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Proton gradient formation in early endosomes from proximal tubules

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Abstract

Heavy endosomes were isolated from proximal tubules using a combination of magnesium precipitation and wheat-germ agglutinin negative selection techniques. Two small GTPases (Rab4 and Rab5) known to be specifically present in early endosomes were identified in our preparations. Endosomal acidification was followed fluorimetrically using acridine orange. In presence of chloride ions and ATP, the formation of a proton gradient (ΔpH) was observed. This process is due to the activity of an electrogenic V-type ATPase present in the endosomal membrane since specific inhibitors bafilomycin and folimycin effectively prevented or eliminated endosomal acidification. In presence of chloride ions (K_m = 30 mM) the formation of the proton gradient was optimal. Inhibitors of chloride channel activity such as DIDS and NPPB reduced acidification. The presence of sodium ions stimulated the dissipation of the proton gradient. This effect of sodium was abolished by amiloride derivative (MIA) but only when loaded into endosomes, indicating the presence of a physiologically oriented Na^+/H^+-exchanger in the endosomal membrane. Monensin restored the gradient dissipation. Thus three proteins (V-type ATPase, Cl^--channel, Na^+/H^+-exchanger) present in early endosomes isolated from proximal tubules may regulate the formation, maintenance and dissipation of the proton gradient.

Keywords: Acidification; ATPase, V-type; Chloride channel; Sodium ion–proton exchanger; Monensin; Rab4; Rab5; (Early endosome); (BBM vesicle); (Proximal tubule)

1. Introduction

Numerous mammalian intracellular organelles, including clathrin-coated vesicles, endosomes and lysosomes have been found to have acidic interiors [1]. Regulation of pH in these organelles plays a critical role in a number of cellular processes, including receptor-mediated endocytosis and secretion. In renal cortical endocytotic vesicles from different species such as rat [2–6], rabbit [7,8] or pig [9] the acidification is driven by V-type ATPase, which energetically accounts for 10–20% of total cellular oxygen consumption in dog kidney proximal tubules [10]. It has been proposed that regulation of endosomal acidification is based upon a chemiosmotic regulatory mechanism [11] and depends on the existence of anion-channels together with proton pumps. Although considerable information regarding the structure and biochemistry of the renal V-type H^+-ATPase have been obtained [12,13] only recently has progress been made regarding the molecular properties of kidney intracellular organelles chloride-channels [14].

The co-existence of V-type ATPase and chloride-channels is universal finding in vacuolar compartments and could be important due to their potential role in the cellular pathophysiology of cystic fibrosis [15]. Subcellular colocalization of CFTR chloride-channels with transferrin receptors and Rab4 GTPases in early endosomes of ductal epithelium has been described [16]. Functional CFTR has also been found in endosomal vesicles from Chinese hamster ovary cells [17] where they may determine endosomal acidification. Chloride-channels have been described in

Abbreviations: BBMV, brush-border membrane vesicles; WGA, wheat-germ agglutinin; MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; KHS, Krebs–Henseleit saline; SDS-PAGE, SDS polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; Baf, bafilomycin A_1; Nig, nigericin; Mon, monensin; CCCP, *m*-chlorophenylhydrazone; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; SITS, 4-acetamido-4'-isothiocyonatostilbene-2,2'-disulfonic acid; DNDS, 4,4'-dinitrostilbene-2,2'-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.

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kidney cortical endosomes from rabbit [7,8,18,19], rat [20] and pig [9] species. Similarly to CFTR, the chloride-channel present in rabbit cortical endosomes was regulated by protein kinase A-dependent phosphorylation. However, the exact nature of the chloride-channels found in renal endosomal vesicles and their participation in pH gradient formation still remains controversial [21]. As well, the presence of a Na^+/H^+-exchanger in renal endosomal vesicles is also a controversial issue. The presence of Na^+/H^+-exchanger was previously described in rat [22] and rabbit [23,24] kidney cortical endosomes. In contrast, endocytic vesicles derived from proximal tubule apical membrane were not shown to contain Na^+/H^+-exchanger [25].

Using a novel technique, the purification of heavy endosomes from dog kidney proximal tubules in suspension was recently presented and the high homogeneity of this endosomal preparation derived from the receptor-mediated endocytosis pathway was demonstrated [26]. In this paper we study the acidification process in heavy endosomes purified from dog kidney proximal tubules in suspension. First, using Rab4 and Rab5 small GTPases as marker proteins, the heavy endosomes were identified as early endosomes. Secondly, we demonstrate that three proteins (V-type ATPase, Cl^--channel and Na^+/H^+-exchanger) are present in early endosomes and may regulate the formation, maintenance and dissipation of the proton gradient. From these data we suggest that the rate of endocytotic transport may be affected by the local cytoplasmic concentrations of sodium and chloride ions as well as by the modulation of activity of the V-type H^+-ATPase, chloride channel and Na^+/H^+-exchanger present on endosomal membrane.

2. Materials and methods

2.1. Materials

Ouabain, oligomycin, nigericin, CCCP, bafilomycin A_1, wheat-germ agglutinin, aprotinin, pepstatin A, chymostatin and phenylmethylsulfonyl fluoride were purchased from Sigma (St.Louis, MO, USA). Concanamycin A (Folimycin) was from Wako BioProducts (Richmond, VA, USA). Acridine orange, SITS, DIDS, DNDS were provided by Molecular Probes (Eugene, OR, USA). NPPB was obtained from Research Biochemicals International (Natick, MA, USA). All reagents for SDS-PAGE and Western blot were from Bio-Rad Laboratories (Hercules, CA, USA). Enzymes and cofactors for enzymatic determinations were from Boehringer-Mannheim (GmbH, Germany). Polyclonal anti-Rab4 and anti-Rab5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horse-radish peroxidase linked anti-rabbit antibody and ECL™ Western blotting detection reagents were obtained from Amersham Life Science (Oakville, ON, Canada).

