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Site-directed antibodies as topographical probes of the gastric H,K-ATPase α-subunit

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Gastric acid is secreted by an ATP-driven H^+ and K^+ exchanger (H,K-ATPase), an integral apical membrane protein of parietal cells. Although the primary structure of the enzyme is known, its higher order structure is uncertain. In order to acquire topographical probes of native, microsomal H,K-ATPase, synthetic peptides corresponding to the 17 amino-terminal (N-peptide) and 16 carboxyl-terminal (C-peptide) residues of pig gastric H,K-ATPase α-subunit were coupled to keyhole limpet hemocyanin (KLH). Rabbits were immunized with peptide-KLH conjugates and their sera were tested for specificity by enzyme-linked immunosorbent assay (ELISA), immunoblotting, and immunocytochemistry. All sera showed high ELISA reactivities with synthetic peptides, peptide-BSA conjugates, and microsomal H,K-ATPase adsorbed to microtiter wells (some titers $> 1:10^4$). Immunoblots of H,K-ATPase resolved by SDS-PAGE showed both N-peptide and C-peptide antibodies reacting with a single 94 kDa band. All sera selectively stained parietal cells in pig gastric mucosal sections. Preimmune sera gave negative or weak signals in all assays. In competition ELISAs, N-peptide antibodies, but not C-peptide antibodies, were displaced from the corresponding bound synthetic peptides by added microsomal H,K-ATPase. One of the N-peptide antibodies inhibited H,K-ATPase activity by more than 50%; binding of this antibody was decreased when ATP or K^+ were bound to the enzyme. These results indicate a cytoplasmically-oriented α-subunit N-terminus which may participate conformationally in the H,K-ATPase catalytic cycle, and suggest that antibodies against synthetic H,K-ATPase peptides are potentially useful probes of native microsomal H,K-ATPase topography.

Introduction

Secretion of protons across the apical membrane of gastric parietal cells is mediated by a magnesium-dependent, potassium-stimulated, proton-transporting adenosine triphosphatase (H,K-ATPase, EC 3.6.1.37) [1]. This enzyme belongs to the class of plasma membrane ATPases which form a phosphorylated intermediate and are inhibited by vanadate. Other members of this class are Na,K-ATPase and Ca^{2+}-ATPase. Like the Na,K-ATPase, the gastric H,K-ATPase consists of an α-subunit which bears the catalytic site, and a β-subunit whose function is unknown. The rat and pig gastric H,K-ATPase α-subunits have been cloned and sequenced [2,3]. Hydropathy analysis [3] predicts a cytosolic N-terminus followed by four hydrophobic α-helices (H1–H4), a hydrophilic cytosolic domain which contains the phosphorylation site [4] and FITC [5] and pyridoxal phosphate [6] binding sites, and finally four more hydrophobic α-helices (H5–H8) preceding the putative cytosolic C-terminus. The rat and rabbit β-subunits have also been cloned and sequenced [7,8]; hydropathy plots predict a single transmembrane domain with uncertain location of N-and C-termini with respect to the membrane.

Direct verification of secondary structure models of the H,K-ATPase α-subunit based on hydropathy analysis awaits acquisition of X-ray diffraction data from α-subunit crystals which are not yet available. Our approach to α-subunit secondary structure is based on measurements of the interactions of site-directed polyclonal antibodies with gastric microsomes enriched in H,K-ATPase. We formed panels of antibodies by immunizing rabbits with synthetic peptides based on the deduced amino acid sequence of the N- and C-termini of the pig gastric H,K-ATPase α-subunit. We verified that the antibodies bound to native H,K-ATPase by

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Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pNPPase, *p*-nitrophenylphosphatase; kDa, kilodaltons; Ig, immunoglobulin; $C_{12}E_8$, octaethylene glycol dodecyl ether; P_i, inorganic phosphate; KLH, Keyhole limpet hemocyanin; BSA, bovine serum albumin; MBS, maleimidobenzoyl-*N*-hydroxysuccinimide ester.

means of competitive enzyme-linked immunosorbent assays (ELISA), immunoblotting, and immunocytochemistry. Antibody binding assays and effects on enzyme activity indicated a cytosolic, conformation-sensitive orientation of the α-subunit N-terminus.

Experimental procedures

Material

Microplates (96-well polystyrene) were obtained from Flow Laboratories. KLH, BSA, and MBS were from Sigma, while dialysis membranes were from Spectrapor. Polyvinylidifluoride membranes (Immobilon-P) were supplied by Millipore, and goat anti-rabbit Ig-horseradish peroxidase conjugate was from Bio-Rad. Sephadex G-10 and CNBr-activated Sepharose 4B were acquired from Pharmacia, and reagents for immuno-cytochemistry (Dako Pap Kit) were obtained from Dako. Precast SDS-PAGE gels, electrophoresis apparatus, and transblotting cells were products of Novex. All other chemicals were of the highest quality available.

Methods

Synthetic peptides. The heptadecapeptide corresponding to the NH_2-terminal amino acid sequence 1–17 (N-peptide), and the hexadecapeptide corresponding to the COOH-terminal amino acid sequence 1019–1034 (C-peptide) of the pig gastric H,K-ATPase α-subunit were synthesized by Dr. Christian Schwabe, Peptide Synthesis and Microsequencing Core Facility, Department of Biochemistry, Medical University of South Carolina, using an Applied Biosystems Model 430A peptide synthesizer using *t*-butoxycarbonyl chemistry. A cysteine residue was added to the carboxyl-terminus of the N-peptide to allow coupling of carrier proteins to cysteines and not to amino groups elsewhere on the peptides. Completion of coupling reactions was verified by the ninhydrin test. The sequence of the N-terminal peptide was Met-Gly-Lys-Ala-Glu-Asn-Tyr-Glu-Leu-Tyr-Gln-Val-Glu-Leu-Gly-Pro-Gly-Cys-OH, while the sequence of the C-terminal peptide was Gly-Val-Arg-Cys-Cys-Pro-Gly-Ser-Trp-Trp-Asp-Gln-Glu-Leu-Tyr-Tyr-OH.

