J. Membrane Biol. 124, 139–149 (1991)
002226319100139K

The Journal of
Membrane Biology

Effect of Sulfhydryl Compounds on ATP-Stimulated H^+ Transport and Cl^- Uptake in Rabbit Renal Cortical Endosomes

Shirley A. Hilden and Nicolaos E. Madias
Department of Medicine, Tufts University School of Medicine, and Division of Nephrology, New England Medical Center, Boston, Massachusetts 02111

Summary. The vacuolar H^+ ATPase is inhibited by N-ethylmaleimide (NEM), a sulfhydryl compound, suggesting the involvement of a sulfhydryl group in this transport process. We have examined the effects of several sulfhydryl-containing compounds on the vacuolar H^+ ATPase of rabbit renal cortical endosomes. A number of such compounds were effective inhibitors of endosomal H^+ transport at 10^{-5}–10^{-6} M, including NEM, mersalyl, aldrithiol, 5,5′ dithiobis (2-nitrobenzoic acid), *p*-chloromercuribenzoic acid (PCMB) and *p*-chloromercuriphenyl sulfonic acid (PCMBS). NEM, mersalyl, aldrithiol and PCMBS had no effect on pH-gradient dissipation, whereas PCMB decreased the pH gradient faster than control. In the absence of ATP, PCMB (10^{-4} M) stimulated endosomal $^{36}Cl^-$ uptake, particularly in the presence of an inside-alkaline pH gradient (pH_{in} = 7.6/pH_{out} = 5.5). This result was not an effect of PCMB on the Cl^--conductive pathway. The less permeable PCMBS did not stimulate $^{36}Cl^-$ uptake. The effects of PCMB were concentration dependent and were prevented by dithioerithritol. ATP-dependent $^{36}Cl^-$ uptake was decreased by addition of PCMB. Finally, PCMB had no effect on $^{45}Ca^{2+}$ uptake. These results support the presence of two functionally important sulfhydryl groups in this endosomal preparation. One such group is involved with ATP-driven H^+ transport and must be located on the cytoplasmic surface of the endosomal membrane. The second sulfhydryl group must reside on the internal surface of the endosomal membrane and relates to a PCMB-activated Cl^-/OH^- exchanger that is functional both in the presence and absence of ATP. This endosomal transporter is similar to the PCMB-activated Cl^-/OH^- exchanger recently described in rabbit renal brush-border membranes.

Key Words sulfhydryl compounds · *p*-chloromercuribenzoic acid · endosomes · Cl^-/OH^- exchange · Cl^- transport · H^+ transport

Introduction

Endosomes, lysosomes and other intracellular organelles contain an H^+ ATPase that transports H^+ into the organelle and creates an acidic interior. Acidification of the vacuolar interior by this H^+ ATPase is considered of critical importance in carrying out a range of organellar functions (Al-Awqati, 1986; Stone & Xie, 1988). The mechanisms mediating the regulation of vacuolar pH are under active investigation. In recent studies, we have described the presence of a Cl^- conductance (Hilden, Johns & Madias, 1988), an H^+/Ca^{2+} exchanger (Hilden & Madias, 1989) and a Na^+/H^+ exchanger (Hilden, Ghoshroy & Madias, 1990) in an H^+ ATPase-bearing endosomal fraction isolated from rabbit renal cortex. We have suggested that these secondary transporters might represent important modulators of the vacuolar H^+ ATPase activity.

Proton transport by the vacuolar H^+ ATPase is inhibited by N-ethylmaleimide (NEM), a sulfhydryl compound, suggesting the involvement of a sulfhydryl group in this transport process. Therefore, in the present studies, we have examined the effects of several sulfhydryl-containing compounds on the vacuolar H^+ ATPase of rabbit renal cortical endosomes. Such compounds have previously been used successfully in characterizing properties of a variety of transport systems. For example, phlorizin binding in intestinal brush-border membrane vesicles (BBMV) has been shown to be inhibited by *p*-chloromercuribenzoic acid (PCMB), but not by the less permeable *p*-chloromercuriphenyl sulfonic acid (PCMBS), suggesting that a relevant sulfhydryl group is exposed on the cytoplasmic surface of the brush-border membrane (Klip, Grinstein & Semenza, 1979). The results of our studies suggest that two transport processes are actually affected by sulfhydryl-containing compounds in renal cortical endosomes. The vacuolar H^+ ATPase activity is inhibited by a number of such reagents, including NEM, the diuretic mersalyl, aldrithiol, PCMB and PCMBS. In addition, PCMB, but not PCMBS, appears to activate a Cl^-/OH^- exchange process that bears similarities to the PCMB-activated Cl^-/OH^- exchanger recently reported in rabbit renal BBMV (Karniski, 1989).

Materials and Methods

Endosomal Membrane Vesicle (Endo-MV) Preparation

This preparation has previously been described (Hilden et al., 1988). Briefly, New Zealand White rabbits were killed with Beuthanasia-D (Burns-Biotec Laboratories, Omaha, NE) and the kidneys were removed. Cortex was separated from medulla, minced and homogenized in a Teflon-glass homogenizer using 35 ml of homogenizing medium containing (in mM): 200 sucrose, 25 K gluconate, 10 N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES), 2 EDTA, 0.1 phenylmethylsulfonyl fluoride (PMSF), pH 7.6 with KOH. Initial homogenization used 10 strokes with a loose-fitting pestle followed by 25 strokes with a tight-fitting pestle. The homogenate from two rabbits was diluted to 140 ml with homogenizing medium and centrifuged for 30 min at 34,800 × *g* (17,000 rpm, Sorvall SS-34 rotor). The pellet (P_1) was used to make brush-border membrane vesicles (BBMV). Mg sulfate (1 M) was added to the supernatant so that the final concentration of Mg sulfate was 14 mM. This mixture was stirred on ice for 20–40 min. The resulting suspension was then centrifuged at 34,800 × *g* for 30 min. The pellet was the endo-MV H^+ ATPase preparation and was resuspended in a small volume for transport or enzyme assays. The characteristics of this preparation have previously been reported (Hilden et al., 1988).

BBMV Preparation

P_1 was resuspended in 140 ml of a medium containing (in mM): 25 K gluconate, 10 HEPES, 2 EDTA, pH 7.6 with KOH by homogenization in a Teflon-glass homogenizer (1–2 strokes). Mn sulfate (1 M) was added so that the final concentration of Mn sulfate was 14 mM. After stirring on ice for 20 min, the resulting suspension was centrifuged at 1,935 × *g* (4,000 rpm, Sorvall SS-34 rotor) for 10 min. The pellet was discarded, and the supernatant was centrifuged at 34,800 × *g* for 30 min. The resulting pellet was the BBMV; it was resuspended in a small volume and treated as needed for a given experiment.

H^+ Transport

H^+ transport was measured with acridine orange (AO) as described previously (Hilden et al., 1988). Endo-MV were suspended in a medium containing (in mM): 100 K gluconate, 100 mannitol, 1 Mg gluconate, 5 HEPES, pH 7.6. The suspension was centrifuged and resuspended in a small volume for transport. Membrane vesicles were left on ice until the assay. Endo-MV (50 μl, 50–200 μg protein) were added to 3 ml of a medium containing (in mM): 100 KCl, 100 mannitol, 1 $MgCl_2$, 5 HEPES, pH 7.6, and 3 μM AO. The fluorescence of this mixture was measured in a Perkin-Elmer model L8-5 fluorescence spectrophotometer (excitation 490 nm, emission 530 nm). After stabilization of fluorescence, 20 μl of 50 mM Mg adenosine-triphosphate (ATP) was added, and the change in AO fluorescence was monitored as a function of time. AO is a fluorescent weak base that accumulates in acidic compartments. Intravesicular dye at high concentrations results in self quenching and, therefore, a decrease in the fluorescent signal. Stimulation of H^+ pumping into vesicles by ATP leads to a decrease in the fluorescence intensity of AO. Inhibitors (small volumes of stock solutions) were added before ATP or after ATP-induced fluorescence changes had stabilized (i.e., after a stable pH gradient had been established). Initial rates of change in AO fluorescence are reported.

