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Characterization of Chloride Channels in Membrane Vesicles from the Kidney Outer Medulla

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Summary. The basolateral membrane of the thick ascending loop of Henle (TALH) of the mammalian kidney is highly enriched in Na^+/K^+ ATPase and has been shown by electrophysiological methods to be highly conductive to Cl^-. In order to study the Cl^- conductive pathways, membrane vesicles were isolated from the TALH-containing region of the porcine kidney, the red outer medulla, and Cl^- channel activity was determined by a ^{36}Cl uptake assay where the uptake of the radioactive tracer is driven by the membrane potential (positive inside) generated by an outward Cl^- gradient. The accumulation of $^{36}Cl^-$ inside the vesicles was found to be dependent on the intravesicular Cl^- concentration and was abolished by clamping the membrane potential with valinomycin. The latter finding indicated the involvement of conductive pathways. Cl^- channel activity was also observed using a fluorescent potential-sensitive carbocyanine dye, which detected a diffusion potential induced by an imposed inward Cl^- gradient. The anion selectivity of the channels was $Cl^- > NO_3^- = I^- \gg$ gluconate. Among the Cl^- transport inhibitors tested, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPAB), 4,4′-diisothiocyano-stilbene-2,2′-disulfonate (DIDS), and diphenylamine-2-carboxylate (DPC) showed IC_{50} of 110, 200 and 550 μM, respectively. Inhibition of ^{36}Cl uptake by NPPAB and two other structural analogues was fully reversible, whereas that by DIDS was not. The nonreactive analogue of DIDS, 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), was considerably less inhibitory than DIDS (25% inhibition at 200 μM). The irreversible inhibition by DIDS was prevented by NPPAB, whereas DPC was ineffective, consistent with its low inhibitory potency. It is proposed that NPPAB and DIDS bind to the same or functionally related site on the Cl^- channel protein.

Key Words TALH · basolateral vesicles · Cl^- transport · DIDS

Introduction

The current model for transcellular NaCl reabsorption in the TALH invokes the concerted operation of a furosemide-sensitive $Na^+/K^+/2Cl^-$ cotransporter with a Ba^{2+} sensitive K^+ channel in the luminal membrane, and the Na^+/K^+ ATPase together with a KCl cotransporter and a Cl^- channel in the basolateral membrane (Greger & Schlatter, 1983*a*,*b*; Burnham, Karlish & Jorgensen 1985; Greger, 1985). Thus Cl^- is transported into the cell from the lumen by an electroneutral cotransport process, driven primarily by the inward Na^+ gradient maintained by the Na^+/K^+ ATPase, and it moves out of the cell down its electrochemical gradient via the basolateral Cl^- channel or the putative KCl cotransporter. In the mouse TALH, the Cl^- channels have been found to be activated by elevation of cytosolic cAMP leading to the suggestion that this might represent a primary site of regulation of NaCl absorption (Schlatter & Greger, 1985). This model is analogous to that proposed for NaCl secretion by secretory epithelia such as the trachea, elasmobranch rectal gland, colon, lacrimal glands, frog cornea, etc., where the primary event is thought to involve activation of apical Cl^- channels mediated by cAMP (Mandel, Dharmathaphorn & McRoberts, 1986; Yanase & Handler, 1986; Frizzell, 1987) or possibly by Ca^{2+} (Frizzell, Rechkemmer & Shoemaker, 1986). Unlike the Cl^- channels of the TALH, those of the secretory epithelia have been studied extensively by single-channel techniques. They are characterized by (i) single-channel conductances of 20–60 pS, (ii) selectivity for Cl^- over Na^+ of about 10 : 1, and (iii) voltage dependence, i.e., activation by depolarizing voltages (Frizzell, 1987). Large conductance apical Cl^- channels of 350 to 450 pS have been found in cultured cells (the A6 and MDCK lines) derived from mammalian and amphibian kidneys (Nelson, Tang & Palmer, 1984; Kolb, Brown & Murer, 1985). The Cl^- channels of salt-secreting and salt-absorbing epithelia may be related, although the extent of this relationship is not known.

Here we present evidence that membrane vesicles derived from the red outer medulla of porcine kidney, which is enriched in TALH, contain Cl^- channels. This was demonstrated by two separate assays, one based on membrane potential-driven

uptake of $^{36}Cl^-$ against its chemical gradient, the other on determination of transmembrane potential with a fluorescent potential-sensitive dye. The channels show anion selectivity and are sensitive to inhibition by reversible blockers structurally related to DPC, as well as by the irreversible inhibitor DIDS. The two types of inhibitors might bind at the same or adjacent sites on the channel.

