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A monoclonal antibody which inhibits H^+/K^+-ATPase activity but not chloride conductance

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A mouse monoclonal antibody was raised against hog gastric membranes. This antibody (95–111 mAb) has a very high affinity for the 95 kDalton band of H^+/K^+-ATPase-enriched membranes, and does not react with Na^+/K^+-ATPase. The epitope is located on the tubulovesicles and canaliculi of the parietal cells. The 95–111 mAb also inhibits the ATP hydrolytic activity, decreases the steady-state phosphorylation level and inhibits the phosphatase activity of H^+/K^+-ATPase, strongly suggesting that the epitope is on the catalytic subunit of H^+/K^+-ATPase. The 95–111 mAb also recognizes rat, rabbit and human gastric H^+/K^+-ATPase. This mAb differs from the H^+/K^+-ATPase-inhibiting mAb previously described (Asano et al. (1987) J. Biol. Chem. 262, 13263–13268), in that it does not inhibit the chloride conductance opened by Cu-*o*-phenanthroline in gastric vesicles.

Introduction

The fundic epithelium of stomach contains a highly active proton secreting H^+/K^+-ATPase which is restricted to the intracytoplasmic and apical membranes of parietal cells [1]. The current theory of the mechanism of HCl secretion, involves, this H^+/K^+-ATPase, a chloride conductance and a potassium conductance [2]. The anion conductance is thought to account for electrogenic chloride secretion and to be regulated by a cAMP-dependent protein kinase [3]. The H^+/K^+-ATPase is thought to account for H^+ secretion and also for intracellular K^+ accumulation. The cation conductance is thought to account for potassium recycling across the secretory membrane [2]. The secretion of HCl probably involves not only the parietal cell secretory membrane transporters, but also cytoskeletal elements and their membrane binding proteins [4]. Membrane preparations containing H^+/K^+-ATPase activity are also enriched in 95 kDa polypeptides. The H^+/K^+-ATPase catalytic subunits are 95 kDa and the other ionic transporters are not yet characterized. Monoclonal antibodies (mAb) against tubulovesicle proteins should therefore be very useful tools for purifying and characterizing parietal cell ionic transporters.

This paper describes a monoclonal antibody specific for H^+/K^+-ATPase according to the following criteria: it is specific for the gastric parietal cell tubulovesicles and canaliculi, it recognizes 95 kDa antigens and it inhibits H^+/K^+-ATPase activities on a mole to mole ratio. This antibody is different from the monoclonal antibodies previously described [5,6] in that it does not inhibit the Cu-*o*-phenanthroline-induced [6], or the ATP-induced chloride conductance in gastric vesicles [3]. This mAb has been used to purify hog gastric vesicles by immunoprecipitation [7], to assay H^+/K^+-ATPase in hog, human, rat and rabbit stomachs (unpublished data) and to study the ontogeny of H^+/K^+-ATPase in rat [28].

Materials and Methods

1. Materials

ATP, PNPP and 2, 6, 10, 14-tetramethylpentadecane (pristane) were purchased from Sigma (St. Louis, MO, U.S.A.). Anti mouse immunoglobulins were purchased from Biosys (Compiègne, France). PVC plates for radioimmunoassays, culture medium (DME) and other culture reagents were obtained from Flow laboratories (Lab System, Les Ulis, France), horse serum was from

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Boehringer-Mannheim France (Meylan, France) $^{125}I^-$ (NaI, 200 mCi/ml) was purchased from Amersham (France).

II. Methods

1. Preparation of H^+/K^+-ATPase membranes. Fresh stomachs were from New Zealand rabbits and Wistar male rats. Fresh hog stomachs were obtained at the local slaughterhouse (Domont abattoirs, Pontoise, France). The subcellular fractions were prepared as previously described [8]. The fundic mucosa was scraped off and homogenized in 0.25 M sucrose, 50 mM Hepes-Tris (pH 7.2). Homogenate was first centrifuged for 10 min at $800 \times g$, then the supernatant was centrifuged at $40\,000 \times g$ for 7 min (rotor SS34, Sorvall RC5C centrifuge) at 4°C. The microsomes were pelleted from the latter supernatant at $100\,000 \times g$ for 30 min (rotor 70Ti, Beckman L5 65 centrifuge) at 4°C. Isopycnic equilibration of microsomes was performed to purify H^+/K^+-ATPase membranes at the 8.3–35% sucrose interface.