Membrane Conductance

The anion permeability of BBMV was demonstrated by measuring the membrane potentials developed by ion gradients. The potential-sensitive dye, 3,3'-dipropylthiadicarboxocyanine iodide ($DiSC_3(5)$), was used to monitor membrane-potential changes in BBMV. For example, to demonstrate a NO_3^- diffusion potential, BBMV were equilibrated in a medium containing (in mM): 150 tetraethylammonium (TEA) gluconate, 1 $MgSO_4$, 50 HEPES, pH 7.5. Brush-border protein (100–200 μg) was added to 3 ml of the same solution along with 10 μl of dye (0.9 mM in ethanol). Cuvettes containing this mixture were placed in an Aminco Bowman spectrofluorimeter where the temperature of the cuvette was regulated at 20°C. After stabilization of fluorescence, stock $TEANO_3$ was added so that the final concentration of $TEANO_3$ was 23 mM. The change in dye fluorescence was monitored. The protein concentration was varied to achieve maximal fluorescence change with a valinomycin-induced potassium-diffusion potential, and this concentration was used for all experimental conditions. Other details are reported elsewhere (Beck & Sacktor, 1978*a,b*). $DiSC_3(5)$ was a gift of Dr. Waggoner (Sims et al., 1974). Results are reported in arbitrary fluorescence units. A decrease in dye fluorescence represents a more negative membrane potential.

$^{36}Cl^-$ Uptake

Membrane vesicles were suspended in a medium containing (in mM): 100 K gluconate, 100 mannitol, 1 EGTA, 1 Mg gluconate, 50 HEPES, pH 7.6. Ten μl MV were added to 100 μl of a medium containing (in mM): 100 K gluconate, 100 mannitol, 1 EGTA, 1 Mg gluconate, 3–5 $^{36}Cl^-$, and 50 HEPES, pH 7.6 or 50 2-[N-morpholino] ethanesulfonic acid (MES), pH 5.5. At different times, washing solution was added, and the resulting mixture was filtered as previously described (Hilden et al., 1988).

Materials

2,2'-Dithiobis (5-nitropyridine) 6,6'-dithiodinicotinic acid, aldrithiol-2 and 4, and S-acetyl thiocholine were purchased from Aldrich Chemical (Milwaukee, WI). 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), and ethylmercurithiosalicylic acid were purchased from Eastman Kodak (Rochester, NY). Other chemicals were purchased from Sigma Chemical (St. Louis, MO). $^{36}Cl^-$ was purchased from New England Nuclear (Wilmington, DE).

All experiments were done at least three times with different membrane preparations. When presenting group results, all experiments performed were averaged, and results are reported as means ± SE.

Results

Effect of Sulfhydryl Compounds on H^+ Transport

N-ethylmaleimide (NEM) is an inhibitor of all known vacuolar H^+ ATPases and has previously been shown by us to inhibit ATP-dependent H^+ transport

Table 1. Effect of sulfhydryl-containing compounds on H^+ ATPase activity in renal cortical endosomes

	H^+ ATPase activity (% of control)			
	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
N-ethylmaleimide (NEM)		80 ± 18	40 ± 16	11 ± 5
Mersalyl	34 ± 12	6 ± 6	3 ± 3	0
Glutathione, oxidized			85 ± 13	10 ± 10
Glutathione, reduced			82 ± 6	0
p-Chloromercuribenzoic acid (PCMB)	93 ± 8	0	0	0
p-Chloromercuriphenyl sulfonic acid (PCMBS)	74 ± 23	0	0	0
Aldrithiol-2	81 ± 28	54 ± 10	16 ± 2	2 ± 2
Aldrithiol-4	55 ± 11	15 ± 8	4 ± 4	6 ± 3
2-Mercaptoethylamine			114 ± 12	60 ± 15
2,4 Dithiopyrimidine			104 ± 4	15 ± 15
Ethylmercurithiosalicylic acid	140 ± 27	76 ± 14	0	0
5,5′ Dithiobis (2-nitrobenzoic acid) (DTNB)	22 ± 5	11 ± 11	0	0
3-Carboxypropyl disulfide			81 ± 14	7 ± 7
2,2′-Dithiosalicylic acid			73 ± 19	0
6,6′-Dithiodinicotinic acid	110 ± 15	16 ± 11	4 ± 4	4 ± 4
2,2′-Dithiobis (5-nitropyridine)			95 ± 19	27 ± 27
S-Acetyl thiocholine				159 ± 55

Endosomes were equilibrated in a solution containing (in mM): 100 mannitol, 100 K gluconate, 5 Mg gluconate, 5 HEPES, pH 7.0. Membrane vesicles (50–75 μl) were added to 3 ml of a medium containing (in mM): 100 mannitol, 100 KCl, 5 $MgCl_2$, 5 HEPES, pH 7.0, and 3 μM acridine orange. Other details of the acridine orange assay are described in Materials and Methods. Means ± SE are reported (n = 3–4).

in rabbit renal cortical endosomes (Hilden et al., 1988). Table 1 shows the concentration dependence of NEM inhibition and the effect of other sulfhydryl-containing compounds on this transport process. As shown in Table 1 and Fig. 1, several of these compounds were effective inhibitors. Of all the reagents tested, the diuretic mersalyl, and 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB) were the most effective inhibitors; both compounds inhibited a large fraction of the H^+ transport activity at 10^{-6} M. PCMB, and its less permeable derivative PCMBS, each inhibited the H^+ ATPase completely at 10^{-5} M. Equal effectiveness as an inhibitor for these two compounds with different permeabilities suggests that the relevant sulfhydryl group on the endosomal H^+ ATPase faces the cytoplasmic surface, thus being equally accessible to both reagents. Aldrithiol-2, aldrithiol-4, and 6,6′-dithiodinicotinic acid were also effective inhibitors at 10^{-5} M, whereas glutathione (in the oxidized or reduced form) inhibited only at mM concentrations.

Figure 1 shows the effect on H^+ transport of several inhibitors (mersalyl, aldrithiol, PCMB, PCMBS and glutathione) when each of these compounds was present prior to the addition of ATP. To examine whether these compounds inhibited the H^+ ATPase itself rather than increasing the proton permeability of the membrane vesicles, the effect of these inhibitors on the dissipation of the established pH gradient was tested and compared with the dissipation attained in the absence of residual ATP (addition of hexokinase + glucose). As can be seen in Fig. 1, mersalyl, aldrithiol and PCMBS did not stimulate the dissipation of the pH gradient. By contrast, addition of PCMB did stimulate H^+ loss in renal cortical endosomes. The effect of glutathione was unique, as the initial rate of dissipation was fast, but a short time later, the pH gradient stabilized at a new steady-state level.

Figure 2 shows the concentration dependence of the PCMB effects. As can be seen, the inhibition of the initial rate of H^+ transport was more sensitive to PCMB than the stimulation of the pH-gradient dissipation. In these studies, 71 nmol PCMB/mg protein had a more enhancing effect on the dissipation of the pH gradient. This amount of PCMB is similar to that used by Karniski (1989) in demonstrating activation of a Cl^-/OH^- exchanger in BBMV. If such an exchanger were present in endosomes, stimulation by PCMB would result in movement of Cl^- out of the vesicle (that originally had entered the vesicle because of the electrogenicity of the H^+ pump) and movement of OH^- into the intravesicular compartment, thereby neutralizing the acidic interior.