Materials and Methods

Preparation of Membrane Vesicles from the Red Outer Medulla of Porcine Kidney

The red outer medullary region was dissected from fresh pig kidneys and a microsomal fraction was prepared as described by Jorgensen (1974). Preparations were made in bulk from approximately 20 kidneys and were kept frozen at −70°C. Membrane vesicles were prepared by thawing frozen red outer medullas in the solution of choice (10 ml/g tissue) followed by homogenization (7 strokes) in a 60-ml Teflon-glass motor-driven homogenizer at 4°C. The homogenate was centrifuged at 7,000 rpm (6,000 × *g*) for 15 min at 5°C, the pellet-containing tissue and cell debris was discarded, and crude membrane vesicles were collected from the supernatant by centrifugation at 18,000 rpm (39,000 × *g*) for 25 min at 5°C. Sealed membrane vesicles (15 mg protein/ml) were enriched by layering 200 μl of the crude vesicles on top of 300 μl of 15% metrizamide (Sigma Chemical Co., St. Louis, MO) dissolved in the appropriate homogenization buffer, and centrifugation for 20 min at maximal speed (13,000 × *g*) in a cooled microcentrifuge (Hettich). The sealed vesicles were recovered from the floating layer on top of the metrizamide cushion (the leaky vesicles settle to the bottom). Recovery was approximately 6 mg crude vesicle protein and 2 to 3 mg sealed vesicle protein per gram wet tissue as determined by the method of Bradford (1976).

Preparation of Anion Exchange Dowex-1 Columns

Dowex-1 × 2 resin, 50–100 mesh, Cl^- form (Fluka, CH), was washed in distilled water, the slurry was titrated to pH 12.5 with NaOH and then washed extensively with distilled H_2O until the pH of the eluate was 6.0. The slurry was then titrated with 50% gluconic acid (from Merck, Munchen, FRG, decolorized with charcoal) to pH 2.8 and left overnight. Excess gluconic acid was then washed out with H_2O until the pH of the eluate was stable at 4.5 to 5. For use in $^{36}Cl^-$ fluxes, approx. 1 ml of resin was packed into pasteur pipettes plugged with Dacron wool. These columns retained 1 ml of 100 mM Cl^- with virtually 100% efficiency as determined with $^{36}Cl^-$ as tracer. Prior to use, the columns were washed with 1 ml of 350 mM sucrose containing 25 mg/ml bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO) to prevent vesicle absorption, followed by 2 ml of 350 mM sucrose.

$^{36}Cl^-$ Uptake Assay

The assay was initiated by loading the membrane vesicles (up to 0.35 ml) onto a chilled Dowex-1 column followed by stepwise elution with 350 mM sucrose. The vesicles were recovered between 0.5 to 1.0 ml elution volume. This step served to replace all extravesicular Cl^- by gluconate. Isotope uptake was initiated by the addition of an aliquot of the vesicles (usually 100 μl or multiples thereof) to an equal volume of 350 mM sucrose, 20 mM MOPS-Tris, at pH 7.3, containing 2.0 mM $Na^{36}Cl$ (Amersham, UK, 0.64 mCi/mmol). At the times indicated, samples of 200 μl of the reaction mixture were loaded on chilled Dowex-1 columns and the vesicles were eluted immediately into scintillation vials with 1 ml of ice-cold 350 mM sucrose. The eluted $^{36}Cl^-$ representing vesicle-trapped isotope was determined by scintillation counting in 10 ml Lumax scintillation fluid (Lumac, Londgraaf, Netherlands). Routinely about 150 μg crude vesicle or 50 μg sealed vesicle protein was used per time point. All experiments were performed at least three times separately.

Solutions and Chemicals

Membrane vesicles were routinely prepared in homogenization solution containing in mM: 100 KCl, 150 sucrose, 2 $MgCl_2$, 1 EGTA and 10 MOPS (Sigma Chemical Co., St. Louis, MO), titrated to pH 7.3 with Trizma base. Where specified, KCl was replaced with 100 mM chloride salt of N-methyl-D-glucamine (NMG.Cl) or 100 mM K-gluconate, (both from Sigma Chemical Co., St. Louis, MO). Bumetanide (20 mM in dimethylsulfoxide (DMSO)) was from Hoffmann-La Roche (Nutley, NJ) and 3,3′-diprophythiodicarbocyanine iodide (di-$SC_3(5)$ from Molecular Probes (Eugene, OR). DIDS and DNDS (20 mM in 10 mM Na-phosphate at pH 7.4) were kindly provided by Dr. Z.I. Cabantchik (Hebrew University of Jerusalem, Israel). DPC, NPPAB, 5-nitro-2-(4-phenylbutylamino)-benzoic acid (NPBAB) and 2-(3-phenylpropylamino)-benzoic acid (PPAB) (20 mM in DMSO) were generous gifts from Dr. R. Greger (Albert-Ludwigs Universitat, Freiburg, FRG). Valinomycin (0.5 mM in 95% ethanol) was from Sigma Chemical Co., St. Louis, MO.