H^+/K^+-ATPase and PNPPase activities were measured as previously described [8]. H^+/K^+-ATPase incubation medium (1 ml) contained 2 mM MgATP, 4 mM phospho*enol*pyruvate, 1 unit/ml pyruvate kinase in 40 mM Hepes-Tris (pH 7.0) and the incubation was carried out in triplicate plus or minus 20 mM KCl. Incubation was started by the adddition of 50 µl of membranes, was run at 37°C and was stopped by 0.5 ml of trichloroacetic acid 10%. Specific activities of H^+/K^+-ATPase membranes were: Mg^{2+}-ATPase, 45 ± 5 µmol P_i mg per h and Mg^{2+}/K^+-ATPase, 150 ± 13 µmol P_i/mg per h (means +S.E, $n = 21$). A specific H^+/K^+-ATPase inhibitor, Schoering 28080 10^{-4} M, inhibited 97% of the K^+-stimulated activity. pNPPase incubation medium, 250 µl, contained 5 mM $MgSO_4$, 5 mM *p*-nitrophenyl phosphate and 40 mM Hepes-Tris (pH 6.9) plus or minus 20 mM KCl. Phosphorylation assays were performed as previously described using 5 µM ATP as substrate [8]. Proteins were measured by the method of Bradford (9), using bovine serum albumin as standard.

Microsomes were prepared from rabbit renal medulla as described by Kaunitz et al. [10]. Intestinal microsomes were prepared in the presence of 1 mM benzamidine, 1 mM PMSF. Microsomes were prepared from rabbit colon mucosa as described by Gustin and Goodman [11]. The Na^+/K^+-ATPase-enriched fraction from rat heart was a gift from L. Lelièvre [12]. The chromaffin granule-enriched preparation of bovine adrenal medulla was a gift from M.P. Roisin and J.P. Henry [13].

2. Salt permeability Changes in light scattering at 550 nm were recorded using a Perkin Elmer LS5B spectrofluorimeter (excitation 550 nm and emission 550 nm). Gastric vesicles (100 µg/ml) were incubated for 10 min at room temperature with 10 µM *o*-phenanthroline, 5 µM $CuSO_4$ [6] and then diluted 10-fold in the spectrofluorimeter cuvette. Vesicle swelling was induced by adding KCl in the medium. Preincubation of the vesicles with 30 µM DIDS inhibited the effect of Cu-*o*-phenanthroline.

Anion exchange was measured at 30°C using $^{125}I^-$ as tracer, and 2 mM NaI in the medium. To separate free from trapped tracer, the samples were filtrated on Dowex columns as previously described (3).

3. Mouse immunization Female Balb/c mice (8–10-week old) were immunized by a first intraperitoneal injection of antigen emulsified with an equal volume of Freund's complete adjuvant. Two boosts of the same antigenic preparation, emulsified with an equal volume of Freund's incomplete adjuvant were given, i.p., 2 weeks and 4 weeks later. The mice were injected intravenously with the antigenic preparation three days before fusion. For 95–111 mAb, 0.5 mg of hog gastric membranes were used as antigens, for 95–A3 mAb, hog gastric membranes were factionated by SDS-PAGE electrophoresis and then transferred to a nitrocellulose sheet. After protein staining with ponceau S, the 95 kDa band was cut off, frozen in liquid nitrogen to be carefully minced, homogenized in PBS and injected in mice as antigenic preparation.

4. Production of monoclonal antibodies. The culture medium was Dulbecco Modified Eagle Medium (DMEM) with penicillin (50 µg/ml), streptomycin (50 µg/ml), glutamine 2 mM, sodium pyruvate 2 mM, and horse serum (10%). Medium 1 contained 0.4 µM aminopterin, 0.1 mM hypoxanthine and 16 µM thymidine.