2.2. Preparation of tubules in suspension

Cortical tubules (> 85% proximal) were prepared from slices of dog renal cortical tissue by collagenase digestion as previously described [27]. Briefly, dogs were anesthetized with pentobarbital sodium (10 mg/kg) and succinylcholine chloride (Anectine, 0.5 mg/kg) and received an intravenous bolus of 20% mannitol (20 ml). Both kidneys were removed and immediately immersed in an ice-cold modified Krebs–Henseleit saline (KHS) containing (in mM): 120 NaCl, 3.2 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 50 mannitol. The renal cortex was sliced with a Stadie Riggs microtome and used for the preparation of cortical tubules using the collagenase digestion procedure [28]. In some experiments the preparation of cortical tubules was made in presence of 0.1 mM MIA. The final suspension of cortical tubules containing around 60 mg wet wt per ml was kept at 4°C in KHS fully gassed with 5%CO_2/95%O_2 until utilization.

2.3. Preparation of BBMV enriched with endosomes

The endosomal population being increased in isolated proximal tubules, the procedure of isolation of endosomes (E) starts by obtaining a BBMV (VE) preparation from such tubules by the conventional magnesium precipitation technique as previously described [29,10]. The suspension of cortical tubules was homogenized in 10 ml of homogenization buffer (Buffer A) (250 mM sucrose, 1 mM EDTA, 18 mM Tris adjusted to pH 7.4 with Hepes) per gram of suspension in the presence of protease inhibitors: 0.1 μM aprotinin, 1 μM pepstatin A, 10 μM chymostatin and 100 μM PMSF. Homogenization was achieved using 20 complete strokes with a glass potter (model C-925, Thomas) equipped with a tight-fitting Teflon pestle.

After the addition of 10 mM $MgCl_2$, the suspension was stirred on ice for 20 min. The suspension was then centrifuged at 7700 × g for 15 min (J-21 centrifuge, JA-20 rotor, Beckman) and the supernatant was centrifuged again at 20000 × g for 30 min. The pellet was homogenized in the resuspension buffer (Buffer B) (150 mM KCl, 5 mM Hepes-Tris, pH 7.4) by aspiration of the sample through a 25-gauge steel needle and recentrifuged for 15 min at 1900 × g. The supernatant was then centrifuged at 31000 × g for 30 min. This pellet containing endosome-enriched BBM Vesicles (VE) was resuspensed in buffer B (1 mg protein/ml) and was used to further separate heavy endosomes from BBM vesicles. Protein concentration was measured after solubilization of the membranes in 0.1% SDS with the Pierce-BCA (bicinchoninic acid) protein assay reagent (Pierce, Rockford, IL, USA) using albumin as standard [30].

2.4. Separation of heavy endosomes from BBM vesicles

The wheat-germ agglutinin (WGA) negative selection technique was used to separate endosomes (E) from BBM

vesicles (V) as previously described [26]. Endosome-enriched BBM Vesicles (VE) (1 mg protein/ml) suspended in buffer B were mixed with WGA creating a VE protein to agglutinin ratio of 10:1. This mixture was left overnight at 4°C with continuous stirring. The suspension was then centrifuged at $14\,500 \times g$ for 3 min. The pellet contained BBM vesicles (V) and the supernatant contained endosomes (E) which were pelleted by a second centrifugation at $48\,000 \times g$ for 30 min. Both E and V were resuspended in buffer B to yield approximately 5–10 mg protein/ml, and the preparation was stored in liquid nitrogen in 50- to 200-μl aliquots until utilization. This material was used to measure enzymatic activities, proton transport and to perform Western blot analyses. The procedure and purity of the preparation are fully described elsewhere [26].

2.5. H^+-ATPase activity

The H^+-ATPase activity of endosomes was taken as the bafilomycin A_1-sensitive moiety of the ATPase activity measured at room temperature (22–24°C) in presence of 0.5 μM oligomycin (inhibition of the mitochondrial ATPase) and 1 mM ouabain (inhibition of the Na^+,K^+-ATPase) using a continuous spectrophotometric assay [10]. Ice-cold endosomes (E) were added in 2 ml ATPase buffer (50 mM Tris-Hepes, 150 mM KCl, 5 mM $MgSO_4$, 2 mM ATP, 0.2 mM NADH, 10 mM phospho*enol*pyruvate, 10 U/ml pyruvate kinase, 6.25 U/ml lactate dehydrogenase adjusted at pH 7.4) to initiate ATP hydrolysis. Stoichiometric NADH production was followed as absorbance measurements monitored using a 'Perkin–Elmer Lambda 3' UV/Vis spectrophotometer at 340 nm. The absorbance was continuously recorded using a 'MacLab/8' (Analog Digital Instruments) connected to a Macintosh SE computer and the MacLab™ Chart v.3.3.4 software.

2.6. ATP-driven acidification

Acidification of the intravesicular space of intact endosomes was followed by measuring the changes in fluorescence of acridine orange at room temperature. Ice-cold BBM vesicles (V), endosomes (E) or a mixture of BBM vesicles and endosomes (VE) were added in 2 ml acridine orange buffer containing an ATP regeneration system (50 mM Tris-Hepes, 150 mM KCl, 5 mM $MgSO_4$, 2 mM ATP, 5 μM acridine orange, 10 mM phospho*enol*pyruvate, 10 U/ml pyruvate kinase adjusted at pH 7.4) to initiate proton transport. Acidification of endosomal vesicles can be similarly measured using purified (E) or unpurified (VE) endosomes since acidification of VE fraction is entirely due to the presence of endosomes in this preparation (Fig. 2) [26]. Therefore acidification experiments were performed with either E or VE fractions as indicated in the figures. Fluorescence measurements were performed using a 'Deltascan Model RFM-2001' spectrofluorimeter (Photon Technology International, South Brunswick, NJ, USA) with excitation at 450 nm (slit width 1 nm) and emission at 525 nm (slit width 2 nm). Fluorescence was recorded using the Oscar™ software. Bafilomycin A_1 (10^{-7} M) was used to inhibit the H^+-ATPase while CCCP (10^{-5} M) and/or nigericin ($2 \cdot 10^{-5}$ M) were used to dissipate the proton gradient.