Determination of Membrane Vesicle Potential with $diSC_3(5)$

The membrane vesicles used in these experiments were prepared in homogenization buffer containing 100 mM K-gluconate as the major salt. Thirty μl of vesicle suspension was added to a stirred disposable cuvette (Sarstedt, FRG) containing 1.5 ml of the appropriate buffer at room temperature, followed by 3 μl of 1 mM $diSC_3(5)$ dissolved in DMSO to give a final concentration of 2 μM. Fluorescence was recorded continuously at excitation 620 nm, emission 670 nm (with 5- and 15-nm slits, respectively) in a Perkin-Elmer MPF-44A fluorescence spectrophotometer.

Results

In order to study the conductive Cl^- pathways in membrane vesicles, an adaptation of the potential-

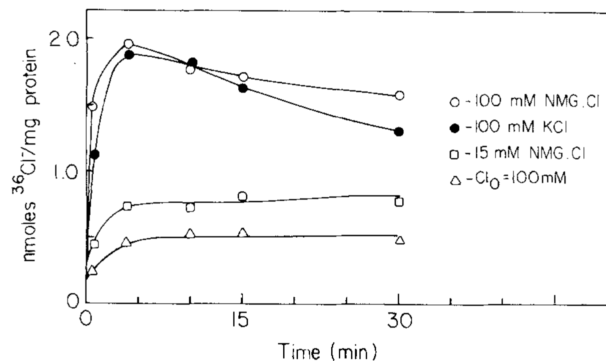

Fig. 1. Dependence of $^{36}Cl^-$ uptake on intravesicular Cl^- and cations. Crude membrane vesicles were prepared from frozen porcine kidney outer medullas in media containing in mM: 100 NMG.Cl, 150 sucrose, 2 $MgCl_2$, 1 EGTA and 10 MOPS-Tris at pH 7.3 (○); 100 KCl, 150 sucrose, 2 $MgCl_2$, 1 EGTA and 10 MOPS-Tris at pH 7.3 (●); 15 NMG.Cl, 320 sucrose, 2 $MgCl_2$, 1 EGTA and 10 MOPS-Tris at pH 7.3 (□). $^{36}Cl^-$ uptake was determined as described in Materials and Methods. Total extravesicular Cl^- concentration was 2.0 mM, except where 100 mM NMG.Cl was added (△). Uptake is expressed as nmol $^{36}Cl^-$ taken up per mg vesicle protein

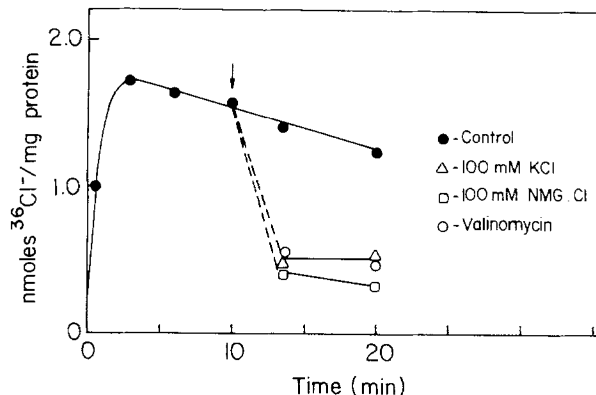

Fig. 2. Effect of valinomycin and external Cl^- on retention of $^{36}Cl^-$ accumulated inside vesicles. Crude membrane vesicles were prepared in medium containing 100 mM KCl as the major salt, and time-dependent $^{36}Cl^-$ uptake was determined. After 10 min, the vesicles were divided into four equal portions as indicated by the arrow. The following additions were made: 100 mM KCl (△), 100 mM NMG.Cl (□), 2.5 μM valinomycin (○), or no addition (●), and the determination of intravesicular $^{36}Cl^-$ content was continued. Addition of ethanol, the vehicle solvent of valinomycin, had no effect