Preparation of peptide conjugates. Peptides were coupled to KLH or BSA using MBS [9]. Before coupling, peptides were desalted by passage over Sephadex G-10, and to ensure coupling through cysteines, free amino groups in the peptides were blocked with citraconic anhydride before MBS treatment (these and subsequent coupling procedures were carried out at room temperature) 10 mg peptide was dissolved in 1 ml 100 mM Tris (pH 8.5) and applied to a 1.0 cm × 17.0 cm Sephadex G-10 column equilibrated in the same buffer. The first peak eluting from the column (10 mg peptide in approx. 2 ml 100 mM Tris, pH 8.5) was incubated with 20 mg citraconic anhydride in 2 ml H_2O, keeping the pH at 8.5. After 1 h, the reaction was stopped by adding 0.4 ml 1.0 M sodium phosphate (pH 7.2). MBS (dissolved in dimethylformamide at 25 mg/ml) was added dropwise with agitation to a final concentration of 5 mg/ml; after 30 min, the solution was made 35 mM in β-mercaptoethanol and incubated for 1 h. KLH or BSA (4 mg in 250 μl PBS, pH 7.4) was added, the solution was incubated for 3 h with stirring, and then uncoupled peptide was removed by overnight dialysis (12000–14000 molecular weight cut-off membranes) against PBS.

Generation of antibodies. Antibodies were raised in adult female New Zealand White rabbits; three rabbits were immunized with N-peptide-KLH conjugate, and three with C-peptide-KLH conjugate. On day 0, each rabbit was bled from the ear marginal vein (preimmune serum), and then received 500 μg of peptide-KLH conjugate emulsified with Freund's complete adjuvant, distributed subcutaneously into multiple dorsal sites. On days 18 and 56, peptide antigen emulsified in Freund's incomplete adjuvant was administered similarly. Rabbits were bled from the ear marginal vein on days 18, 28, 42, 70 and 105, the sera were made 0.02% in sodium azide, and were stored aliquoted at −20°C. For some assays, immunoglobulin G (IgG) was isolated from the sera by protein A-Sepharose chromatography as described previously [10].

N-peptide-specific antibody was recovered from immune IgG by peptide affinity chromatography. N-peptide-BSA conjugate was coupled to CNBr-activated Sepharose 4B according to the manufacturer's protocol. The affinity column (2 ml bed-volume) was equilibrated in 50 mM Tris-HCl (pH 7.4), and then 100 μl HKαN2-IgG (15 mg/ml) in the same buffer was applied. After a pre-elution wash with 10 mM phosphate (pH 8.0), specific antibody was eluted with 3 column volumes of 100 mM triethylamine (pH 11.5), collected into 1/20 volume 1 M phosphate (pH 6.8) and dialysed overnight against 50 mM Tris-HCl (pH 7.4).

Preparation of H,K-ATPase. Gastric microsomal vesicles enriched in H,K-ATPase activity were prepared by differential and sucrose/Ficoll step gradient centrifugation of pig gastric mucosal homogenates. Briefly, pig stomachs obtained at a slaughter-house within 1 h post-mortem were washed with ice-cold 0.25 M sucrose and the fundus was dissected from the cardiac and antral regions. All subsequent procedures were at 4°C. The mucosa was flooded with saturated NaCl, and the surface mucus and superficial cells wiped off with paper towels. The mucosa was scraped from the underlying connective tissue, suspended (10% w/v) in isolation buffer (0.25 M sucrose, 20 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), and disrupted by two 10-s bursts at maximum power in a Tissumizer (Tekmar, Cincinnati, OH). The homogenate was centrifuged at 20000 × *g* for 30 min,

and the supernatant was centrifuged at 10000 × *g* for 1 h. The resulting microsomal pellet was resuspended in isolation buffer, and applied to a discontinuous gradient of 7% w/v Ficoll and 34% sucrose (both in isolation buffer). After 3 h at 32500 rpm (Sorvall AH 629 rotor), the microsomal band (G1) recovered from the 7% Ficoll interface was resuspended to 10 mg/ml in 15 mM Pipes-Tris (pH 6.8), diluted 1:1 with cold 60% sucrose, and stored in 0.5 ml aliquots at −70°C. GI microsomes are about 0.1 μm in diameter, are enriched in H,K-ATPase activity, and more than 80% of their protein content bands at 94 kDa by SDS-PAGE.

Enzyme activity. K^+-stimulated ATPase activity was measured in 200 μl reaction volumes containing 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 2 μg H,K-ATPase, plus or minus 20 mM KCl. After addition of 20 mM ATP-Tris (final concentration), the enzymatic reaction proceeded for 15 min at 37°C; inorganic phosphate release was quantitated by the method of Forbush [11], adapted for 96-well microplates. The sensitivity of K^+-stimulated ATPase activity to ionophores was measured by addition of 10^{-5} M valinomycin or 10^{-5} M nigericin (final concentration) to the assay reaction volumes. To measure the effects of antibodies on H,K-ATPase activity, immune IgG or peptide-specific IgG were added to ATPase assay mixtures at room temperature 15 min before addition of ATP.