X63.Ag.8.653 myeloma cells were fused with mouse splenocytes as described by Köhler and Milstein [14]. The fusion was performed in the presence of 50% (w/v) polyethylene glycol, using $2 \cdot 10^8$ spleen cells and $2 \cdot 10^7$ myeloma cells. Medium 1 was used to select hybridomas after fusion. Hybridoma cells were distributed in 24-well costar plates (10^6 cells/well) and grown for 2 weeks in a growth promoter-enriched medium 1. All wells were tested and reactive hybridoma were cloned twice by limiting dilution (0.5 cell/0.2 ml per well) using 96 well plates. Spleen cells from 4 week-old Balb/c mice were used as feeder cells during the cloning. The clones were frozen at −80°C and then stored in liquid nitrogen. Ascite tumors were produced in mice as previously described [15]. 95–111 ascite fluid contained 20 mg protein/ml and 6.6 mg mAb/ml. The immunoglobulin class was determined by RIA, using rat immunoglobulins raised against mouse immunoglobulin isotypes.

5. Radioimmunoassays. Antigenic fractions were diluted to 20 µg protein/ml with 50 mM carbonate-bicarbonate buffer (pH 9.6) and 100 µl of the diluted solutions were incubated overnight in PVC plate wells

at 0–4°C and then decanted. The free plastic sites were then saturated with 200 µl of PBS-BSA (1%) (phosphate-buffered saline-bovine serum albumin) for 1 h at room temperature; 50-µl aliquots of culture supernatant were incubated in each well for 2 h at room temperature. The plates were rinsed three times with PBS-BSA (0.1%) and, 100000 cpm of ^{125}I anti-mouse IgG were added (50 µl) and incubated for 2 h at room temperature. The plates were again rinsed three times with PBS-BSA (0.1%) and cut out; radioactivity was counted in gamma-counter.

6. Electrophoresis and Western blots. The technique described by Laemmli [16], with minor modifications, was used [10]. The stacking gel was 5% acrylamide, the separating gel was a linear 5–20% polyacrylamide gradient. Proteins were transferred from the acrylamide gel to nitrocellulose sheets as described by Burnette [17]. Western blots were probed with monoclonal antibodies using a modification of the procedure described by Towbin et al. [18]. When ascitic fluids were tested, controls were run using ascitic fluid from myeloma cell injection.

7. Histochemical localization of antigens.

(a) Tissue preparation. Samples of fundus, colon and kidney were obtained from sodium pentobarbital-anesthetized New Zealand rabbits. They were fixed for 4 h in freshly prepared 4% paraformaldehyde, frozen in isopentane cooled with liquid nitrogen and placed in OCT (Tissue-tek, Miles Scientific). 5-µm sections were cut on a cryostat and placed on gelatin-coated slides. The slices were air-dried (2 to 12 h), dipped in acetone for 10 min, air-dried and either used immediately or stored at −80°C.

(b) Immunolabeling. Gold-silver technique: Gold particles were prepared according to Frens [19] and to Garaud et al. [20]. A mixture of 1% sodium citrate and 4% tetrachloroauric acid was boiled to obtain a clear orange-red solution. Gold particles were then coupled to immunoglobulins: gold solution was diluted 1/3 in 10% NaCl, 5 mM carbonate buffer (pH 9.7), and stirred vigorously in the presence of immunoglobulins. The optimal immunoglobulin concentration was determined as the maximal quantity which kept the gold solution red; too high a concentration turned the solutions to purple and blue. Excess immunoglobulins were discarded by centrifuging the gold-labeled IgG for 30 min at 50000 × *g*. The pellet was rinsed three times with carbonate buffer, and the gold-labeled IgG was stored at −20°C in 50% glycerol.

The cryostat sections were rinsed for 10 min in buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 1/1000 normal rabbit serum) and incubated overnight at 0–4°C with the 95–111 mAb and for 1 h with the gold-labeled antispecies immunoglobulins. Slides were then rinsed three times in 50 mM Tris (pH 7.6), 150 mM NaCl and once in distilled water, and dipped in 10% (w/v) citrate buffer (pH 3.5), 0.2% (w/v) hydroquinone (Fotopur, Merck)

8. Cytochemical localization of antigens. Tissue samples were fixed for 3 h at room temperature in 4% paraformaldehyde, incubated for 1 h at 40°C in 10% gelatin in PBS, post-fixed in 4% paraformaldehyde for 1 h at room temperature and rinsed with PBS. Tissue sections (45 µm) were then cut (Vibratome Oxford), treated with 0.1% saponin for 5 min [21], incubated with normal serum for 20 min and then with the mAb overnight at 4°C. Immune complexes were revealed using peroxidase-labeled anti-mouse immunoglobulins (Biosys France, ref BI 2413) and diaminobenzidine-H_2O_2 as substrate. Tissue slices were then post-fixed in 1% osmic acid in 0.1 M phosphate buffer (pH 7.4), dehydrated and embedded in LX112.