2.7. SDS-polyacrylamide gel electrophoresis and Western blotting

Electrophoresis was performed with 12% SDS-Tris-glycine-polyacrylamide gels (SDS-PAGE) according to Laemmli [31]. Equal protein aliquots (20 μg protein) of BBM vesicles (V) and heavy endosomes (E) were applied to gel wells and submitted to electrophoresis using a conventional electrophoresis cell (Mini-ProteanII, Bio-Rad Laboratories). A graphite electroblotter system (Milli-Blot™, Millipore, Bedford, MA, USA) was used to transfer proteins from the gels to Immobilon-P PVDF membranes (Millipore, Bedford, MA, USA). Before immunoanalysis, non-specific binding sites were blocked by exposing the membrane to 5% nonfat dry milk for 1 h. The membranes were incubated with a first antibody against Rab4 (1:500) or Rab5 (1:500) and then exposed to peroxidase-conjugated anti-rabbit (1:10 000) IgG for 1 h. The membranes were then washed three times in Tris-buffered saline-Tween solution (5 mM Tris-HCl, 15 mM NaCl, 0.3% Tween 20, pH 7.0) and covered with ECL detection reagents for 1 min. Luminograms were obtained by exposing Fuji RX film to membranes for 2–10 s. All procedures were performed at room temperature.

3. Results

The endosomes prepared from isolated proximal tubules in suspension were previously defined as heavy endosomes [26]. Here we performed experiments to functionally characterize these endosomes. Markers, such as small GTPases Rab4 and Rab5, known to be specifically associated with early endosomes [32–34] were used. SDS-PAGE analysis of brush-border membrane vesicles (V) and heavy endosomes (E) (Fig. 1, Panel 1) demonstrates the presence of low molecular weight proteins (18–30 kDa) in these fractions. Western blot analysis with anti-Rab4 (Fig. 1, Panel 2) and anti-Rab5 (Fig. 1, Panel 3) revealed the presence of these small GTPases in endosomes and BBM vesicles.

The presence of proteins capable of establishing or modulating endosomal acidification (V-type ATPase, chloride channel and Na^+/H^+-exchanger) was tested by following their function and by using specific inhibitors. The acidification of early endosomes was examined in presence of ATP (and of an ATP-regenerating system) by the acridine orange fluorescence technique. Upon addition of endosomes (E), a steep acidification was observed (Fig. 2, curve 3). This process was bafilomycin-sensitive as well as

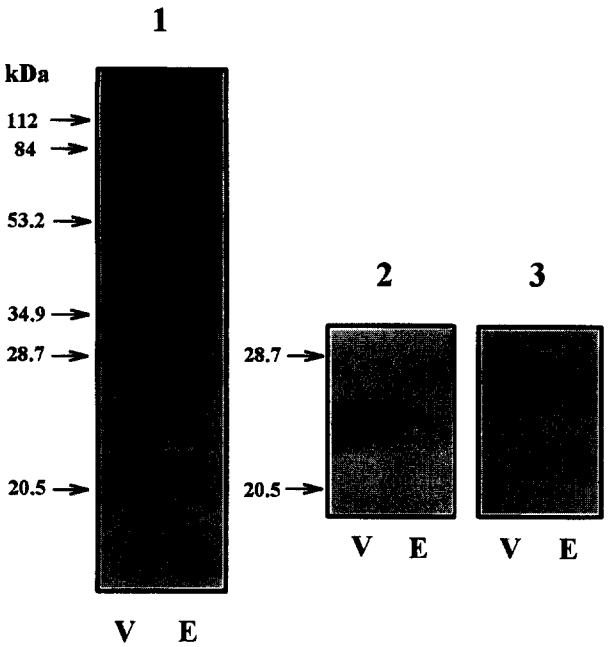

Fig. 1. Identification of Rab4 and Rab5 in heavy endosomes prepared from proximal tubules in suspension. Coomassie blue staining (Panel 1) of total proteins (20 μg) of BBM vesicles (V) and heavy endosomes (E) from dog kidney proximal tubules in suspension after SDS-PAGE and electroblotting to Immobilon-P PVDF membrane. Western blot analysis to detect the presence of specific markers of early endosomes, Rab4 (Panel 2) and Rab5 (Panel 3) small GTPases, in BBM vesicles (V) and heavy endosomes (E).

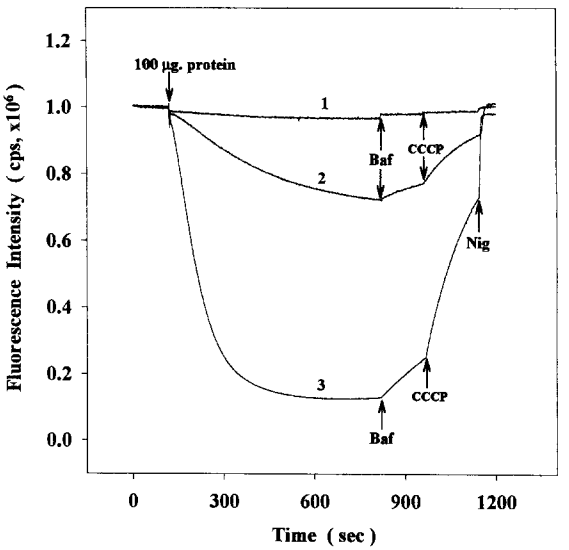

Fig. 2. Acidification of early endosomes prepared from cortical tubules in suspension. ATP-dependent acidification observed in endosomes before (Curve 2) and after (Curve 3) separation from BBM vesicles (Curve 1) using wheat-germ agglutinin negative selection technique. The effects of 10^{-7} M bafilomycin (Baf), 10^{-5} M *m*-chlorophenylhydrazone (CCCP) and $2 \cdot 10^{-5}$ M nigericin (Nig) are indicated by arrows. Adapted from Marshansky et al. [26] with permission.

oligomycin and ouabain-insensitive indicating that a V-type proton ATPase was at play. The proton gradient was dissipated by proton ionophore CCCP and/or by the potassium/proton exchanger nigericin. The same amount of BBM vesicles (V) obtained after WGA treatment was not capable of ATP-dependent acidification (Fig. 2, curve 1). Therefore, the acidification observed with the mixture VE was entirely due to the presence of endosomes (Fig. 2, curve 2).