PCMB-Activated Cl^-/OH^- Exchanger

Figure 3 presents observations consistent with a PCMB-activated Cl^-/OH^- exchange in renal cortical endosomes. When a pH gradient was established

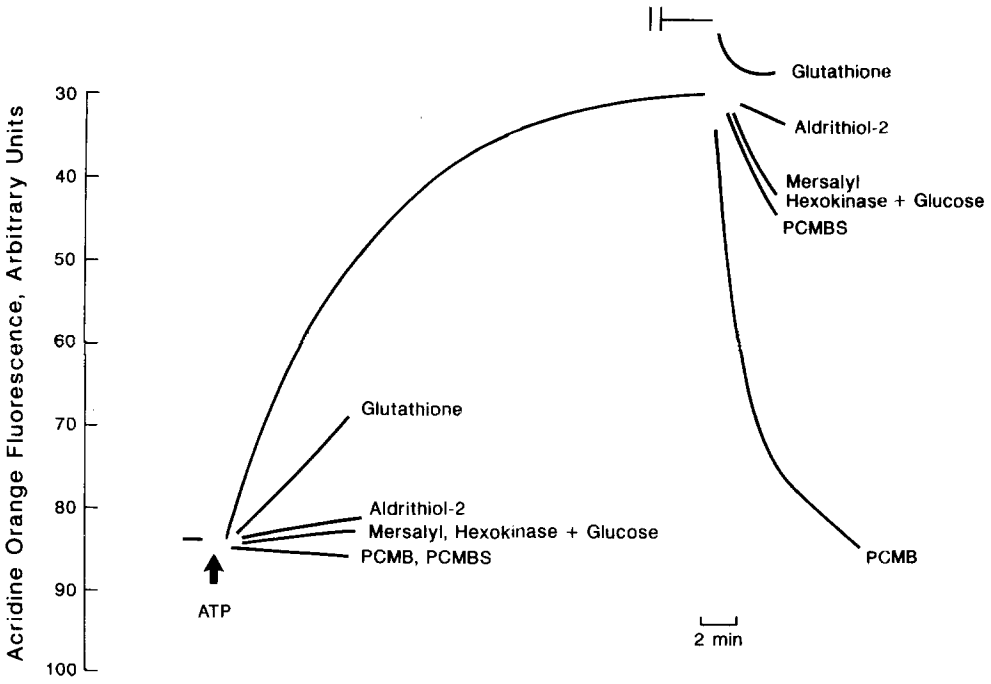

Fig. 1. Effect of sulfhydryl-containing compounds on ATP-driven H^+ transport in renal cortical endosomes. Membrane vesicles were equilibrated in a medium containing (in mM): 100 K gluconate, 100 mannitol, 1 EGTA, 1 Mg gluconate, 5 HEPES, pH 7.6. Vesicles (50 μl) were added to 3 ml of a medium containing (in mM): 100 KCl, 100 mannitol, 1 EGTA, 1 $MgCl_2$, 5 HEPES, pH 7.6, and 3 μM acridine orange. Other details of the acridine orange assay are described in Materials and Methods. Inhibitors were added before the addition of ATP or after the establishment of the maximum pH gradient. The concentration of inhibitor was: glutathione, 1×10^{-3} M; PCMB, PCMBS, mersalyl, 2×10^{-5} M; aldrithiol-4, 1×10^{-5} M. The concentration of PCMB used resulted in 189 nmol PCMB/mg protein. Sufficient hexokinase and glucose were added to hydrolyze all the original ATP. A representative experiment is shown.

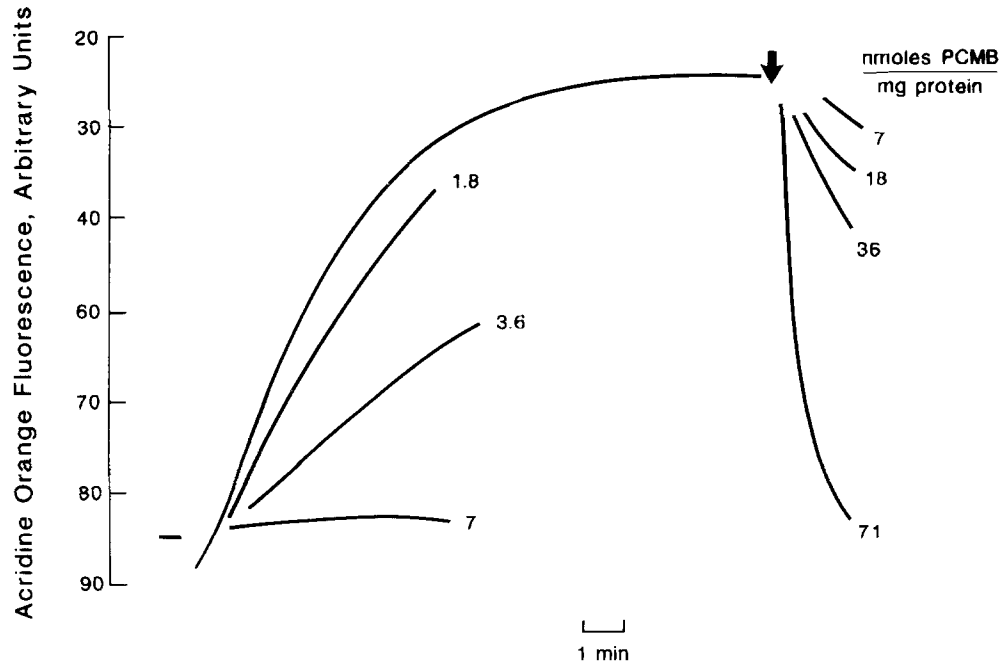

Fig. 2. Concentration dependence of PCMB inhibition of H^+ transport and pH-gradient dissipation in renal cortical endosomes. Experimental details are as described in Fig. 1 and in Materials and Methods. At a PCMB concentration of 10^{-5} M, 36 nmol PCMB/mg protein were present. A representative experiment is shown.

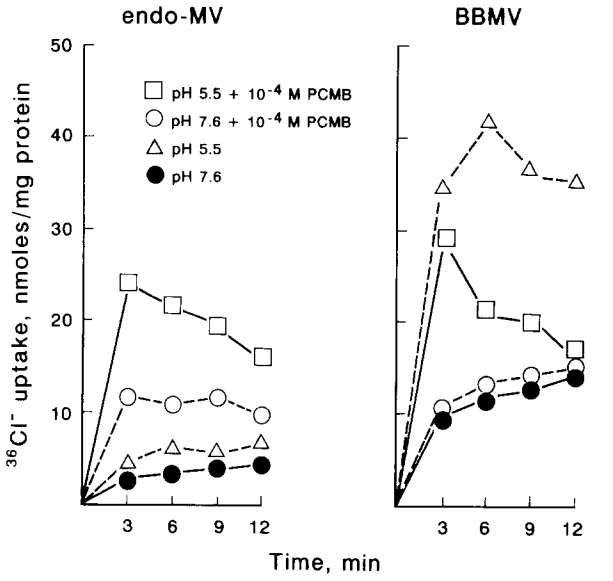

Fig. 3. Effect of PCMB and a pH gradient on $^{36}Cl^-$ uptake in renal cortical membrane vesicles. Endosomes (endo-MV) or BBMV were equilibrated in a medium containing (in mM): 100 K gluconate, 100 mannitol, 1 EGTA, 1 Mg gluconate, 50 HEPES, pH 7.6. Membrane vesicles (10 μl) were added to 100 μl of a medium containing (in mM): 100 K gluconate, 100 mannitol, 1 EGTA, 1 Mg gluconate, 6.1 $^{36}Cl^-$, and 50 MES, pH 5.5, or 50 HEPES, pH 7.6 ± PCMB. Endosomes were exposed to 94 nmol PCMB/mg protein at 10^{-4} M PCMB. At 10^{-4} M PCMB, BBMV were exposed to 75 nmol PCMB/mg protein. Other experimental details are described in Materials and Methods. Representative experiments are shown.