ELISA-based binding assays. The solid phase enzyme-linked immunosorbent assay (ELISA) was the primary assay for measuring interactions of antibodies present in immunized rabbit antiserum with the H,K-ATPase and with synthetic peptides. Assays were carried out in 96-well polystyrene plates at room temperature, with the plates rinsed five times with phosphate-buffered saline (PBS) between each of the sequential reagent incubations. Antigens were coupled to the plates by incubating microsomal H,K-ATPase, synthetic peptide, or peptide-BSA conjugates (one μg antigen in 100 μl 0.1 M Na_2CO_3, pH 9.6) in the wells for 1 h. Non-specific protein adsorption sites in the wells were then blocked by the addition of 200 μl 5% BSA/PBS, 0.02% sodium azide for 45 min. Primary antibodies in 100 μl dilution buffer (1% BSA/PBS, 0.02% sodium azide) were incubated in the wells for 1 h, followed by 100 μl secondary antibody (1:500 dilution of alkaline phosphatase conjugated-goat anti-rabbit antibody in dilution buffer), also for 1 h. Signal was developed by addition of 100 μl 1.0 mg/ml *p*-nitrophenyl phosphate in 1.0 M diethanolamine (pH 9.8) to the wells for 30 min. Quantitation of the signal

TABLE I

Development of antibodies in rabbits immunized with synthetic peptides based on N-terminus or C-terminus of H,K-ATPase α-subunit

Three N-peptide-immunized rabbits and three C-peptide-immunized rabbits were bled on the days indicated, and the sera were tested by ELISA for immune reactivity with appropriate synthetic peptide, peptide-BSA conjugate, or microsomal H,K-ATPase as the bound antigen.

Bound antigen	ELISA absorbance at 405 nm [a]					
	Preimmune	day 18	day 28	day 42	day 70	day 105
N-peptide sera						
N-peptide	0.092	0.010	0.106	0.143	0.311	1.117
	0.009	0.036	0.219	0.285	0.402	1.408
	0	0.004	0.034	0.071	0.237	1.034
N-peptide-BSA	0.112	0.113	0.918	1.124	1.181	1.389
	0.079	0.539	0.979	1.106	1.453	1.334
	0.041	0.086	0.513	0.952	1.442	1.336
H,K-ATPase	0.062	0.030	0.170	0.200	0.173	0.654
	0.175	0.259	0.337	0.478	0.485	0.950
	0.043	0.055	0.994	0.109	0.166	0.466
C-peptide sera						
C-peptide	0	0.031	0.542	0.538	0.783	1.315
	0	0.643	1.528	1.369	1.290	1.641
	0	0.617	1.556	1.388	1.171	1.643
C-peptide-BSA	0.118	0.137	1.167	1.384	1.113	1.066
	0.091	1.093	1.282	1.289	1.241	1.242
	0.097	1.083	1.293	1.110	1.307	1.358
H,K-ATPase	0.163	0.191	0.191	0.237	0.273	0.690
	0.199	0.222	0.273	0.339	0.340	0.780
	0.179	0.305	0.272	0.276	0.296	0.531

[a] Data are shown as the absorbances at 405 nm of ELISA reaction products, and represent the means of triplicate measurements of each serum sample.

was by measurement of the absorbance of the wells at 405 nm in a Titertek Multiskan plate reader (Flow Laboratories). For competition ELISAs, synthetic peptides were plated in microtiter wells and blocked with BSA as described above. In separate sets of microtiter wells, antibodies diluted in 50 mM Tris-HCl (pH 7.2), were incubated for 1 h with H,K-ATPase microsomes suspended in the same buffer, then transferred to the peptide-coated wells for quantitation by ELISA.

Immunoblotting. Microsomes prepared from pig gastric mucosa as detailed above were resolved by SDS-PAGE (4%–20% acrylamide gradient), and the resulting gel patterns were transferred electrophoretically to polyvinyldifluoride membranes at 300 mA for 1 h in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 10% methanol, pH 11.0 transfer buffer. The sheets were incubated for 1 h with 3% nonfat dry milk in 20 mM Tris, 150 mM NaCl (pH 7.5) (TBS), then for 1 h with varying dilutions of rabbit antisera in 1% nonfat dry milk, 1% Tween 20/TBS (TTBS). The sheets were washed 3 times in TTBS, and incubated for 1 h with 1:2500 dilution in TTBS of goat anti-rabbit Ig conjugated to horseradish peroxidase. After washing as above, the color reaction was developed by addition of 0.05% 4-chloro-naphthol, 0.015% H_2O_2 in TBS/15% methanol for 30 min.

Immunocytochemistry. Pieces of pig and rat gastric mucosa (2 mm × 10 mm) from the acid-secreting region on the greater curvature of the stomach were fixed for 1 h in Bouin's fixative, then embedded in paraffin, sectioned at 5 μm, and deparaffinized by standard procedures. Tissue sections were treated with 3% H_2O_2 for 5 min, then washed three times in PBS. Primary antibody binding to the sections at 1:25 dilution was assessed using the peroxidase-anti-peroxidase technique and phase-contrast microscopy (Zeiss IM 35).

Results

Binding assays

In the course of immunization with synthetic peptide-KLH conjugates, rabbit sera developed specific antibody binding activity towards synthetic peptides alone, peptide-BSA conjugates, and H,K-ATPase-enriched pig gastric microsomes. These immunoreactivities were measured by ELISA with the appropriate antigens adsorbed to microtiter plates, and are shown in Table I. Two of three rabbits immunized with C-peptide-KLH conjugate promptly (within 18 days) developed high levels of specific binding activity towards C-peptide and C-peptide-BSA conjugate. In comparison, development of activity towards microsomal H,K-ATPase was slow, with binding activity after 105 days approximating only half that measured against peptide alone or peptide-BSA conjugate. N-peptide-KLH conjugates elicited comparably high levels of activity against N-peptide and N-peptide-BSA conjugate, and again much slower development of activity against H,K-ATPase. Due to their relatively high titers, antisera HKαN2 and HKαC2 were the primary focus of further immunochemical assays.

