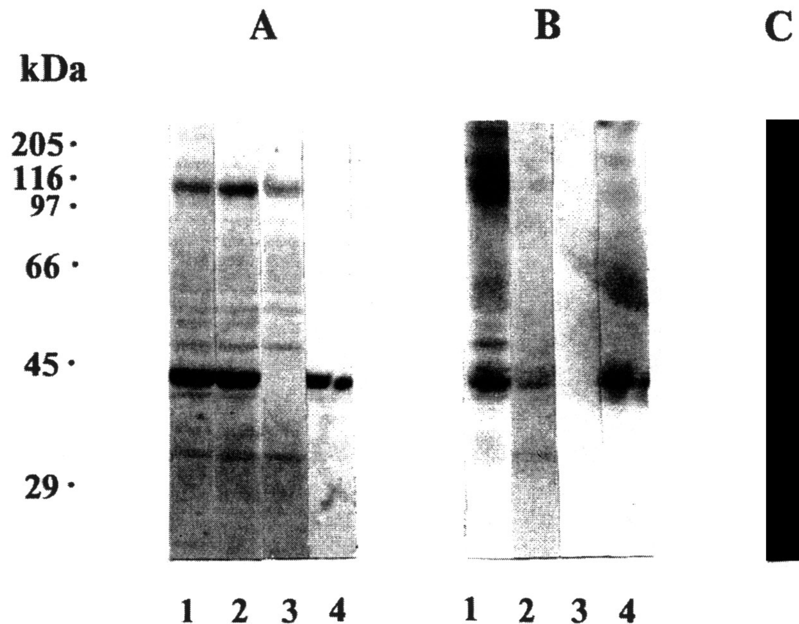

Fig. 1. Phosphorylation of H,K-ATPase in microsomes from rabbit gastric mucosa by PKA. (A) Separation of the microsomal fraction from rabbit gastric mucosa after protein phosphorylation by PKA using SDS polyacrylamide gel electrophoresis; the gels were stained with Coomassie Brilliant Blue. Phosphorylation was carried out as described in Methods in the presence of: lane 1—8 μg microsomal protein, 2 μg PKA, 0.2% v/v Triton X-100; lane 2—8 μg microsomal protein, 2 μg PKA; lane 3—8 μg microsomal protein, 0.2% v/v Triton X-100; lane 4—2 μg PKA, 0.2% Triton X-100. (B) Autoradiogram of the gel in Fig. 1A. (C) Separation of FITC-labeled microsomal proteins from rabbit gastric mucosa using SDS polyacrylamide gel electrophoresis (after treatment with 0.2% v/v Triton X-100, 8 μg microsomal protein was loaded per lane). Labeling of microsomes with FITC was as described in Methods. The photograph of the gel was made in a transilluminometer UV light box using a camera fitted with green filter.

microsomes with Triton X-100 but in the absence of added PKA and hence is the control situation. A number of protein bands are seen with the major component of the microsomal fraction being a protein with M_r about 100 kDa. Its content in different microsome preparations ranged 25–40% of the total protein. Figure 1A, lane 4 shows an incubation with PKA and Triton alone (minus the microsomes) and thus pinpoints the position of the PKA catalytic subunit (44 kDa protein) which is also seen in lanes 1 and 3.

In the absence of added PKA, no ^{32}P incorporation onto proteins occurred indicating no endogenous phosphorylation by any kinases that might be present in the microsomal preparation (Fig. 1B, lane 3). Incubation of microsomes with [γ-^{32}P]ATP + PKA led to low incorporation of ^{32}P into several proteins as shown in the autoradiogram of Fig. 1B (lane 2). One of these proteins was the PKA catalytic subunit itself as well as some other minor bands.

The addition of Triton X-100 to the incubation medium resulted in enhanced phosphorylation of several proteins in the microsomal fraction (Fig. 1B, lane 1). Again, one of these was PKA itself. Several new phosphoproteins were also radiolabeled (compare Fig. 1B, lanes 1 and 2). One of the new phosphoproteins present in incubations with detergent was a 100-kDa protein. This protein band showed the greatest radioactivity incorporation of any of the endogenous proteins in the microsomal preparation.

It is known that the main protein component of the microsomal fraction from gastric mucosa with M_r about 100 kDa is the H,K-ATPase α-subunit [1]. However, this fraction may be contaminated by Na,K-ATPase. Molecular mass of Na,K-ATPase α-subunit is also close to 100 kDa. Furthermore, Na,K-ATPase α-subunit can be phosphorylated by PKA in the presence of Triton [12, 13].