When the sensitivity of endosomal acidification to bafilomycin A_1 was examined using increasing concentrations of the inhibitor (10^{-11} to 10^{-8} M) an apparent K_i was calculated to be 0.38 nM (Fig. 3A, curve 1). A comparable apparent K_i of 0.3 nM (Fig. 3B, curve 1) was calculated for bafilomycin A_1 during measurement of V-type ATPase activity. The sensitivity of acidification and

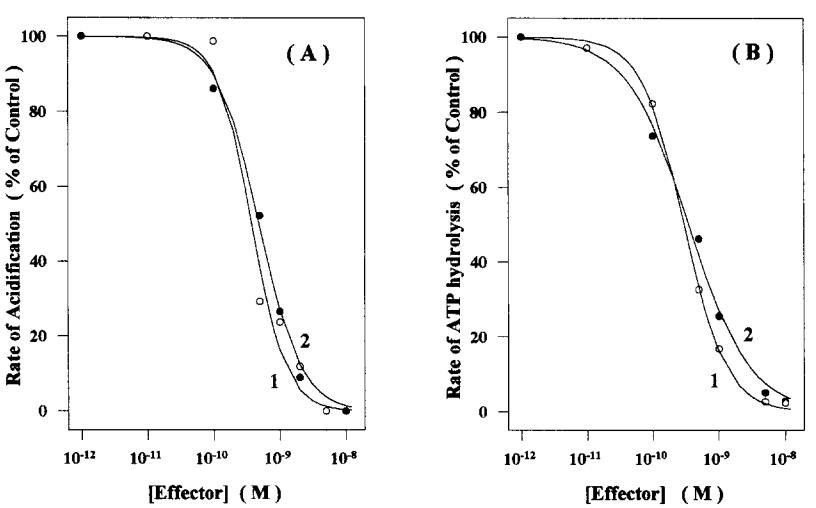

Fig. 3. Apparent K_i of bafilomycin and folimycin on the proton pump measured as initial rate of acidification and ATP hydrolysis. Initial rate of acidification of endosomes prepared from proximal tubules was measured in presence of increasing concentrations of bafilomycin (Panel A, curve 1) or folimycin (Panel A, curve 2). V-type ATPase hydrolyzing activity of early endosomes was measured in absence of detergent and in presence of different concentrations of bafilomycin (Panel B, curve 1) and folimycin (Panel B, curve 2).

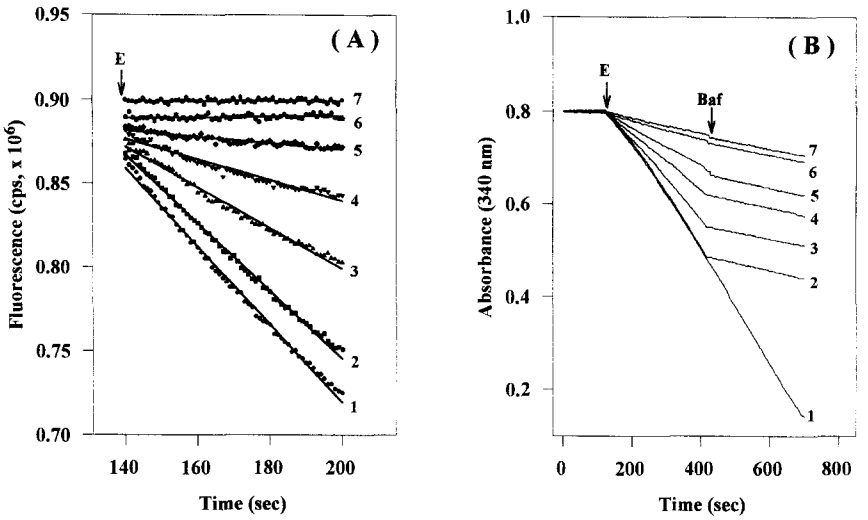

Fig. 4. Inhibition of early endosomal acidification and ATPase activity by concanamycin A (folimycin), a novel V-type ATPase inhibitor. The effect of increasing concentrations of folimycin on initial rate of acidification (Panel A) and on ATPase activity (Panel B) of endosomes prepared from proximal tubules is demonstrated. Early endosomes (E) (50 μg protein) were added into incubation medium containing increasing concentrations of folimycin (nM) (curve 1, 0; curve 2, 0.1; curve 3, 0.5; curve 4, 1; curve 5, 2; curve 6, 5; curve 7, 10). The effect of 10^{-7} M bafilomycin (Baf) is indicated by the arrow.

ATPase activity to concanamycin A (folimycin), a new specific inhibitor of proton V-type ATPase [35,36], was also examined. Concanamycin A inhibited both acidification (Fig. 4A) and ATPase activity (Fig. 4B) at concentrations comparable to that observed for bafilomycin A_1. The apparent K_i of concanamycin A was calculated to be 0.49 nM for acidification (Fig. 3A, curve 2) and 0.35 nM for ATPase activity (Fig. 3B, curve 2).

The next experiment was designed to test the effect of chloride ions on acidification. In the presence of 150 mM KCl and ATP, a steep endosomal acidification is observed (Fig. 5, curve 4). This process is entirely absent when bafilomycin A_1 (10^{-7} M) is present in the incubation medium (Fig. 5, curve 1). When endosomes are incubated in absence of chloride ions (substitution of potassium chloride by potassium gluconate), a significant diminution of acidification was observed (Fig. 5, curve 2). Re-introduction of chloride ions during experiment stimulates acidification (Fig. 5, curve 3).

The dose–response curve relating acidification to chloride concentration established by a stepwise increment of chloride concentration allows to calculate (using the initial (150 s) rate of acidification monitored as the slope of fluorescence extinction) (Fig. 6A) an apparent K_m of 30 mM for chloride ions (Fig. 6B). The chloride did not affect specific bafilomycin-sensitive ATP hydrolysis in the presence of a detergent [10]. Thus chloride ions are required to obtain an optimal acidification because of its effect on endosomal membrane potential and not through a direct effect on the proton pump.