Titers of N-peptide and C-peptide antibodies with respect to synthetic peptides, peptide-BSA conjugates, and pig gastric microsomes enriched in H,K-ATPase, compared to the binding of preimmune serum to the same antigens, were measured by ELISA, and a representative set of data for the N-peptide antibody HKαN2 and the C-peptide antibody HKαC2 is shown in Fig. 1. Titers of C-peptide antibodies against synthetic peptide and conjugate (expressed as the dilution of antibody yielding half-maximal binding to antigen) were approximately equivalent (1:8192) and somewhat higher than the corresponding titers for N-peptide antibodies (1:512 for N-peptide, and 1:4096 for N-peptide-BSA conjugate). Titers of both N- and C-peptide antibodies against native H,K-ATPase adsorbed to microplate wells were equivalent (1:512). Preimmune sera showed no specific binding to either synthetic peptides or peptide-BSA conjugates, and marginal reactivity with native H,K-ATPase, which could reflect low levels of gastritis-associated autoimmune antibodies against endogenous H,K-ATPase.

Immunocytochemistry

Since H,K-ATPase mediates acid secretion by the gastric parietal cell, we sought to confirm by light microscopic immunocytochemistry that both N- and C-peptide antibodies would label the appropriate cell type in gastric mucosa. In the pig, N- and C-peptide antibodies labelled parietal cells exclusively, as shown in Fig. 2 for the antibodies HKαN2 and HKαC2. Labelling was most dense with the three C-peptide antibodies and HKαN2 (+ + + +); the remaining N-peptide antibodies, HKαN1 and HKαN3, labelled parietal cells less intensely (+ +). In the rat, N-peptide antibodies did not label parietal cells, or any other cells; the C-peptide antibodies HKαC2 and HKαC3 gave dense labelling of parietal cells, while HKαC1 labelled parietal cells more weakly (+). In both pig and rat, labelling was distributed throughout the cytoplasm of parietal cells, with somewhat more dense labelling on the apical side of the cells. At the magnifications possible on the light microscope, localization of labelling to tubulovesicular or other membranes could not be confirmed. Nuclei were not labelled. When pig and rat gastric sections were exposed to preimmune sera, no labelling was apparent in any cell type in either mucosa (shown for pig gastric mucosa in Fig. 2, a).

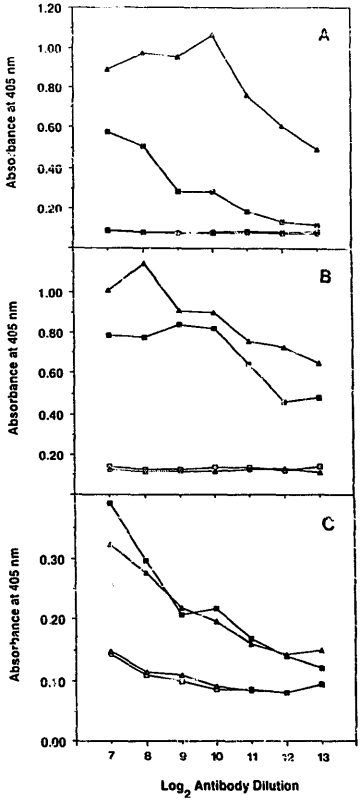

Fig. 1. Antibody titers against synthetic peptide, peptide-conjugate, and microsomal H,K-ATPase. Microtiter wells were coated with N- or C-peptide (A), N- or C-peptide-BSA conjugate (B), and H,K-ATPase-enriched gastric microsomes (C), and then incubated with serial twofold dilutions (abscissa, starting with 1:128 dilution) of HKαN2 (□, preimmune; ■, postimmune) or HKαC2 (△, preimmune; ▲, postimmune). Antibody binding was measured by ELISA at 405 nm (ordinate, see Methods).

Immunoblotting

Separation of the proteins present in H,K-ATPase-enriched microsomes by means of SDS-PAGE on 4%–20% acrylamide gels followed by staining with Coomassie blue showed two prominent polypeptide bands, one at 94 kDa and another at 42 kDa (Fig. 3, lane a). The former band corresponds to the H,K-ATPase α-subunit, while the latter band is actin. In addition, numerous bands of intermediate and lower molecular weight whose identity is unknown were evident. Both N-and C-peptide antibodies were shown to bind only to the 94 kDa polypeptide, with no cross-reactivity with other bands on the gel (Fig. 3, lanes N2 and C2). The C-peptide antibodies appeared to exhibit lower affinity for denatured and reduced H,K-ATPase α-subunit; in order to obtain comparable immunoblot signal strength, we loaded five times more microsomal protein onto electrophoretic gels for anti-C-peptide blots than for anti-N-peptide blots. At the dilutions of sera yielding the signal shown in Fig. 3 (1:1000 for HKαN2 and 1:2000 for HKαC2), parallel SDS-PAGE immunoblots of the same amounts of sheep kidney Na,K-ATPase showed no signal anywhere on the blots. At dilutions of 1:500, N-peptide antibodies still showed no reactivity with Na,K-ATPase immunoblots, while two of the C-peptide antibodies (HKαC2 and HKαC3) showed a barely visible signal at 94 kDa, presumably with the Na,K-ATPase α-subunit (data not shown). Preimmune sera at dilutions greater than 1:200 showed no reactivity with either H,K-ATPase or Na,K-ATPase immunoblots. Both N- and C-peptide antibodies retained their reactivity with tryptic peptides of the H,K-ATPase α-subunit (manuscript in preparation).