Results

1. Antibodies

The monoclonal antibody 95–111 was found to be an IgG with a 55 kDa heavy chain and a 28 kDa light chain after SDS-mercaptoethanol denaturation. The 95–A3 mAb was also an IgG.

2. Antigen characterization

Western blots demonstrated that the 95–111 and 95–A3 mAb specifically recognized a 95 kDa band in H^+/K^+-ATPase-enriched membranes (Fig. 1). Detergent was required to solubilize the antigen, suggesting its intramembrane location. The 95–111 mAb antigen was present in all the species tested, hog, man, rabbit and rat, suggesting that the epitope was located in a highly conserved area of the molecule. The 95–A3 was tested in hog, rabbit and rat where the antigen was also detected.

Tissue distribution was examined by immunohistochemistry. Glutaraldehyde (0.2–1%) fixation destroyed reactivity to both mAb's while no fixation and fixation with 2 to 4% paraformaldehyde did not. The antigen distributions on unfixed or 4% paraformaldehyde-treated tissues were the same. Many, but not all the fundic glandular cells were stained, reactive cells were abundant from the middle part of the gland down to the base (Fig. 2). They were large cells, often protruding from the glands, suggesting that they were parietal cells. Several different embedding media (LR White, LX112, nanoplast or Lowicryl) were tested: all inhibited the 95–111 mAb immunoreactivity. Preembedding reactions were used for immunocytochemistry studies. Staining was specific for parietal cells: no chief cells or mucous cells were stained. The staining was restricted to the tubulovesicles and the canaliculi of parietal cells (Fig. 3).

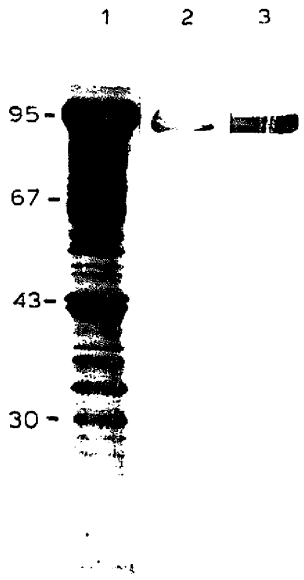

Fig. 1. Western blots of hog gastric subcellular fraction using 95–111 and 95–A3 mAb. SDS-PAGE was run with a 1.5 mm thick slab gel. Standard migration is shown on the left margin (units are kDa). Lane 1 (20 μg of hog gastric microsomes) shows the profiles of Coomassie blue-stained proteins on SDS-PAGE. A crude membrane fraction was preferred to the sucrose gradient-purified fraction to test the antibody specificity. Proteins were transferred to nitrocellulose sheets: lane 2 (5 μg of hog gastric microsomes) was incubated with 95–A3 mAb (0.9 μg ascite/ml) in PBS (pH 7.4); lane 3 (5 μg of hog gastric microsomes) was incubated with 95–111 mAb (0.1 μg ascite/ml) in 40 mM Tris (pH 7.4). Several controls were run (1) using 20 μg of liver microsomes/lane with 95–111 mAb and 95–A3 mAb, (2) using 5 μg of hog gastric microsomes/lane and an ascitic fluid from myeloma cell injection. Immune complexes were revealed using peroxidase-labeled anti-mouse IgG, diaminobenzidine and H_2O_2. All controls were negative.

3. 95–111 mAb and 95–A3 mAb specificity

The epitopes of 95–111 and 95–A3 mAb were not detected in the colonic mucosa, in the small intestine mucosa, in the liver or the kidney by radioimmunoassay or by immunohistochemistry.

The antigen molecular weight, its cellular localization and its selectivity for the fundic epithelium suggested that H^+/K^+-ATPase could be the antigen. Therefore the reactivity against other ATPases was explored. Radioimmunoassays of 95–111 mAb with Na^+/K^+-ATPase membranes were negative. Furthermore, the absence of basolateral membrane staining in all the tissues also suggested that the 95–111 and the 95–A3 mAb did not recognize Na^+/K^+-ATPase. Radioimmunoassays with NEM-sensitive H^+-ATPase from bovine chromaffin granule membrane preparations were aiso negative.