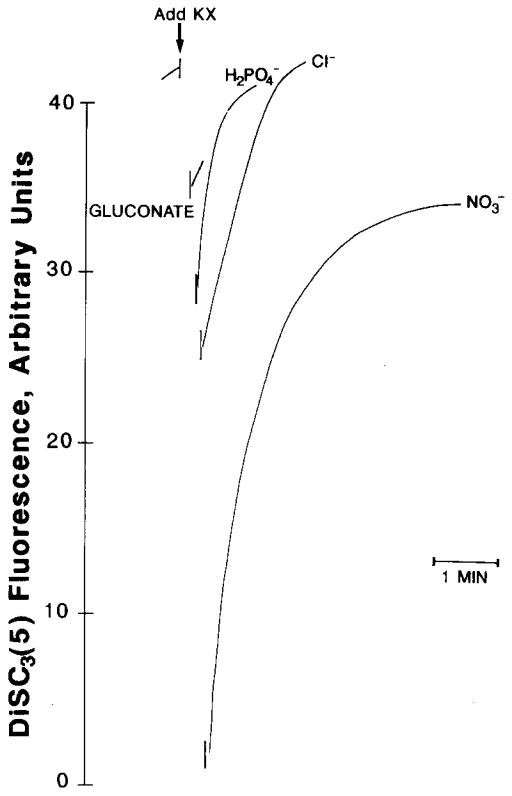

Fig. 4. Changes in fluorescence of potential-sensitive dye, $DiSC_3(5)$ in response to gradients of different anions in BBMV. Vesicles were equilibrated in a 150 mM K^+ gluconate medium as described in Materials and Methods. An aliquot of these membranes was transferred to the same medium containing $DiSC_3(5)$. After stabilization of fluorescent signal, stock salt solutions were added so that the final concentration of KX was 23 mM. Representative experiments are shown.

across the endosomes (pH_{in} 7.6/pH_{out} 5.5) there was little stimulation of $^{36}Cl^-$ uptake. However, in the presence of PCMB, the uptake of $^{36}Cl^-$ was stimulated, both in the presence of a pH gradient (pH_{in} 7.5/pH_{out} 5.5) and in its absence (pH_{in} = pH_{out} = 7.6). No stimulation was seen at a PCMB concentration of 10^{-5} M, but stimulation was observed at a concentration of 10^{-4} M (equivalent to 94 nmol PCMB/mg protein).

The effect of PCMB on $^{36}Cl^-$ uptake by BBMV was different. At concentrations of 10^{-5} or 10^{-4} M, PCMB had no effect on $^{36}Cl^-$ uptake in the absence of a pH gradient. Yet, in the presence of a pH gradient, PCMB stimulated $^{36}Cl^-$ uptake compared with the uptake seen in the absence of a pH gradient. It is notable, however, that the $^{36}Cl^-$ uptake in the presence of only the pH gradient was even higher. Previous work on pH gradient-stimulated $^{36}Cl^-$ uptake in BBMV has emphasized that part of this uptake might be potential stimulated through a Cl^- channel rather than uptake through an electroneutral Cl^-/OH^- exchange (Warnock & Yee, 1981; Liedtke & Hopfer, 1982; Schwartz, 1983; Shiuan & Weinstein, 1984; Seifter, Knickelbein & Aronson, 1984; Ives, Chen & Verkman, 1986; Baum, 1987; Forsyth & Gabriel, 1988). In this regard, Fig. 4 presents an experiment that demonstrates the presence of a Cl^- conductance in BBMV. The magnitude of membrane potentials induced by gradients of different ions can be used to describe the basic membrane permeability to these ions. Waggoner and associates have developed cyanine dyes, which can be used to monitor membrane potentials in vesicular systems that are too small to penetrate with microelectrodes (Sims et al., 1974). One of the dyes, $DiSC_3(5)$, has previously been used to measure potentials in BBMV (Beck & Sacktor, 1978*a,b*). BBMV are not very permeable to K^+ or Na^+, as fluorescence changes seen in the presence of K^+ or Na^+ gradients are equal to those seen when K^+_{in} = K^+_{out} (*data not shown*). In Fig. 4, $DiSC_3(5)$ has been used to describe the magnitude and duration of negative membrane potentials developed in BBMV in response to gradients of different anions. The results suggest NO_3^- >

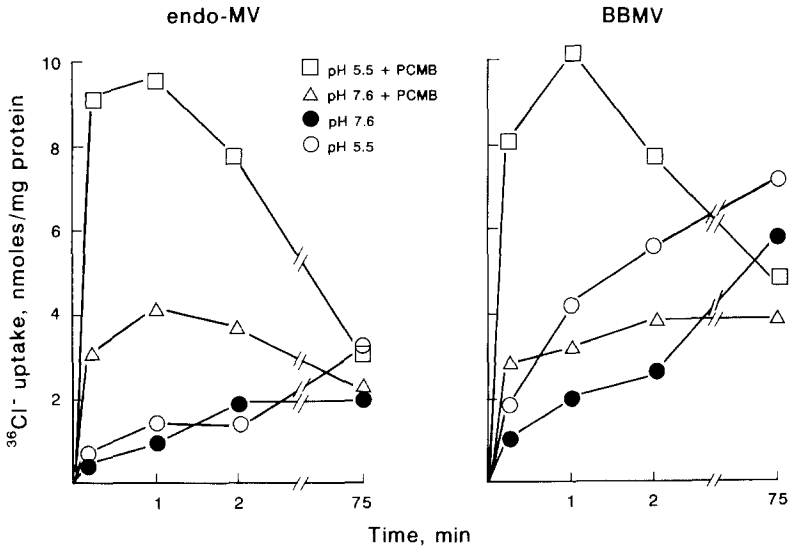

Fig. 5. Effect of PCMB and a pH gradient on $^{36}Cl^-$ uptake in renal cortical endosomes or BBMV in the presence of valinomycin to short circuit the membrane potential. Endosomes (endo-MV) or BBMV were equilibrated in a medium containing (in mM): 100 K gluconate, 100 mannitol, 1 EGTA, 1 Mg gluconate, 50 HEPES, pH 7.6. Membrane vesicles (10 μl) were added to 100 μl of a medium containing (in mM): 100 K gluconate, 100 mannitol, 1 EGTA, 1 Mg gluconate, 2.9 $^{36}Cl^-$, and 50 MES, pH 5.5, or 50 HEPES, pH 7.6 ± 10^{-4} M PCMB in the presence of valinomycin. Other experimental details are described in Materials and Methods. Representative experiments are shown.

$Cl^- > H_2PO_4^- >$ gluconate as the permeability sequence. The permeability to SCN^- and vanadate could not be tested due to an apparent interference with dye fluorescence. Finally, HCO_3^- induced ambiguous changes that could not be interpreted. Attempts have previously been made to demonstrate the presence of a similar anion conductance in our endosomal preparation with negative results (Hilden et al., 1988).