Competition assays

The specificity of the N-peptide antibodies for the N-terminus of native, microsomal H,K-ATPase was confirmed by competition ELISAs in which N-peptide or C-peptide was bound to ELISA microtiter wells, and incubated with N- or C-terminal antibodies in the presence of H,K-ATPase-enriched microsomes. Fig. 4 shows the results of competition ELISAs for antibodies HKαN2 and HKαC1. As the amount of native, microsomal H,K-ATPase incubated with N-terminal antibody increased, less antibody was left bound to the N-peptide on the microtiter plate. This experiment establishes three important points. Firstly, it shows that antibodies binding specifically to synthetic N-peptide are displaced by intact H,K-ATPase, indicating that the antibodies recognize a competing epitope, presumably the N-terminus, on the H,K-ATPase. Microsomes enriched in Na,K-ATPase did not displace N-peptide antibodies from bound N-peptide. Secondly, the experiment demonstrates that antibody HKαN2 recognizes the native conformation of the H,K-ATPase N-terminus, as it exists on the surface of catalytically-active vesicular membranes. The ELISA data shown in Fig. 1c also shows reactivity of antibodies with H,K-ATPase vesicles, but only after these have been ad-

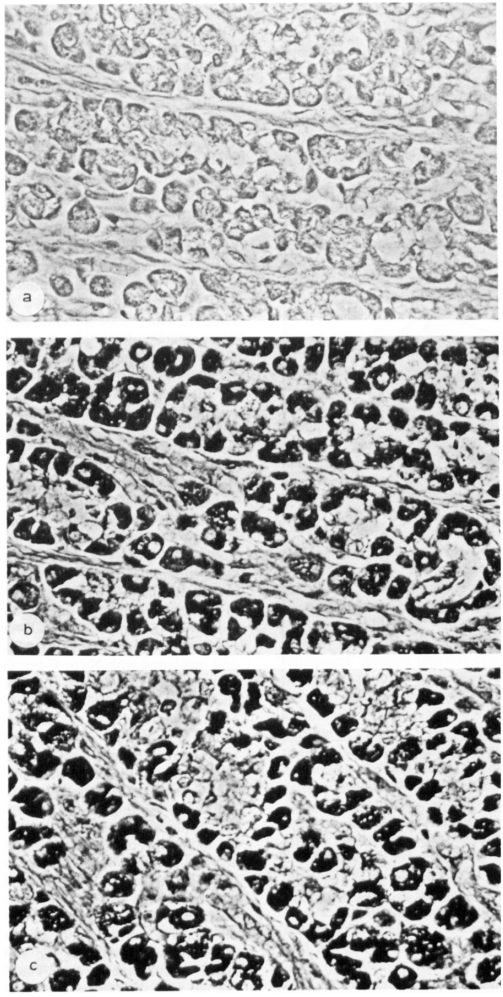

Fig. 2. Immunocytochemical reactivity of antibodies with gastric mucosa. Pig gastric mucosal sections were prepared as described in Methods, and incubated for 1 h with 1:25 dilutions of: (a) rabbit preimmune serum; (b) HKαN2 antiserum; and (c) HKαC2 antiserum. Magnification is 400 X.

sorbed to the polystyrene surface of the ELISA plates, with uncertain effects on the orientation and conformation of the H,K-ATPase and its N-terminus. Thirdly, if the majority of H,K-ATPase-enriched microsomes are oriented with their cytoplasmic surface on the outside, this experiment establishes that the N-terminus of the H,K-ATPase α-subunit is exposed on the cytoplasmic side of the parietal cell apical membrane.

In competitive binding assays as shown in Fig. 4, preincubation of native H,K-ATPase microsomes in 5 mM ATP or 100 mM KCl, inducing an E_1 or an E_2 conformation, respectively, had no significant effect on the competitive binding of HKαN2 to the enzyme at low concentrations of added H,K-ATPase (1 to 2.5 μg/ml); at higher concentrations of added H,K-ATPase (5 and 10 μg/ml), both E_1 and E_2 forms of

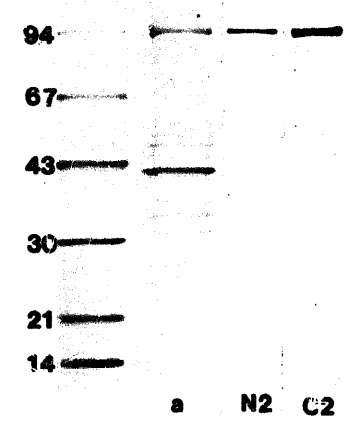

Fig. 3. Immunoblot reactivity of antibodies with gastric microsomal proteins. Pig gastric microsomes enriched for H,K-ATPase as described in Methods were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with N- or C-peptide-specific rabbit antisera. Signal detection was by means of HRP-linked goat anti-rabbit Ig and 4-chloronaphthol/H_2O_2. Lane a: 5 μg of GI gastric microsomes stained in the gel with Coomassie blue. Lanes N2 and C2: PVDF replicas of 1 μg SDS-PAGE-resolved gastric microsomes probed with a 1:2000 dilution of antibody HKαN2 (N2), and a 1:1000 dilution of antibody HKαC2 (C2). Molecular weight standards (phosphorylase *b*, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; soybean trypsin inhibitor, 21000; lysozyme, 14000) are displayed on the left.