4. Inhibition of H^+/K^+-ATPase

The 95–111 mAb inhibited H^+/K^+-ATPase. Inhibition was specific as it was not obtained with other anti-95 kDa mAb, such as 95–A3 mAb and as it was dependent upon the 95–111 mAb concentration (Fig. 4). The rate of K^+-dependent ATP hydrolysis was 50% inhibited in the presence of 7 μg mAb/ml using 50 μg membrane/ml. Mean maximal inhibition was 58 ± 4% (mean ± S.E. (n + 13)). Maximal phosphatase inhibition was obtained at the same concentration of antibody, but the percent inhibition was less (25 ± 1%(n = 10)) (Fig. 4). The steady-state phosphorylation level of H^+/K^+-ATPase was also inhibited by 62% (n = 2) in the presence of 8 μg of mAb/ml.

5. Effect of 95–111 mAb on chloride permeability

As previously described by Takeguchi et al. [22], changes in light scattering can be used to monitor

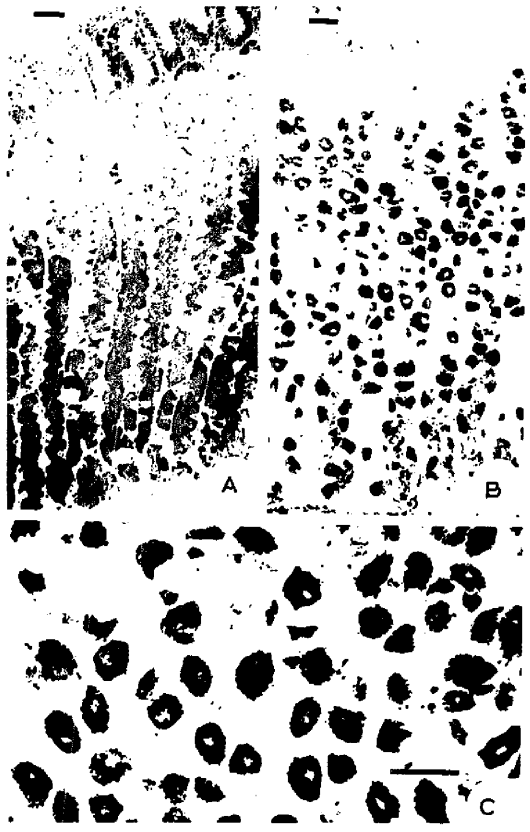

Fig. 2. Histochemical localization of 95–111 mAb antigens. The rabbit mucosa was fixed in 4% paraformaldehyde as specified in Methods, frozen and sectioned. Tissue sections were treated with 95–111 mAb using the gold technique as described in Methods. (A) Control tissue showing gland height (Toluidine blue staining); (B) tissue incubated with 95–111 mAb; (C) a detail of mAb-treated mucosa. Bars represented 0.1 mm.