In order to further investigate the nature of the chloride effect, inhibitors of various chloride channels were tested for their inhibitory effect on the stimulation of acidification induced by chloride. Fig. 7, curve 1 shows acidification without chloride and Fig. 7, curve 2 shows acidification stimulated by the addition of KCl. When NPPB 100 μM (Fig. 7, curve 3) or DIDS 100 μM (Fig. 7, curve 4) were introduced before KCl, the stimulation of acidification was significantly suppressed. Other chloride channel inhibitors had no effect (SITS) or less pronounced in-

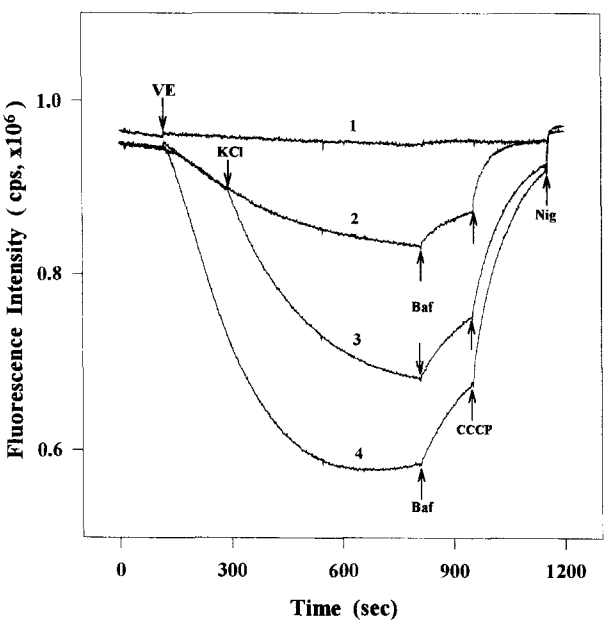

Fig. 5. Effect of chloride ions on the ATP-dependent endosomal acidification. Acidification of endosomes prepared from cortical tubules observed with 100 mM KCl in absence (4) or presence (1) of bafilomycin or with 100 mM K-gluconate (2). The effect of 50 mM KCl added during acidification to medium containing 50 mM K-gluconate is shown on curve 3 by the arrow. The effects of 10^{-7} M bafilomycin (Baf), 10^{-5} M *m*-chlorophenylhydrazone (CCCP) and $2 \cdot 10^{-5}$ M nigericin (Nig) are also indicated by the arrows.

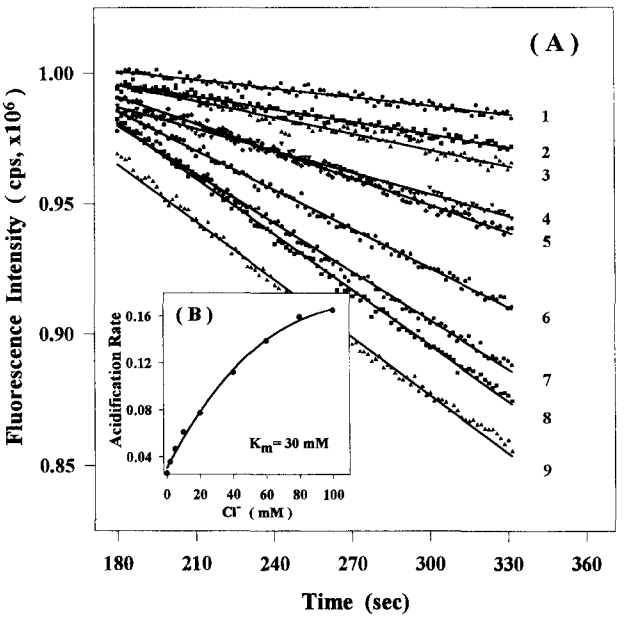

Fig. 6. Effect of chloride ions on the initial rate of endosomal acidification. The initial rate of ATP-dependent acidification of endosomes incubated in the presence of increasing concentrations of chloride ions (mM) (curve 1, 0; curve 2, 2; curve 3, 3; curve 4, 10; curve 5, 20; curve 6, 40; curve 7, 60; curve 8, 80; curve 9, 100) is shown in Panel A. A dose–response plot is shown in Panel B.

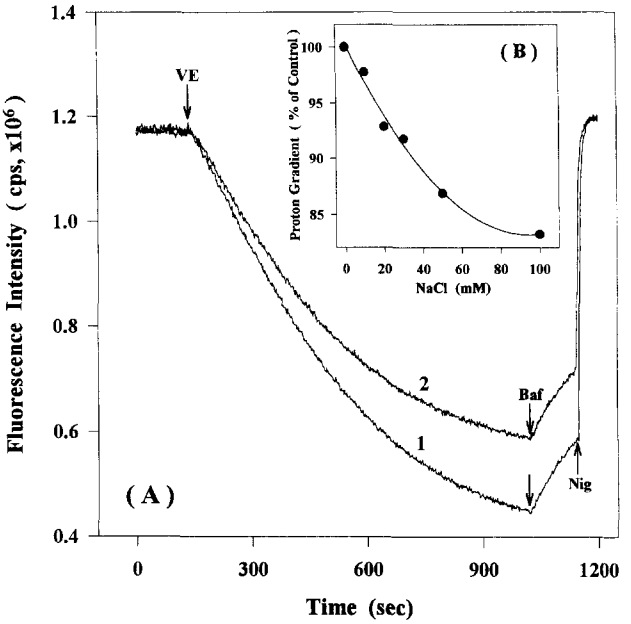

Fig. 8. Effect of sodium ions on the establishment of a proton gradient. Endosomal acidification observed without (curve 1) or with (curve 2) sodium in incubation medium (Panel A). Panel B presents the dissipation of the proton gradient obtained in presence of different concentrations of sodium ions. The effects of 10^{-7} M bafilomycin (Baf) and $2 \cdot 10^{-5}$ M nigericin (Nig) are indicated by the arrows.

hibitory effect (DNDS) (not shown). These observations suggest that the effect of chloride on endosomal ATP-dependent acidification is mediated by the presence of a NPPB/DIDS-sensitive chloride channels in endosomal membrane.