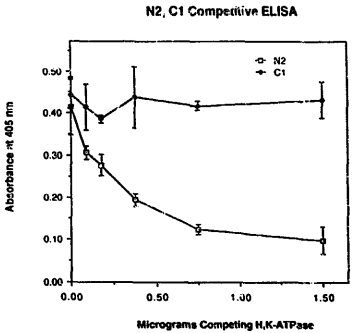

Fig. 4. Competitive ELISA with synthetic peptide and microsomal H,K-ATPase. 1 μg aliquots of N- or C-peptides were plated into microplate wells, blocked with BSA, and incubated with 100-μl aliquots of either HKαN2 or HKαC1 (1:1000 dilutions) containing increasing amounts of microsomal H,K-ATPase, as shown on the abscissa. After 1 h, microplate wells were emptied and processed by ELISA as described in Methods.

the H,K-ATPase bound 30% less HKαN2 antibody than the native H,K-ATPase (data not shown).

Analogous competition ELISAs were carried out with the C-peptide antibodies, in order to confirm their specificity for the C-terminus of native H,K-ATPase. However, despite extensive manipulations of antigen and antibody concentrations, displacement effects similar to those observed for HKαN2 were not found when C-peptide antibodies were incubated with native, microsomal H,K-ATPase in the presence of bound C-peptide (shown in Fig. 4 for antibody HKαC1). Confirmation that C-peptide antibodies specifically recognized H,K-ATPase was obtained with a modified competition ELISA, whereby C-peptide antibodies were first incubated with increasing amounts of C-peptide-BSA bound to microtiter plate wells, and were then removed and incubated with standard amounts of bound microsomal H,K-ATPase. Fig. 5 shows the result of such an experiment for antibody HKαC3, compared to HKαN1. The data clearly indicate that both C-peptide and N-peptide antibodies interacting with epitopes on microsomal H,K-ATPase (adsorbed to polystyrene microtiter wells) are specifically removed by prior incubation with synthetic C- or N-peptides. The complete absence of C-peptide antibody competitive effects with intact microsomal H,K-ATPase (i.e., not adsorbed to polystyrene wells), could result from the C-terminus being luminally oriented, that is, being exposed on the inside surface of the vesicles, and therefore not accessible to antibody. The C-peptide

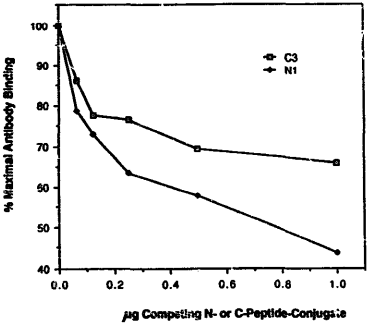

Fig. 5. Modified competitive ELISA with C-peptide antibody. Increasing amounts of N- or C-peptide-BSA conjugates (abscissa) were plated into microtiter wells and incubated with HKαN1 (1:1024 dilution) or HKαC3 (1:16,000 dilution), as appropriate. After 1 h, the antibodies were aspirated and transferred into microtiter wells previously incubated with 1 μg microsomal H,K-ATPase, and subsequently blocked with BSA. Antibody binding to these wells was assayed by ELISA as described in Methods.

antibody competitive ELISAs (as shown in Fig. 4) were repeated with microsomal H,K-ATPase solubilized in 0.125% $C_{12}E_8$ (a concentration found not to affect ELISA reactions), and no competitive effects were observed. Given that our C-peptide antibodies appeared not to recognize the C-terminus of native H,K-ATPase, this negative result precluded assignment of the C-terminus to the luminal or cytoplasmic side of the membrane.

Effects of antibodies on enzyme activity

Representative specific activities of freshly-prepared gastric microsomal vesicles in 20 mM KCl were 22.7 μmol P_i/mg protein per h, 38.0 μmol P_i/mg protein per h with 10^{-5} M valinomycin (a K^+ uniport ionophore), and 124.7 μmol P_i/mg protein per h with 10^{-5} M nigericin (a K^+/H^+ antiport ionophore). This ionophore-dependent stimulation of K^+-ATPase specific activity is interpreted in terms of an intravesicular K^+ binding site which becomes accessible to extravesicular K^+ only in the presence of K^+ ionophores; that is, the majority of the vesicles (82%) are oriented with their luminal or extracytosolic side facing inwards. Since the site-directed antibodies appeared to act cytosolically, that is, on the outside surface of microsomal vesicles, we measured their effect on the K^+-stimulated activity of gastric microsomes enriched in H,K-ATPase. Fig. 6a shows the effects of protein A-purified N-terminal and C-terminal antibodies on K^+-stimulated AT-Pase activity in such vesicles. Maximal activity refers to the difference between Mg^{2+}-stimulated ATPase and Mg^{2+},K^+-stimulated ATPase activity in the absence of antibodies. The N-terminal antibody consistently inhibited enzyme activity in a dose-dependent manner, while no significant inhibition of activity was induced in the presence of C-terminal antibody. The small reaction volume of the ATPase assay as adapted for microtiter plates (200 μl) limited the amount of immune IgG that could be added to the wells, preventing measurement of the maximal extent of enzyme inhibition. This limitation was partially overcome by using peptide affinity-purified HKαN2 IgG, as shown in Fig. 6b. H,K-ATPase activity was maximally inhibited by 55% by this antibody fraction, a result consistent with involvement of the H,K-ATPase N-terminal domain in catalytic and/or transport activity of the enzyme.