Hence, to confirm that the 100-kDa phosphoprotein was the α-subunit of H,K-ATPase, additional experiments were done. We measured the ATPase activity of the microsomal fraction from rabbit gastric mucosa. Total ATPase activity in the presence of 20 mM KCl was 42.2 ± 5.9 μmol/hr/mg protein whereas basal Mg-dependent ATPase was 15.2 ± 1.5 μmol/hr/mg protein. The addition of NaCl (100 mM) in the presence of 20 mM KCl inhibited K-stimulated ATPase activity. Ouabain (1 mM) had no effect on ATPase activity in a medium with KCl alone or with NaCl + KCl. Thus, the data show that the microsomal fraction from rabbit gastric mucosa is enriched in H,K-ATPase and devoid of Na,K-ATPase.

Next, we treated microsomes with low concentrations of the fluorescent dye, FITC, that specifically labels only catalytic subunits of P-type ATPases [15]. Incubation of microsomes with FITC (5μM, 1 hr) resulted in the loss of about 70% of H,K-ATPase activity. The addition of 10 mM KCl to FITC-labeled microsomes led to 10–15% quench of the fluorescence whereas 10 mM of choline chloride had no effect (*data not shown*). K^+ is known to quench fluorescence of FITC-labeled H,K- and Na,K-ATPase [10, 14] but since Na,K-ATPase was lacking in this fraction, we concluded that FITC labeled the α-subunit of H,K-ATPase.

Figure 1C shows the separation of proteins in FITC-labeled microsomes by SDS polyacrylamide gel electrophoresis. It can be seen that only one protein band includes the fluorescence label. The electrophoretic mobility of this labeled protein corresponded to that of the high abundance, ^{32}P-labeled 100 kDa protein (Fig. 1A and B, lane 1) that was phosphorylated by PKA in the presence of Triton X-100.

Together, all these data demonstrate that H,K-ATPase α-subunit can be phosphorylated by PKA in detergent containing medium.

Although cAMP-mediated protein phosphorylation is known to be involved in the activation of HCl secretion, there has been no information to date concerning the phosphorylation of H,K-ATPase by PKA. Nevertheless, a study of protein phosphorylation in parietal cells treated with histamine showed that about 20 different proteins were phosphorylated and 100 kDa protein was among them [6]. On the other hand, it was shown that the α1-subunit of Na,K-ATPase, that may be phosphorylated *in vivo* after activation of PKA [13], is also a target for PKA *in vitro* in the presence of detergents [12, 13] or after reconstitution of Na,K-ATPase into liposomes [16]. *In vivo* and in the presence of Triton X-100 *in vitro* PKA phosporylates the only serine residue (Ser-943) in Na,K-ATPase α-subunit from *Bufo marinus* kidney [13].

The homology of amino acid sequences between the α1-subunit of Na,K-ATPase and of H,K-ATPase is about 70% and this is sufficiently high to consider these proteins to be isoforms [17]. Analysis of the C-terminal amino acid sequence of the H,K-ATPase α-subunit revealed the existence of Ser-952 located in a conserved consensus sequence RRLS(952) that is conventional for PKA phosphorylation. It is likely, then, that this serine residue of the H,K-ATPase α-subunit is the one that is phosphorylated by PKA in the presence of detergent in our experiments.

Phosphorylation of H,K-ATPase α-subunit by PKA in the medium containing detergent may cause some doubts about the physiological significance of this phosphorylation. However, the occurrence of PKA-mediated phosphorylation of Na,K-ATPase *in vivo* and the dependence of this phosphorylation on detergent *in vitro* suggests that the conformation of Na,K-ATPase (as well as H,K-ATPase) is changed *in vitro* compared to the conformation of the pumps in the living cells. The presence of Triton alters protein conformation *in vitro* in a way that makes the phosphorylation site available to PKA. This suggestion is supported by the known location of Ser-943 that is the target for PKA phosphorylation in Na,K-ATPase and the putative target of phosphorylation in H,K-ATPase (Ser-952). According to the 10-transmembrane topology model of the α-subunit proposed for both ATPases, this serine residue is located in a short cytoplasmic loop which joins the 8th and 9th transmembrane segments [18]. This region of Na,K-ATPase α-subunit has been shown to have a very labile conformation [19]. One can suggest that the change in H,K- or Na,K-ATPase conformation induced by various factors *in vivo* (e.g., regulatory proteins) may be associated with a change in the accessibility of this site for PKA.

In conclusion, our findings suggest that H,K-ATPase might be a physiological substrate for PKA *in vivo*. Further studies should be directed to the identification of the phosphorylated residue(s), confirmation of the occurrence of phosphorylated and dephosphorylated forms of the enzyme *in vivo*, and clarification of the physiological relevance of phosphorylation to enzyme function.

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