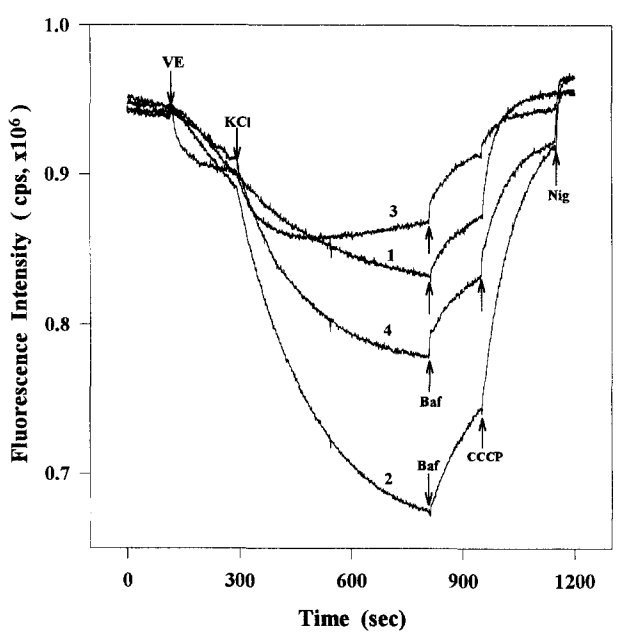

Fig. 7. Effect of chloride channels inhibitors on chloride-stimulated endosomal acidification. The acidification observed with (curve 2) or without (curve 1) chloride and following addition of the chloride channels inhibitors NPPB 100 μM (curve 3) or DIDS 100 μM (curve 4) are presented. The effects of 10^{-7} M bafilomycin (Baf), 10^{-5} M *m*-chlorophenylhydrazone (CCCP) and $2 \cdot 10^{-5}$ M nigericin (Nig) are indicated by the arrows.

When ATP-dependent endosomal acidification was studied in absence (Fig. 8A, curve 1) or presence (Fig. 8A, curve 2) of sodium ions the rate of acidification and the final steady-state proton gradient was reduced in the presence of 100 mM sodium. The dose–response curve (Fig. 8B) indicates a maximal reduction of 20% of the net acidification. Addition of amiloride or of the derivative of amiloride MIA, to the incubation medium, did not suppress the effect of sodium, even after a preincubation of 120 min (not shown). In contrast, if MIA is added during the preparation of proximal tubules (collagenase treatment), and is therefore trapped into the intra-endosomal compartment, a strong effect of MIA on endosomal acidification is observed in endosomes incubated in presence of sodium. Indeed, acidification of endosomes prepared from tubules not exposed to MIA and incubated in presence of 100 mM NaCl was less pronounced (Fig. 9A, curve 1) than that of endosomes prepared from tubules exposed to MIA (Fig. 9A, curve 2). Monensin reduced acidification in the presence of sodium (but less so with potassium and not with choline), indicating the well known specificity of this artificial Na^+/H^+-exchanger [37] in our experimental conditions (Fig. 9B). Monensin acts here as an exogenous Na^+/H^+-exchanger unaffected by MIA. The dose–response study of the effect of monensin (Fig. 10A, curves 1–8) shows that monensin effectively diminished both the initial rate of acidification (Fig. 10B, curve 2) and the

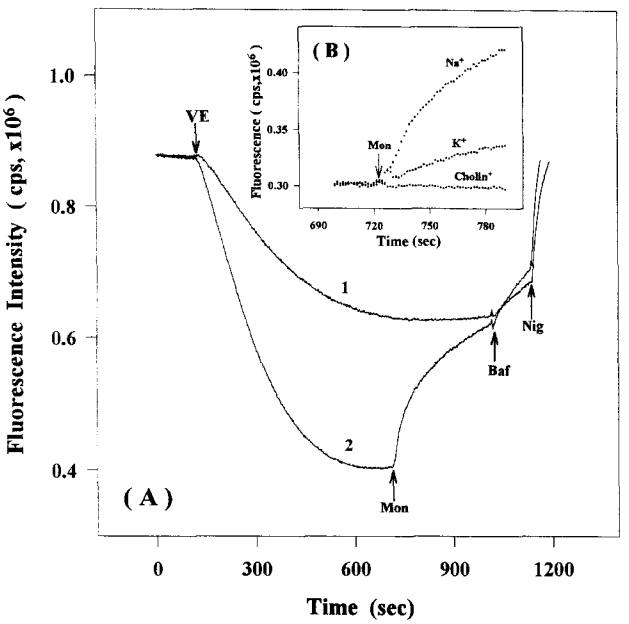

Fig. 9. Effect of MIA loaded into endosomes on the establishment of a proton gradient. Endosomal acidification observed without (Panel A, curve 1) or with (Panel A, curve 2) MIA loading in the intra-endosomal compartment through incubation with the proximal tubules prior to the preparation of endosomes. The effects of $3 \cdot 10^{-6}$ M monensin (Mon), 10^{-7} M bafilomycin (Baf) and $2 \cdot 10^{-5}$ M nigericin (Nig) are indicated by the arrows. Panel B shows the effect of monensin on the dissipation of a previously established endosomal proton gradient in the presence of 100 mM NaCl (Na^+), KCl (K^+) or CholinCl ($Cholin^+$).