In Fig. 5, valinomycin was added to membrane vesicles featuring equal internal and external concentrations of K^+ ($K^+_{in} = K^+_{out} = 100$ mM), in order to short circuit any membrane potentials developed in the presence of a pH gradient. In endosomes, $^{36}Cl^-$ uptake in the presence or absence of PCMB or a pH gradient was similar to the pattern observed in the absence of valinomycin, i.e., $^{36}Cl^-$ uptake was highest in the presence of both PCMB and a pH gradient (*compare* Fig. 3 with 5). As can be seen in Fig. 5, less stimulation occurred in the presence of added PCMB without a pH gradient, and no stimulation occurred in the presence of just a pH gradient. In the case of short-circuited BBMV, the greatest stimulation also occurred in the presence of both PCMB and a pH gradient, but some stimulation was observed in the presence of either a pH gradient or PCMB alone. Our observation that the pH gradient-stimulated $^{36}Cl^-$ uptake in BBMV was greater in the absence of valinomycin than in its presence (Figs. 3 and 5) taken together with the demonstration of a Cl^- conductance in these vesicles (Fig. 4) agrees with previous suggestions that a major part of this uptake is driven by a membrane potential (Schwartz, 1983; Seifter et al., 1984).

The stimulation of $^{36}Cl^-$ uptake by PCMB was specific, as this compound did not stimulate $^{45}Ca^{2+}$ uptake. As shown in Fig. 6, $^{45}Ca^{2+}$ uptake by endosomes was virtually the same whether PCMB was present or not. Stimulation of $^{45}Ca^{2+}$ uptake by the vesicles was possible, as ATP did stimulate this uptake in the present studies and in previous observations from our laboratory (Fig. 4 of Hilden & Madias, 1989). Like the response in endosomes, PCMB had no effect on $^{45}Ca^{2+}$ uptake by BBMV (Fig. 6).

The effects of PCMB on the pH-gradient dissipation and $^{36}Cl^-$ uptake were not seen in the presence of the thiol-reducing reagent, dithioerithritol (DTE). Figure 7 shows that DTE alone had no effect on the pH-gradient dissipation, but it could prevent the stimulation of H^+ efflux (OH^- influx) induced by PCMB. Similarly, Fig. 8 demonstrates that DTE also prevented the stimulation of $^{36}Cl^-$ uptake induced by PCMB in both endosomes and BBMV. These results suggest that PCMB reacts with a reduced sulfhydryl group of a transporting protein residing in both endosomes and BBMV.

As noted above, PCMBS inhibited ATP-stimulated H^+ transport, but it did not stimulate dissipation of an established pH gradient (Fig. 1). Figure 9 shows that PCMBS did not stimulate $^{36}Cl^-$ uptake by either endosomes or BBMV. Because PCMBS is substantially less permeable than PCMB, these results taken together with the effects of PCMB described above, suggest the presence of two sulfhydryl groups in renal cortical endosomes with different localization and functional properties: one group is important for ATP-stimulated H^+ transport and is located on the cytoplasmic surface of the endosome, and the second group is important for Cl^- (Figs. 3 and 5) and H^+ (OH^-) flux (Fig. 1) and is located on the inside surface of the membrane facing the endosomal interior.

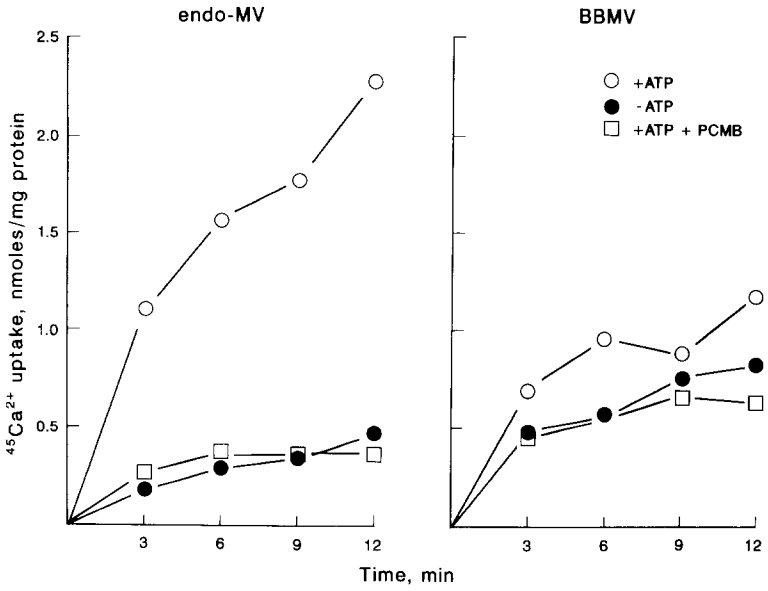

Fig. 6. Effect of PCMB on $^{45}Ca^{2+}$ uptake in renal cortical endosomes or BBMV. Endosomes (endo-MV) or BBMV were equilibrated in a medium containing (in mM): 100 KCl, 100 mannitol, 1 EGTA, 1 Mg gluconate, 50 HEPES, pH 7.0. Membrane vesicles (10 μl) were added to 100 μl of a medium containing (in mM): 100 KCl, 100 mannitol, 1 EGTA, 1 Mg gluconate, 50 HEPES, pH 7.0, and 0.5 $^{45}CaCl_2$. The concentration of ATP was 2.5 mM and that of PCMB 10^{-4} M. Other experimental details are discussed in Materials and Methods. Representative experiments are shown.

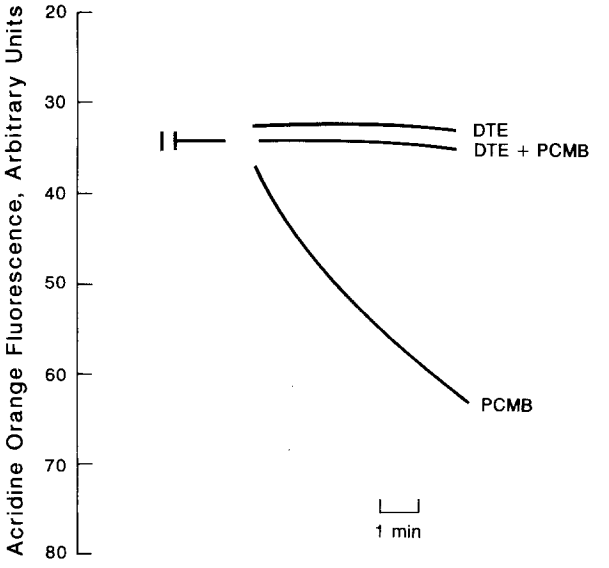

Fig. 7. Effect of dithioerithritol (DTE) on PCMB-induced dissipation of a pH gradient in renal cortical endosomes. A pH gradient was established after ATP addition as described in Fig. 1 and in Materials and Methods. Following stabilization of the pH gradient (as estimated by acridine orange fluorescence), DTE (2×10^{-5} M) was added in the presence or absence of PCMB (10^{-5} M). Other experimental details are described in Materials and Methods. A representative experiment is shown.

A PCMB-activated Cl^-/OH^- exchange has recently been reported in BBMV (Karniski, 1989). Our results are consistent with the presence of a similar PCMB-activated Cl^-/OH^- exchanger in renal cortical endosomes. If so, there should be an effect of PCMB on the ATP-stimulated Cl^- uptake previously observed in these endosomes (Hilden et al., 1988). Indeed, as can be seen in Table 2, addition of PCMB to endosomes incubated in the presence of ATP, returned $^{36}Cl^-$ uptake to the level seen in the absence of ATP.