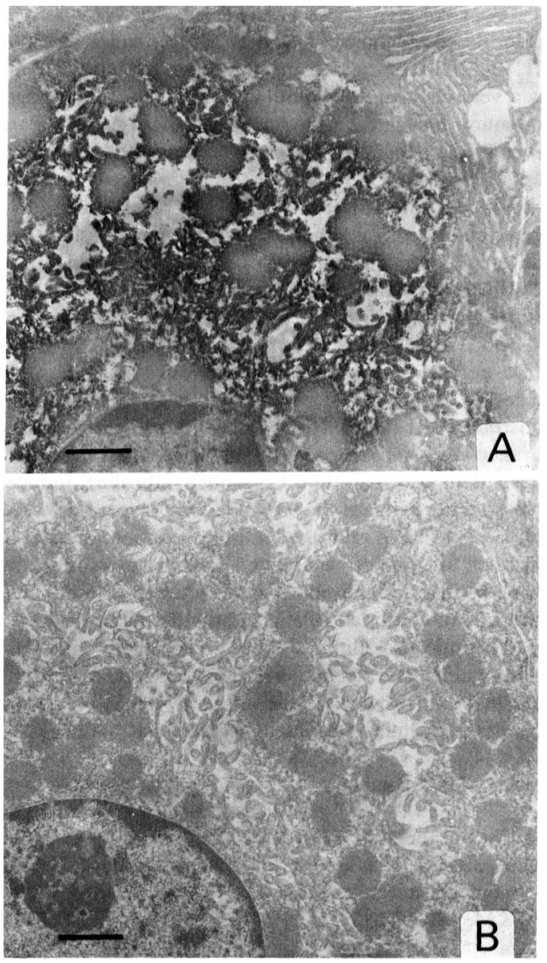

Fig. 3. Cytochemical localization of 95–111 mAb antigens. As specified in Methods, the pieces of fresh rabbit fundic mucosa were fixed in 4% paraformaldehyde, rinsed in sucrose, frozen and 5 μm sections were cut. The sections were incubated with saponin and then with (A) or without (B) 95–111 mAb. The immune complexes were revealed with DAB-H_2O_2 using a peroxidase-labeled anti-mouse IgG. The tissue sections were then embedded and cut for electron microscopy examination (bars represent 1 μm).

changes in vesicle volumes. After the addition of salt to H^+/K^+-ATPase membrane-containing medium, light scattering first rapidly increased (14 ± 3%) due to vesicle shrinking and then decreased due to salt and water uptake. In the absence of Cu-*o*-phenanthroline, KCl and NaCl additions induced a slow decrease of light scattering. After the membranes were treated with Cu-*o*-phenanthroline, KCl induced a faster decrease suggesting that the salts were now permeant (Fig. 5). In agreement with the results of Takeguchi et al. [22] KCl and NaCl had the same effect. DIDS 10 μM inhibited the salt induced-light scattering. In contrast to the

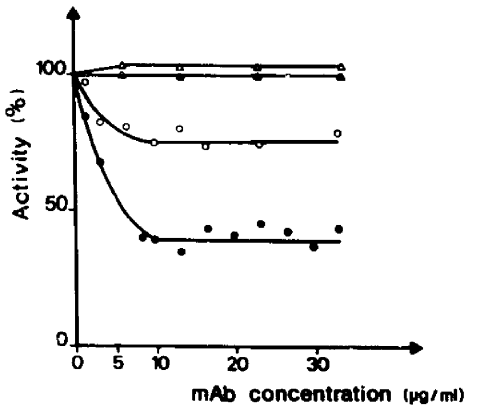

Fig. 4. Effect of the 95–111 and 95–A3 mAb on H^+/K^+-ATPase activity. Hog gastric membranes were incubated overnight at 4°C with various concentrations of 95–111 or 95–A3 mAb. The K^+-stimulated ATPase activities and the K^+-stimulated PNPPase activities were measured as described in Methods. ●———●, K^+-ATPase with 95–111 mAb; ○———○, K^+-PNPPase with 95–111 mAb; ▲———▲, K^+-ATPase with 95–A3 mAb; △———△, K^+-PNPPase with 95–A3 mAb.

monoclonal antibody described by Asano et al. [6], neither the 95–111 mAb nor the 95–A3 mAb inhibited the light scattering changes induced after Cu-*o*-phenanthroline treatment.

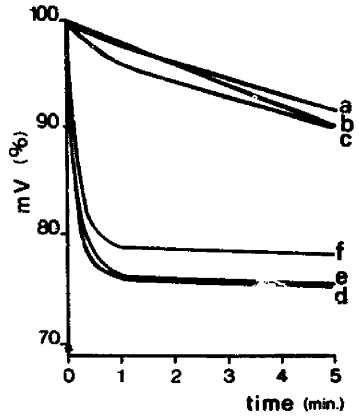

Fig. 5. Effects of mAb on Cu-*o*-phenanthroline-triggered chloride conductance. The hog gastric membranes were diluted to 0.1 mg/ml with 0.2 M sucrose, 10 mM Hepes (pH 7), and preincubated for 10 min at room temperature with 10 μM *o*-phenanthroline and 5 μM $CuSO_4$. The reaction was stopped by adding 0.1 mM EDTA. The 95–111 mAb and the 95–A3 mAb (20 μg ascite/ml) were incubated with the Cu-*o*-phenanthroline-treated membranes or with the untreated membranes at room temperature for 10 min prior to assay; then 100 μl of membranes were added in the spectrofluorimeter cuvette to 0.9 ml of 10 mM Hepes (pH 7.4), 0.2 M sucrose. Swelling was induced by adding 100 μl of 2 M KCl, in the absence of Cu-*o*-phenanthroline treatment (a), in the absence of Cu-*o*-phenanthroline treatment with 95–A3 (b) and with 95–111 (c), after Cu-*o*-phenanthroline treatment (d), after Cu-*o*-phenanthroline treatment plus 95–A3 mAb (e), and after Cu-*o*-phenanthroline treatment plus 95–111 mAb (f).