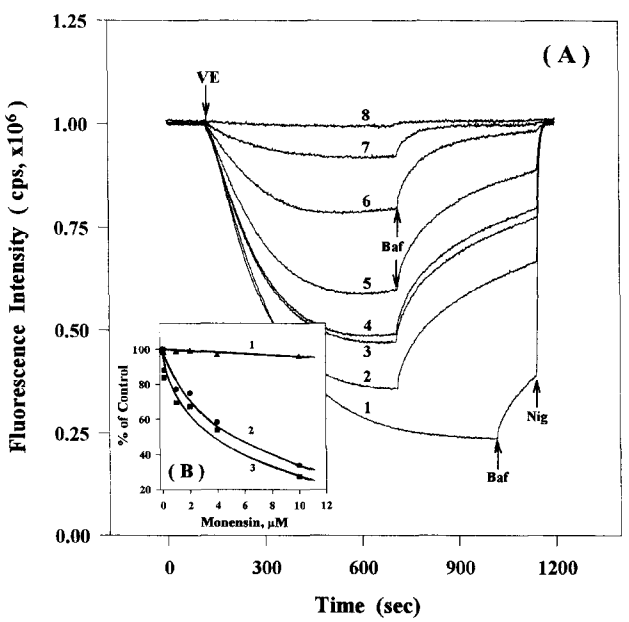

Fig. 10. Effect of Na^+/H^+-exchanger monensin on ATP-driven endosomal acidification. The endosomal acidification observed with increasing concentrations of monensin (μM) (curve 1, control; curve 2, 0.1; curve 3, 1; curve 4, 2; curve 5, 4; curve 6, 10; curve 7, 20; curve 8, 40) is presented (Panel A). The effects of 10^{-7} M bafilomycin (Baf) and $2 \cdot 10^{-5}$ M nigericin (Nig) are indicated by the arrows. Panel B shows the effect of increasing concentrations of monensin on the ATPase activity (curve 1), the initial rate of acidification (curve 2) and the steady-state proton gradient formation (curve 3) in early endosomes.

steady-state proton gradient formation (Fig. 10B, curve 3) in early endosomes. However, monensin did not affect the ATPase activity of the V-type H^+-ATPase (Fig. 10B, curve 1). This demonstrates that the effect of monensin on acidification was due to Na^+/H^+-exchange and not to a direct effect on the proton pump.

4. Discussion

Reabsorption of filtered proteins such as albumin, γ-globulin and proteins of small molecular weight by the proximal tubules relies largely on a receptor-mediated endocytosis process. Receptor-mediated endocytosis is proposed to be regulated, on the one hand, by the formation of a proton gradient in clathrin-coated and endosomal vesicles [1,38,39] and on the other hand, by small GTP-binding proteins [40–42]. We have recently described a novel method to isolate heavy endosomes from proximal tubules in suspension [26]. Our results showed that the large endosomal population observed in isolated proximal tubules arise from the stimulation of protein reabsorption in tubules, because of the presence of proteins in the extracellular medium. Two different experimental approaches, western blot analysis and small-particle flow cytometry analysis, were used to demonstrate the presence of extracellular proteins entrapped in purified heavy endosomes [26]. Indeed, immunochemical analysis indicated that both albumin and collagenase A, originally present in extracellular medium during the preparation of proximal tubules, were found in purified heavy endosomes. Small-particle flow cytometry analysis demonstrated the presence of loaded FITC-albumin molecules in up to 92% of purified heavy endosomes indicating high homogeneity of endosomal fraction derived from the receptor-mediated endocytosis pathway [26]. In the present paper, we examine the nature of these endosomes and the possible regulation of their acidification capacity.

Until recently the lack of specific markers of early or late endosomes made the functional identification of endosomal preparations difficult. However, recent studies on the localization of small GTP-binding proteins have confirmed that early and late endosomes have distinct distribution of Rab proteins [40–42], which could regulate different stages of fusion processes. It has been demonstrated that Rab4 and Rab5 are associated with the cytoplasmic face of the plasma membrane and early endosomes, while Rab7 and Rab9 are restricted to late endosomes [32–34]. Using Western blot analysis, the presence of Rab4 and Rab5 GTPases has been demonstrated in endosomes as well as in BBM vesicles from proximal tubules in suspension. Interestingly, Rab5 is present in similar quantities in endosomes and brush-border membrane vesicles, while Rab4 is greatly diminished in endosomes in comparison with BBM vesicles. Thus, heavy endosomes isolated from proximal tubules in suspension and derived from the recep-

tor-mediated endocytosis pathway [26] can be functionally identified as early endosomes.

The wheat-germ agglutinin negative selection technique allows to separate pure BBM vesicles (V) and pure endosomes (E) from the mixture (VE) prepared with conventional magnesium precipitation technique applied to isolated tubules. Both V and E originate from the BBM (same enrichment with BBM markers [26]) and remain under their physiological configuration. Indeed, they present their proton pumps under physiological polarity. Thus spontaneous acidification can only be observed with E upon ATP addition while a comparable bafilomycin sensitive ATPase activity is found with both membrane fractions when measured in the presence of a detergent [26]. The proton pump appears to be in a comparable physiological state in BBM and endosomes since: (1) the bafilomycin-sensitive ATPase activity is comparable in V and E when the membranes are solubilized with a detergent; (2) the apparent K_i for bafilomycin and folimycin measured for both acidification or ATPase activities of tubular endosomes is comparable to that measured in BBM preparations from intact renal cortex with predominant BBM vesicles [43].

The establishment of a pH gradient within the endosomes is a cardinal feature for the endocytotic reabsorption cycle. We have studied both the accumulation of proton in the endosomal space and the dissipation of the proton gradient as possible regulatory targets for this process. Clearly, the activity of the proton pump may regulate rate of the acidification while the efflux of protons should influence the amplitude of the pH gradient. Altering the activity of the pump with bafilomycin or folimycin immediately influences or abolishes the rate of endosomal acidification. Thus physiologic regulators of the activity of the proton pump may modulate the rate of endosomal acidification in situ. Identification and partial purification of cytosolic activators of V-type ATPase have been earlier described [44,45].

Since proton accumulation determines the formation of inside-positive membrane potential, we have examined the effect of chloride on this process. We have demonstrated that in the absence of chloride the acidification is impaired and this can be reversed by re-introduction of chloride ions. We know from literature [10] and from past experience that chloride has no effect on the proton pump per se. Chloride only prevents the formation of the inhibitory membrane potential. Since this effect probably involves chloride transport through some kind of chloride channels, we have examined the effect of several inhibitors of these channels on endosomal acidification. Both NPPB and DIDS prevented the stimulation of acidification arising from chloride addition while SITS had no effect. Thus our data clearly shows the presence of chloride channels together with proton pumps in these endosomes. We propose that the regulation of the activity of chloride channels [18,19] may thus influence endosomal acidification in kidney proximal tubules in situ.