Discussion

The results of these studies provide evidence in support of the presence of two functionally important sulfhydryl groups in rabbit renal cortical endosomes. One such group is critically involved with ATP-driven H^+ transport in endosomes and confers the NEM-sensitivity characteristic of all vacuolar H^+ ATPases (Al-Awqati, 1986; Stone & Xie, 1988). In addition to NEM, this group can interact with a number of other sulfhydryl-containing compounds, including mersalyl, aldrithiol, DTNB, PCMB, PCMBS and glutathione, thereby resulting in variable degrees of inhibition of ATP-stimulated H^+ transport (Fig. 1 and Table 1). Such inhibition might occur physiologically, for physiological levels of glutathione were inhibitory. This sulfhydryl group must be located on the external surface of the endosomal membrane (i.e., facing the cytoplasm), as PCMB and PCMBS were equally effective inhibitors of H^+ ATPase activity. The second sulfhydryl group relates to the function of an endosomal PCMB-activated Cl^-/OH^- exchanger. Addition of ATP to endosomes stimulates intravesicular H^+ transport via the electrogenic proton pump with subsequent uptake of Cl^- via a Cl^- channel (Hilden et al., 1988). Following the establishment of these pH and Cl^- gradients, exposure to PCMB activates a Cl^-/OH^- exchanger that induces Cl^- efflux (Table 2) in ex-

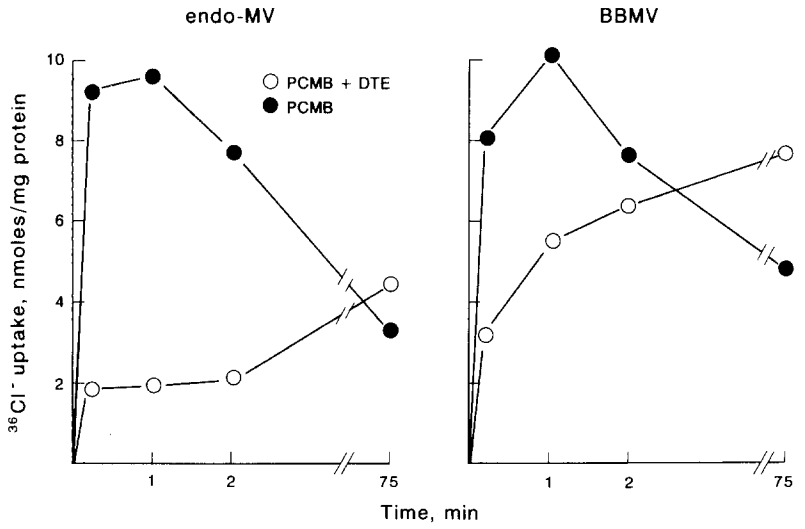

Fig. 8. Effect of dithioerithritol (DTE) on PCMB-stimulated $^{36}Cl^-$ uptake in renal cortical endosomes (endo-MV) or BBMV. Experimental details are reported in Fig. 3 and in Materials and Methods. Membrane vesicles were equilibrated in a medium of pH 7.6 and transferred to a transport medium of pH 5.5. The DTE concentration was 2×10^{-4} M and that of PCMB 10^{-4} M. Representative experiments are shown.

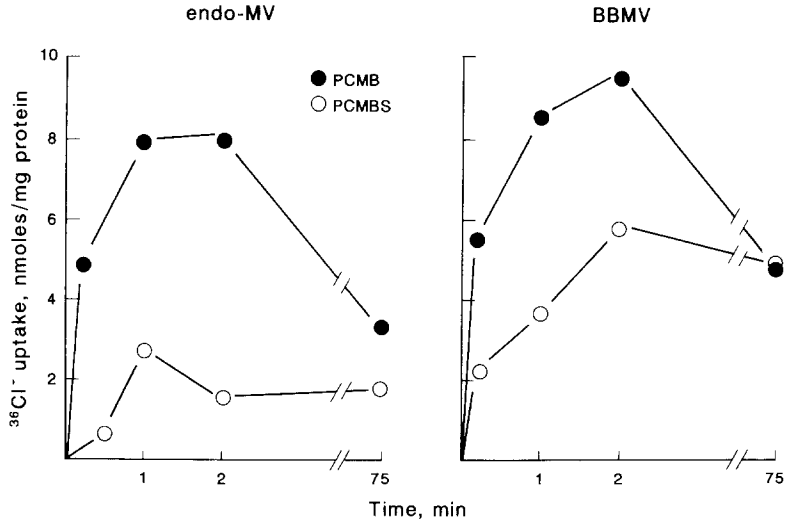

Fig. 9. Effect of PCMB or PCMBS on $^{36}Cl^-$ uptake in renal cortical endosomes (endo-MV) or BBMV. Membrane vesicles were equilibrated in a medium of pH 7.6 and transferred to a transport medium of pH 5.5 containing 10^{-4} M PCMB or PCMBS in the presence of valinomycin. Other experimental details are described in Fig. 5 and in Materials and Methods. Representative experiments are shown.

change for OH^- entry into the endosome (i.e., apparent H^+ loss, Figs. 1 and 2). However, activation of this Cl^-/OH^- exchange by PCMB can occur even in the absence of ATP; indeed, endosomal Cl^- uptake was stimulated by PCMB when no ATP was present, and PCMB stimulation was greater in the presence of an inside-alkaline pH gradient (Figs. 3 and 5), thus supporting the conclusion that this Cl^- flux reflects a Cl^-/OH^- exchanger. The effects of PCMB on the pH-gradient dissipation and $^{36}Cl^-$ uptake vanished in the presence of the thiol-reducing reagent, DTE (Figs. 7 and 8), and they were specific, as no stimulation of $^{45}Ca^{2+}$ uptake was observed (Fig. 6). In contrast to PCMB, activation of Cl^-/OH^- exchange was not effected by mersalyl, aldrithiol or PCMBS. The latter finding indicates that the relevant sulfhydryl group must be located on the internal surface of the endosomal membrane (i.e.,

Table 2. Effect of PCMB on ATP-dependent $^{36}Cl^-$ uptake in renal cortical endosomes

	$^{36}Cl^-$ uptake, % of control
−ATP	100
+ATP	244
+ATP + PCMB	113

Endosomes were equilibrated in a solution containing (in mM): 100 K gluconate, 100 mannitol, 1 EGTA, 1 Mg gluconate, 50 HEPES, pH 7.0. Membrane vesicles (50 μl) were added to 0.5 ml equilibration medium + 5 mM $^{36}Cl^-$ ± 2.5 mM ATP. After incubation for 9 min, $^{36}Cl^-$ uptake was measured as described in Materials and Methods. After 9 min of incubation, PCMB was added to ATP-containing vesicles. Three min after PCMB had been added, $^{36}Cl^-$ uptake was measured. A representative experiment is reported.

facing the endosomal interior) or be embedded in the membrane, thus making itself inaccessible. Because physiological concentrations of glutathione had an initial rapid effect of decreasing the prevailing pH gradient followed by its stabilization at a new steady-state level (Fig. 1), this Cl^-/OH^- exchanger might be activated under physiological conditions to regulate the pH gradient established by endosomes (or a specific fraction of endosomes).