As we previously demonstrated, anion-conductance of gastric microsomes can also be opened by a protein kinase-mediated MgATP stimulation [3]. In the present work, the rate of $^{125}I^-/I^-$ exchange by fresh hog microsomes was stimulated 5.2-fold in the presence of 2 mM MgATP. No difference in the basal and the MgATP-stimulated anion exchange was observed if the microsomes were preincubated for 15 min at room temperature with 0 to 200 μg of 95–111 ascite/ml.

Discussion

The results demonstrate that the 95–111 mAb is specific for the H^+/K^+-ATPase of gastric parietal cells of several species including man. the 95–A3 mAb is also specific for parietal cell tubulovesicles and canaliculus and stain the 95 kDa polypeptides of H^+/K^+-ATPase-enriched fractions. Smolka et al. [5] first produced monoclonal antibodies against the 95 kDa band of H^+/K^+-ATPase membranes. These antibodies were used to show that the 95 kDa band is heterogeneous but none of the antibodies inhibited H^+/K^+-ATPase activity. Since then, Asano et al. [6] have described an anti-95 kDa monoclonal immunoglobulin which inhibited the ATPase activity and the chloride conductance induced by Cu-*o*-phenanthroline in gastric vesicles. The 95–111 mAb inhibited all aspects of H^+/K^+-ATPase activity, phosphorylation and phosphatase but did not inhibit the chloride conductance. Two polyclonal antibodies [23,24] have also been shown to inhibit the H^+/K^+-ATPase. Amazingly, all the antibodies so far reported to inhibit H^+/K^+-ATPase (Refs. 6,23,24 and the present study) inhibit more the ATPase activity than the pNPPase activity. Such a finding could reflect a particularity of the ATPase structure. Furthermore, in every case, inhibition of H^+/K^+-ATPase activity was only partial (Refs. 6,23 and the present study). This might be due to a restricted access of the immunoglobulin to the ATPase molecule. Indeed, freeze-fracture studies suggest that there is a very high density of H^+/K^+-ATPase in the tubulovesicles and the canaliculus [25] and, attempts to bind the mAb to all H^+/K^+-ATPase molecules could be impossible. In agreement with this hypothesis is the report of Takaya et al [24] who completely inhibited the solubilized H^+/K^+-ATPase showing that the inhibition of H^+/K^+-ATPase can be total when the enzyme is in a soluble form. The 95–111 mAb is very efficient to inhibit the ATPase, it produces 50% ATPase inhibition with 0.13 μg mAb/μg of protein, whereas Asano et al. [6] used 1 μg mAb/μg and whereas 0.3 μg/μg [24] and 20 μg/μg [23] of the polyclonal antibodies were required. Of course, the lower efficacy of polyclonal antibodies could be due to the presence of non inhibitory immunoglobulins in the sera.

From the dual effect of their mAb on ATPase and chloride conductance, Asano et al. [6], suggested that H^+/K^+-ATPase carried the chloride conductance. This is a very challenging hypothesis but, to our knowledge, no anion movement was attributed to H^+/K^+-ATPase even in the uncoupled cation movements [26]. If it were linked to the ATPase, the gastric chloride conductance should be different from the cAMP-regulated electrogenic chloride conductances found in many tissues, and different from the chloride conductances which are modified in cystic fibrosis because the H^+/K^+-ATPase is specifically gastric [1]. Nevertheless, the hypothesis of Asano et al. [6] disregards the fact that several of the 95 kDa polypeptides in gastric membranes could be only chloride channels. Indeed, Smolka et al. [5] have demonstrated that the 95 kDa polypeptides of gastric membranes are heterogeneous [5] and Saccomani et al. [27] have demonstrated that Cu-*o*-phenanthroline induces crosslinking between the 95–100 kDa polypeptides. The H^+/K^+-ATPase and the chloride channel might be distinct, but very closely related proteins, crosslinked by Cu-*o*-phenanthroline and it is difficult to rule out that the dual inhibition of Asano's antibody might be due to interpeptide crosslinking. Thus, it is likely that only purification and characterization of gastric chloride channel will provide the definitive evidence on its dependence or independence towards H^+/K^+-ATPase.

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