The dissipation of the protons may also affect the overall pH gradient. Endosomes originating from the BBM

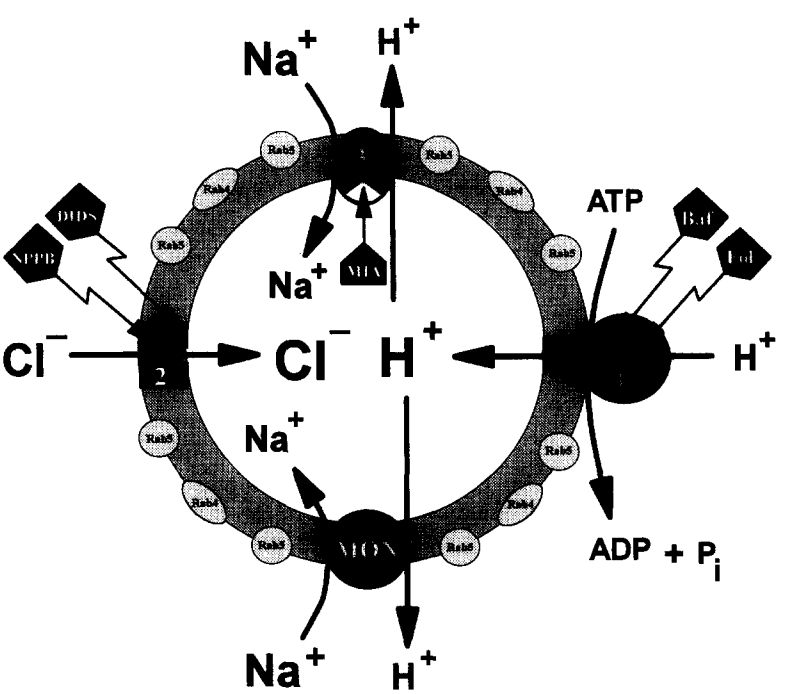

Fig. 11. Schematic representation of formation, maintenance and dissipation of the proton gradient in early endosomes from dog kidney proximal tubules. Three proteins, V-type ATPase (1), Cl^--channel (2) and Na^+/H^+-exchanger (3), are postulated to take part in the regulation of steady-state proton gradient formation in early endosomes. Markers of early endosomes, small GTPases Rab4 and Rab5, are also shown. Sites of action of specific inhibitors of these proteins, bafilomycin (Baf), folimycin (Fol), DIDS, NPPB and MIA as well as artificial Na^+/H^+-exchanger monensin (Mon) are indicated.

may present an active Na^+/H^+-exchanger. We have examine the effect of sodium on endosomal acidification. A modest (20%) reduction of acidification was induced in our endosomal preparation by the presence of 100 mM sodium. To demonstrate that this effect is related to proton efflux driven by sodium influx catalyzed by this Na^+/H^+-exchanger, we have examined the effect of an amiloride derivative (MIA) on acidification in the presence of sodium. MIA had no effect when presented to the endosomal incubation medium. However, when MIA was trapped in the intra-endosomal space, by preparing the proximal tubular suspension in presence of MIA, a profound effect was found. Indeed, the acidification process was considerably stimulated by MIA. This effect was fully reversed by introducing a MIA insensitive artificial Na^+/H^+-exchanger, monensin. Monensin was able to fully dissipate the proton gradient. The naturally occurring Na^+/H^+-exchanger was less effective than monensin in quenching the gradient although we have not established that its activity was maximal in our preparation. Thus, (1) the sodium/proton-exchanger is polarized in the endosomal membrane, presenting the MIA binding site to the extracellular leaflet of the membrane located inside of endosomes; (2) that the activity of this exchanger, which is physiologically regulated [46,47] may modulate acidification of endosomes; (3) the local concentration of sodium around endosomes may also modulate the acidification of endosomes. This latter parameter is influenced by the rate of luminal sodium-coupled cotransport as well as by the activity of the basolateral Na^+,K^+-ATPase.

The experimental findings described in our paper are summarized on Fig. 11. Basically, heavy endosomes derived from the receptor-mediated endocytosis pathway of proximal tubules are shown as early endosomes with Rab4 and Rab5 small GTPases associated with their membrane. Three transport proteins (V-type ATPase, Cl^--channel and Na^+/H^+-exchanger) are present in early endosomes and may regulate the formation, maintenance and dissipation of the proton gradient. On the one hand, the V-type ATPase is sensitive to bafilomycin and folimycin while the Cl^--channel is inhibitable by DIDS and NPPB. On the other hand, the Na^+/H^+-exchanger is sensitive to MIA when loaded into endosomes and its action could be replaced by monensin.

In conclusion, the early endosomes purified from dog proximal tubules in suspension [26] share some common features with endosomal fractions prepared from intact cortex of rat [2–6], rabbit [7,8] and pig [9] species in regard to the presence of essential elements (V-type ATPase, Cl^--channel and Na^+/H^+-exchanger) of acidification machinery. However, the advantages of our endosomal preparation isolated from proximal tubules in suspension in comparison to renal cortex endosomes include: (1) the purification process is simple, efficient and provides a purification of early endosomes of known proximal origin; (2) the endosomal preparation is homogeneous with up to 92% of purified early endosomes derived from the receptor-mediated endocytosis pathway [26]; (3) the early endosomes from proximal tubules can be easily loaded with different molecules such as inhibitors (MIA), proteins (albumin and collagenase A) [26] and probably with other macromolecules of interest such as antibodies and fluorescent tracers.

We have recently demonstrated [48] that interfering with the establishment of endosomal proton gradient by ATP-depletion, bafilomycin application or by quenching the proton gradient with NH_4Cl, suppress the endocytotic transport of albumin in proximal tubules in suspension. Thus the regulation of endosomal acidification through alteration of the activity of V-type ATPase, chloride channel and Na^+/H^+-exchanger in situ may affect this important physiological process. Coupled studying of tubular transport and of the function of endosomal preparations offers a unique opportunity to investigate this system under normal and pathological conditions.

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