Discussion

Antibodies elicited by immunization with synthetic peptides have been widely applied to questions to native protein structure and function [12]. This study

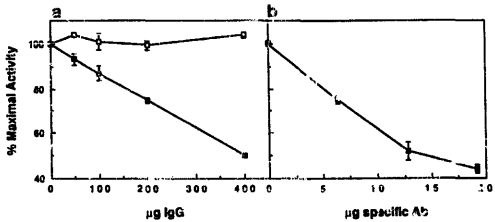

Fig. 6. Effect of N-peptide and C-peptide antibodies on K^+-stimulated ATPase activity. Enzyme activity was measured as specified in Methods, and is shown as a percentage of the K^+-stimulated activity measured in the absence of antibody. (a) Inhibition of H,K-ATPase activity by IgG obtained from antisera HKαN2 (■) and HKαC2 (□) by protein A-affinity purification. (b) Inhibition of H,K-ATPase activity by specific HKαN2 activity obtained from HKαN2 IgG by N-peptide-affinity purification.

describes some properties of antisera directed against synthetic peptide-KLH conjugates based on the primary structure of pig gastric H,K-ATPase N- and C-termini. In choosing KLH as the carrier protein for immunization, we followed Ball and Loftice [13], who found that antisera raised against five unconjugated synthetic peptides from the α-subunit of Na,K-ATPase did not react with the native enzyme, while four of seven antisera raised against synthetic peptides coupled to KLH, including the N-terminal peptide, did react. Our finding of differences in the properties of antisera from rabbits immunized with the same conjugates (Table I) may reflect genetic differences amongst rabbits and the selection of differing dominant clonal responses. The varied immune responses to the synthetic peptides emphasizes the importance of immunizing several animals when antisera with specific binding properties are sought.

Our major objective in generating site-specific antibodies to the gastric H,K-ATPase is to study topography of the enzyme with respect to the apical membrane of the gastric parietal cell. Anomalies have arisen in the model of H,K-ATPase structure deduced from hydropathy plots. Tai et al. (1988) [14] obtained two peptides (25 kDa and 15 kDa) from extended tryptic digestion of rat H,K-ATPase in the presence of Zwittergent 3–14. Both peptides contained the FITC-binding site of the parent molecule, and also bound concanavalin A (Con A), showing that mannose-containing carbohydrate substituents reside on the peptides. FITC binds to Lys-517, which hydropathy analysis places in the midst of the 52 kDa hydrophilic domain on the cytosolic side of the apical membrane. Since protein glycosylation occurs co- and post-translationally in the RER and Golgi apparatus where glycosyltransferases are found, it is unclear how cytosolically-oriented domains of the H,K-ATPase can be glycosylated. In the same vein, Hall et al. (1989) [15] found that a pair of peptides (33 kDa and 31 kDa) were released from membrane-bound pig H,K-ATPase with partial tryptic digestion. Again, both peptides bound FITC and Con A, making Asn-492 a probable acceptor site for N-linked glycosylation. Immunogold Con A electron microscopy showed the majority of staining to be on the luminal side of H,K-ATPase vesicles, and labelling of intact and permeabilized vesicles with UDP-galactosyl transferase showed greater labelling of H,K-ATPase on the luminal (inside) surface of the vesicles. Thus, Asn-492 appears to be luminally-oriented, implying that the hydrophilic 22-residue peptide Lys-496–Lys-517, thought to be cytosolically-oriented, may in fact cross the membrane.

Since the interactions of gastric microsomal vesicles with site-directed antibodies described in this study were interpreted in terms of H,K-ATPase orientation, it was important to establish the sidedness of our vesicle preparations. The evidence that our microsomal vesicles are oriented with their cytoplasmic surfaces facing outward comes from previously published reports using the same preparation of vesicles, and from our own measurements of ionophoric latency of K^+-stimulated ATPase activity in our vesicles. Firstly, electron microscopy of gastric vesicles fixed in 2% gluteraldehyde and 1% tannic acid revealed dense thickening of the outer surface of vesicles [16], indicating that most of the membrane protein content of these vesicles was associated with external face of the bilayer [17]. Secondly, electron microscopy of gastric vesicles labelled with wheat germ agglutinin or concanavalin A colloidal gold conjugates revealed more than 70% of the gold particles associated with the inner surface of the vesicles, indicating a preferential lumen-inside (cytosol-out) vesicle orientation [15]. Thirdly, hypotonic lysis of the vesicles does not lead to increased pNPPase activity (a partial reaction of the H,K-ATPase), while H,K-ATPase activity is increased only by 25%, wholly accounted for by improved access of K^+ to the luminal side of the enzyme [18]. These results indicate the presence of ATP-binding sites (which must be cytosolic) on the outside surface of the vesicles. Lastly, measurements of proton and potential gradients in such vesicles in response to ATP and ionophores confirm intravesicular acidification; that is, the luminal face of the ATPase faces inwards [19]. Our measurements of K^+-stimulated ATPase activity in freshly-prepared gastric microsomal vesicles showed that activity was increased by a factor of 1.6 with valinomycin, and by a factor of 4.5 with nigericin. These data confirm the impermeability of vesicles to K^+ and the intravesicular location of the K^+-binding site on the H,K-ATPase (since the enzyme requires K^+ on the luminal side of the apical membrane for full activity), indicating again that the cytoplasmic side of the H,K-ATPase is extravesicular. In addition to confirming the ionic integrity of the vesicles, and therefore impermeability of the vesicles to antibodies, these data suggest that 82% of the vesicles were oriented cytoplasmic side-out; since up to 18% of vesicles may be oppositely-oriented or fragmented, we cannot exclude the possibility that interactions of site-specific antibodies with this fraction of vesicles contribute in some degree to our binding data.