Similarities exist between this endosomal exchanger and the PCMB-activated Cl^-/OH^- recently described in rabbit renal BBMV (Karniski, 1989) and confirmed in the present studies. Both exchangers are stimulated by PCMB in the presence of a pH gradient (Fig. 5), but not by PCMBS (Fig. 9), suggesting that the sulfhydryl group resides on the internal surface of the vesicular membrane. In both cases, the PCMB effects are expressed at the 10–100 nmol PCMB/mg protein concentration range and are prevented by DTE (or dithiothreitol) (Fig. 8). Such similarity should not be taken, however, to indicate contamination of one membrane preparation by the other. At least two sets of observations dispel this concern by identifying properties unique to each membrane preparation. First, in endosomes, PCMB interacts with the pH and Cl^- gradients established by ATP hydrolysis. Thus, as shown in Figs. 1 and 2, the pH gradient established by the H^+ ATPase was reversed by PCMB and, as shown in Table 2, the stimulation of Cl^- uptake by ATP virtually vanished upon addition of PCMB. Since BBMV do not exhibit ATP-induced pH and Cl^- gradients, this PCMB-induced reversal of ionic gradients must be due to endosomes and not BBMV contaminants. Second, BBMV are reported to feature a pH gradient-stimulated Cl^- uptake that, in large part, represents the expression of a Cl^- channel driven by the pH gradient-derived membrane potential (Schwartz, 1983; Seifter et al., 1984; Figs. 3 and 5). In accord with this formulation, $^{36}Cl^-$ uptake by BBMV was substantially reduced when a valinomycin-induced short circuit eliminated any membrane potentials (*compare* Fig. 3 with 5). Indeed, if both a Cl^- channel and a PCMB-activated Cl^-/OH^- exchanger coexist on the same membrane, results like those obtained for BBMV in Fig. 3 are understandable: reduction by PCMB of the pH gradient that drives the membrane potential (by promoting the influx of Cl^- in exchange for OH^-) led to a decrease in Cl^- uptake. Such a reduction of Cl^- uptake by PCMB is not expressed by the endosomal membrane preparation, thus reinforcing the conclusion that BBMV contaminants do not account for the PCMB effects observed in renal endosomes. In fact, there is precedent for similarity, but not identity, in the properties of a transporter residing on both renal endosomes and BBMV. We (Hilden et al., 1990) and others (Gurich & Warnock, 1986; Sabolic & Brown, 1990) have previously described a Na^+/H^+ exchanger in renal endosomes that behaves like the BBMV Na^+/H^+ exchanger except for the fact that it features insensitivity to amiloride. In conclusion, our present results taken together with our previous observations (Hilden et al., 1988) suggest the presence of several different Cl^- transport systems in these two membrane fractions. In endosomes, there exist an ATP-regulated Cl^- channel and a PCMB-activated Cl^-/OH^- exchanger. In BBMV, a Cl^- conductive flux (primarily expressed in the presence of a pH gradient-induced membrane potential) and a PCMB-activated Cl^-/OH^- exchanger are evident.

Given the numerous similarities in the properties of the PCMB-activated Cl^-/OH^- exchanger residing in endosomes and BBMV, one might well anticipate that the endosomal transporter originates from the apical membrane during the process of endocytosis. Yet, the pattern of the PCMBS *vs.* PCMB sensitivity observed in the two membrane preparations does not actually suggest the physical transfer of this transporter during the membrane trafficking between the two domains. As noted, the data suggest that the PCMB-sensitive site in BBMV resides on the internal (or cytoplasmic) surface of the membrane. If indeed this transporter maintained its membrane orientation during endocytosis, the PCMB-sensitive site should remain cytoplasmic, thus residing on the external surface of the endosomal membrane. However, the PCMBS insensitivity observed suggests that the PCMB-sensitive site faces the endosomal interior. These results might indicate that there is no physical transfer of this transporter between the two domains during endocytosis. Alternatively, the PCMB-sensitive site of the Cl^-/OH^- exchanger might be embedded in the membrane; the PCMBS insensitivity thus reflecting the inaccessibility of the PCMB-sensitive site to PCMBS. In the latter case, the exchanger would maintain its membrane orientation, move along with the bilayer during endocytosis and remain inaccessible to PCMBS in both vesicle preparations. Further work will be required to discriminate between these possibilities.

Other sulfhydryl-containing transporters have been identified in the proximal tubule. Sulfhydryl-reactive heavy metals, such as Hg^{2+}, Cu^{2+} and Au^{3+}, cause changes in K^+ and Ca^{2+} fluxes of proximal tubule suspensions (Kone, Brenner & Gullans, 1990). In this system, NEM and PCMBS induce changes in K^+ flux, whereas Ca^{2+} flux remains unchanged. Hg^{2+} reduces *p*-aminohippurate transport by renal basolateral membranes (Ansari, Thakran & Berndt, 1990). Phlorizin binding by BBMV is inhibited by PCMB, but not by PCMBS (Klip et al., 1979).

Studies with DTE have suggested that one disulfide bond is closely associated with the glucose-binding site of the phlorizin-sensitive glucose transporter in renal BBMV (Turner & George, 1984).

Evidence in support of regulation of transporters by interacting with crucial sulfhydryl groups has also been produced in systems other than the kidney. In bovine heart mitochondria, sulfhydryl-containing reagents change the aspartate/glutamate and ADT/ATP carriers from obligate antiporters to uniporters (Durks et al., 1990). Another example relates to the gastric H^+,K^+ ATPase. Resting parietal cells feature intracellular H^+,K^+ ATPase-bearing tubulovesicles. Proton transport is not readily expressed in these tubulovesicles because of the low K^+ and Cl^- conductances. Following stimulation to secrete acid, however, the tubulovesicles migrate to the cell surface, and the K^+ and Cl^- conductances are now open. Of interest, treatment of tubulovesicles from unstimulated parietal cells with the S-S crosslinking reagent, Cu^{2+}-o-phenanthroline, results in the opening of a Cl^- conductance (Takeguchi et al., 1983; Takeguchi & Yamazaki, 1986).

The results of our studies suggest an anion permeability pattern of $NO_3^- > Cl^- > H_2PO_4^- >$ gluconate in BBMV. This pattern compares with that of $SCN^- \geq NO_3^- > Cl^- > CH_3COO^- \geq$ cyclamate obtained by Liedtke and Hopfer (1977, 1982) using H^+ uptake as the detection method. The use of potentials as a means of measuring ion conductances might underestimate anion permeability, because it does not detect electroneutral movements (such as the Cl^-/OH^- exchanger that has been suggested for BBMV). It does, however, predict the relative effectiveness of anion gradients in stimulating electrogenic transporters. In fact, utilizing $DiSC_3(5)$ fluorescence, Beck and Sacktor (1978*b*) found the following effectiveness of anions in stimulating Na^+-glucose transport: $SCN^- \geq NO_3^- > Cl^- > PO_4^{3-}$, methionate, SO_4^{2-}, etc. Our results also suggest that the electrogenic cation permeability is very low. Again, this is an underestimate of total cation permeability, because it does not measure electroneutral movements (such as Na^+/H^+ exchange) or cation movements linked to compounds not included in the assay (e.g., Na^+-glucose cotransport). Nonetheless, these results clearly predict that in the intact kidney cells, Cl^- will be closer to equilibrium with the net electrochemical forces across the brush-border membrane than Na^+ or K^+. This prediction is in fact confirmed by electrophysiological studies in proximal tubule cells of the intact kidney (Edelman et al., 1978; Sohtell, 1978). Of note, a finite Na^+ conductance in BBMV has been shown by other means. Utilizing differences in the rate of potential decay (Warnock & Yee, 1982) and changes in H^+ gradients (Liedtke & Hopfer, 1977), a measurable Na^+ conductance has been suggested. In the presence of a membrane potential, $^{22}Na^+$ uptake was shown to increase (Warnock & Yee, 1982). On the basis of these and previous reports, the following sequence of conductance is suggested for BBMV: $SCN^- \geq NO_3^- > Cl^- > Na^+ > H^+$.