The ability of intact microsomes enriched in H,K-ATPase to compete with an α subunit N-terminal synthetic peptide for antibody HKαN2 (Fig. 4) shows that the α-subunit N-terminal domain is located on the outer surface of the microsomes. The α-subunit N-terminus is therefore located on the cytoplasmic side of the parietal cell apical membrane. Confirmatory evidence for sidedness of the N-terminus of rat H,K-ATPase has previously appeared in abstract form [20]; in that study, immunoelectron microscopic localization

of an antibody against a synthetic peptide corresponding to the rat H,K-ATPase amino terminal sequence demonstrated that the N-terminus faced the cytoplasm. We cannot deduce the sidedness of the C-terminus of the H,K-ATPase from our competitive ELISA data. The lack of competitive effects with C-peptide antibodies in such experiments, using both intact and solubilized microsomes, suggests either that C-peptide antibodies do not recognize the native conformation of the σ subunit C-terminal domain, or that this domain is shielded by hydrophilic domains of the α- or β-subunits of the H,K-ATPase (even in the presence of a non-denaturing detergent), and thus is sterically unable to interact with C-terminus-specific antibodies. Ball and Loftice [13], using antibodies against a synthetic peptide based on the C-terminus of Na,K-ATPase α-subunit found a similar lack of reactivity of C-peptide antibodies with native Na,K-ATPase, although the same antibodies gave a positive signal on electroblotted α-subunit. Thus, specific binding of our C-peptide antibodies to: (a) microsomes adsorbed to polystyrene microplate wells; (b) H,K-ATPase α-subunit resolved by SDS-PAGE and transferred to PVDF membrane; and (c) H,K-ATPase fixed in situ in gastric mucosal sections, is not necessarily predictive of antibody binding to the native enzyme. This finding emphasizes the importance of verifying experimentally that synthetic peptide-based antibodies recognize the native antigen, particularly when such antibodies are being used as probes of native conformation. This caveat notwithstanding, our C-peptide antibodies are potentially useful probes of α-subunit C-terminus sidedness, since they are immunocytochemically reactive and thus lend themselves to immunogold electron microscopy. Evidence that the H,K-ATPase C-terminus is cytosolically oriented has recently appeared in abstract form [21]. In that study, digestion of surface iodinated (^{125}I) intact microsomal vesicles with carboxypeptidase Y reduced ^{125}I counts in the α-subunit by 24%; this result was interpreted in terms of selective removal from the outer surface of vesicles of the two α-subunit C-terminal tyrosines, which must therefore face the cytosol. With the closely related Na,K-ATPase, Antolovic et al. [??] have recently shown using synthetic peptide-based affinity-purified antibodies that both the N- and C-termini of the α-subunit are cytoplasmically oriented.

Polyclonal antibody [23,24] and monoclonal antibody [25–28] inhibition of gastric H,K-ATPase has been reported previously, although the location of epitopes associated with inhibition was not specified. In addition to effects on H,K-ATPase activity, the polyclonal antibodies and two monoclonals [25,27] also inhibited *p*-nitrophenylphosphatase activity; one monoclonal antibody [25] was reported to inhibit chloride conductance associated with gastric microsomes. Our N-terminus-specific antibody HKαN2 dose-dependently inhibits K^+-stimulated ATPase activity in gastric microsomes enriched in H,K-ATPase; the specificity of this effect is reinforced by the inability of our C-peptide antibody HKαC2 to inhibit activity, consistent with this antibody's non-recognition of the native enzyme. The effects of HKαN2 on pNPPase activity, phosphoenzyme formation, and chloride conductance remain to be examined. For now, the inhibitory effect of this site-directed antibody on H,K-ATPase activity suggests that the N-terminal domain may play a role in the catalytic mechanism of the enzyme. Alternatively, antibody binding to the N-terminus may simply restrict access of physiological ligands to their binding sites elsewhere on the enzyme, thereby inhibiting activity. This interpretation is not supported by our observation that HKαN2 binding to native α subunit decreases by 30% when the physiological ligands ATP and K^+ are bound to the enzyme. Our results are consistent therefore with the N-terminus undergoing conformational changes as the enzyme goes through its catalytic cycle. The possibility remains that conformational changes elsewhere in the enzyme may also restrict the antibody's access to its N-terminal epitope. Inhibition of H,K-ATPase activity by our N-peptide antibody contrasts with the lack of inhibition of Na,K-ATPase by the corresponding N-peptide antibodies [22], a disparity which may reflect the different ion binding and transduction functions of N-terminal domains in the two enzymes.

Previous studies with monoclonal antibodies to gastric H,K-ATPase have shown them to be useful probes of the enzyme at both the cellular and molecular level. Such antibodies have clarified the location and secretagogue-stimulated migration of H,K-ATPase within the gastric parietal cell [29], have shown the existence of H,K-ATPase-like structures in renal cortical collecting duct intercalated cells [30], colonic epithelial cells [31], and non-pigmented epithelial cells of the ciliary body [32], have led to development of an ELISA-based assay of H,K-ATPase in human gastric biopsies [33], and have resulted in purification of H,K-ATPase messenger RNA and its translation in a cell-free system [34]. In addition, antibodies are unique immunocytochemical markers of gastric parietal cells [35,36]. The present study extends the armory of immunochemical probes of the H,K-ATPase by verifying that polyclonal antibodies directed against synthetic H,K-ATPase peptides interact with native enzyme, and are consequently important probes of H,K-ATPase topography and the conformational transitions undergone by the enzyme during catalysis.

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