In summary, our results add a new component to the panel of transport systems heretofore identified in renal cortical endosomes. This panel now includes the vacuolar H^+ ATPase, a Cl^- channel, an H^+/Ca^{2+} exchanger, a Na^+/H^+ exchanger and a PCMB-activated Cl^-/OH^- exchanger. Each of these molecules might represent an important substrate for the regulation of endosomal acidification. Indeed, studies have been reported on the regulation of vacuolar H^+ ATPase activity by cAMP (Gurich & DuBose, 1989) and the regulation of Cl^- flux in renal endocytic vesicles by protein kinase A (Bae & Verkman, 1990).

This study was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-38155.

Part of this work has been reported previously in preliminary form (*Biophys. J.* **37**:219*a*, 1982; *Fed. Proc.* **46**:272, 1987; and *Proc. Am. Soc. Nephrol.* **23**:651, 1990).

References

Al-Awqati, Q. 1986. Proton-translocating ATPases. *Annu. Rev. Cell Biol.* **2**:179–199

Ansari, R.A., Thakran, R.S., Berndt, W.O. 1990. The effects of mercuric chloride on transport by brush border and basolateral membrane vesicles isolated from rat kidney. *Toxicol. Appl. Pharmacol.* **106**:145–153

Bae, H.-R., Verkman, A.S. 1990. Protein kinase A regulates chloride conductance in endocytic vesicles from proximal tubule. *Nature* **348**:637–639

Baum, M. 1987. Evidence that parallel Na^+-H^+ and Cl^--$HCO_3(OH^-)$ antiporters transport NaCl in the proximal tubule. *Am. J. Physiol.* **252**:F338–F345

Beck, J.C., Sacktor, B. 1978*a*. Membrane potential-sensitive fluorescence changes during Na^+-dependent D-glucose transport in renal brush border membrane vesicles. *J. Biol. Chem.* **253**:7158–7162

Beck, J.C., Sacktor, B. 1978*b*. The sodium electrochemical potential-mediated uphill transport of D-glucose in renal brush border membrane vesicles. *J. Biol. Chem.* **253**:5531–5535

Durks, T., Salentin, A., Heberger, C., Kramer, R. 1990. The mitochondrial aspartate/glutamate and ADP/ATP carrier switch from obligate counter exchange to unidirectional transport after modification SH-reagents. *Biochim. Biophys. Acta* **1028**:268–280

Edelman, A., Curci, S., Samarzija, I., Fromter, E. 1978. Determination of intracellular K^+ activity in rat kidney proximal tubular cells. *Pfluegers Arch.* **378**:37–45

Forsyth, G.W., Gabriel, S.E. 1988. Chloride ion transport into pig jejunal brush-border membrane vesicles. *J. Physiol.* **402**:555–564

Gurich, R.W., DuBose, T.D., Jr. 1989. Heterogeneity of cAMP effect on endosomal proton transport. *Am. J. Physiol.* **257:**F777–F784

Gurich, R.W., Warnock, D.G. 1986. Electrically neutral Na^+-H^+ exchange in endosomes obtained from rabbit renal cortex. *Am. J. Physiol.* **251:**F702–F709

Hilden, S.A., Ghoshroy, K.B., Madias, N.E. 1990. Na^+-H^+ exchange, but not Na^+-K^+-ATPase, is present in endosome-enriched microsomes from rabbit renal cortex. *Am. J. Physiol.* **258:**F1311–F1319

Hilden, S.A., Johns, C.A., Madias, N.E. 1988. Cl^--dependent ATP-driven H^+ transport in rabbit renal cortical endosomes. *Am. J. Physiol.* **255:**F885–F897

Hilden, S.A., Madias, N.E. 1989. H^+/Ca^{2+} exchange in rabbit renal cortical endosomes. *J. Membrane Biol.* **112:**131–138

Ives, H.E., Chen, P.Y., Verkman, A.S. 1986. Mechanism of coupling between Cl^- and OH^- transport in renal brush-border membranes. *Biochim. Biophys. Acta* **863:**91–100

Karniski, L.P. 1989. Activation of Cl^-/OH^- exchange by para-chloromercuribenzoic acid in rabbit renal brush-border membranes. *J. Membrane Biol.* **112:**59–66

Klip, A., Grinstein, S., Semenza, G. 1979. Distribution of sulfhydryl groups in intestinal brush border membranes. *Biochim. Biophys. Acta* **558:**233–245

Kone, B.C., Brenner, R.M., Gullans, S.R. 1990. Sulfhydryl-reactive heavy metals increase cell membrane K^+ and Ca^{2+} transport in renal proximal tubule. *J. Membrane Biol.* **113:**1–12

Liedtke, C.M., Hopfer, U. 1977. Anion transport in brush border membranes isolated from rat small intestine. *Biochem. Biophys. Res. Commun.* **76:**579–585

Liedtke, C.M., Hopfer, U. 1982. Mechanism of Cl^- translocation across small intestinal brush-border membrane. II. Demonstration of Cl^--OH^- exchange and Cl^- conductance. *Am. J. Physiol.* **242:**G272–G280

Sabolic, I., Brown, D. 1990. Na^+ (Li^+)-H^+ exchange in rat renal cortical vesicles with endosomal characteristics. *Am. J. Physiol.* **258:**F1245–F1253

Schwartz, G.J. 1983. Absence of Cl^--OH^- or Cl^--HCO_3^- exchange in the rabbit renal proximal tubule. *Am. J. Physiol.* **245:**F462–F469

Seifter, J.L., Knickelbein, R., Aronson, P.S. 1984. Absence of Cl-OH exchange and NaCl cotransport in rabbit renal microvillus membrane vesicles. *Am. J. Physiol.* **247:**F753–F759

Shiuan, D., Weinstein, S.W. 1984. Evidence for electroneutral chloride transport in rabbit renal cortical brush border membrane vesicles. *Am. J. Physiol.* **247:**F837–F847

Sims, P.J., Waggoner, A.S., Wang, C.H., Hoffman, J.F. 1974. Studies on the mechanisms by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. *Biochemistry* **13:**3315–3330

Sohtell, M. 1978. Electrochemical forces for chloride transport in the proximal tubules of the rat kidney. *Acta Physiol. Scand.* **103:**363–369

Stone, D.K., Xie, X.-S. 1988. Proton translocating ATPases: Issues in structure and function. *Kidney Int.* **33:**767–774

Takeguchi, N., Joshima, R., Inoue, Y., Kashiwagura, T., Morii, M. 1983. Effects of Cu^{2+}-o-phenantholine on gastric (H^+-K^+)-ATPase. *J. Biol. Chem.* **258:**3094–3098

Takeguchi, N., Yamazaki, Y. 1986. Disulfide cross-linking of H^+, K^+-ATPase opens Cl^- conductance, triggering proton uptake in gastric vesicles. *J. Biol. Chem.* **261:**2560–2566

Turner, R.J., George, J.M. 1984. Characterization of an essential disulfide bond associated with the active site of the renal brush-border membrane D-glucose transporter. *Biochim. Biophys. Acta* **769:**23–32

Warnock, D.G., Yee, V.J. 1981. Chloride uptake by brush border membrane vesicles isolated from rabbit renal cortex. *J. Clin. Invest.* **67:**103–115

Warnock, D.G., Yee, V.J. 1982. Sodium uptake mechanisms in brush-border membrane vesicles prepared from rabbit renal cortex. *Biochim. Biophys. Acta* **684:**137–140

Received 4 March 1991; revised 11 June 1991