driven isotope uptake method developed by Garty, Rudy & Karlish (1983) was used. This method has been recently applied to Cl^- channels in vesicles from bovine kidney cortex and trachea (Landry et al., 1987). It is generally applicable to ion channels in membrane vesicles and has been used for Na^+ channels from toad bladder (Garty, Asher & Yeger, 1987) and bovine kidney (Sariban-Sohraby & Benos, 1986) and K^+ channels in the TALH (Burnham et al., 1985; Klaerke, Karlish & Jorgensen, 1987). The assay makes use of the principle that the generation of an outward concentration gradient for Cl^- will produce an inward positive potential in those vesicles that contain Cl^- channels. This potential will drive the uptake of trace amounts of $^{36}Cl^-$ against the Cl^- chemical gradient. The amount of $^{36}Cl^-$ taken up should depend on the magnitude of the outward Cl^- gradient. This is shown in Fig. 1. Vesicles containing 100 mM Cl^- showed an initial uptake rate of 1.64 nmol $^{36}Cl^-$/mg protein/min compared to 0.52 nmol/protein/min in vesicles containing 15 mM Cl^-. The calculated Nernst potentials generated by the outward Cl^- gradient (assuming that Cl^- conductance predominates) would be +100 mV in the former and +52 mV in the latter. With a 50% reduction in driving force, the $^{36}Cl^-$ uptake would be expected to be reduced by half rather than by 68% as found. However, if the channels are voltage sensitive then a decrease in the membrane potential might have an additional inhibitory effect on the isotope accumulation. The isotope uptake was independent of the internal cation as it was similar in KCl- or NMG.Cl-loaded vesicles. This indicates that the permeability of the vesicles to Cl^- significantly exceeds that to K^+, which is consistent with electrophysiological observations of the basolateral membrane of TALH cells (Greger & Schlatter, 1983*a*). Excess cold Cl^- (100 mM) strongly inhibited $^{36}Cl^-$ uptake. Since 100 mM gluconate had little effect (*see* Fig. 5*A*) the inhibition by Cl^- must have been due to isotope dilution, as well as collapse of the Cl^- potential.

If the uptake and retention of $^{36}Cl^-$ is maintained by an inside positive potential, then dissipation of this potential should result in efflux of the isotope from the vesicles. As shown in Fig. 2, the K^+ ionophore valinomycin in the presence of an outward K^+ gradient (K_i = 100 mM, K_o contributed by vesicle buffer = 10 mM) created an opposing potential to that generated by the outward Cl^- gradient, producing a rapid loss of accumulated $^{36}Cl^-$ from the vesicles. Addition of 100 mM Cl^-, which brings the Cl^- potential to zero, produced a similar effect. These results indicate that the uptake and retention of $^{36}Cl^-$ in the vesicles involves a conductive rather than an electroneutral pathway.

In order to confirm the presence of Cl^- channel activity in the vesicles, changes in membrane po-

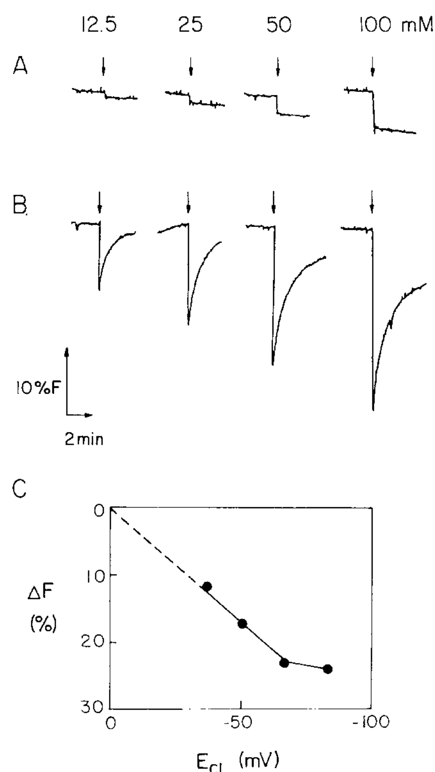

Fig. 3. Effect of Cl^- on the membrane potential of the vesicles as determined by $diSC_3(5)$. A 30-μl aliquot of sealed membrane vesicles was prepared in 100 mM K-gluconate, 150 mM sucrose, 2 mM $MgCl_2$ and 1 mM EGTA. Ten mM MOPS-Tris at pH 7.3 was added to a stirred cuvette containing the same medium at room temperature, followed by 2 μM $diSC_3(5)$. When the fluorescence signal became stable, indicating equilibration of the dye, the fluorescence was adjusted to 70% (0 to 100% scale). Where indicated by arrow, salt solution from 1 M stock was added to the cuvette to give final concentrations of 12.5, 25, 50 and 100 mM. The salts added were Na-gluconate (*A*) and NaCl (*B*). Scale indicates 10% fluorescence. (*C*) Plot of change in fluorescence reading (ΔF) against the calculated Cl^- equilibrium potential (E_{Cl}) in mV, obtained from the traces in (*B*). The values of ΔF were corrected for dilution of cuvette contents by the added salt solution

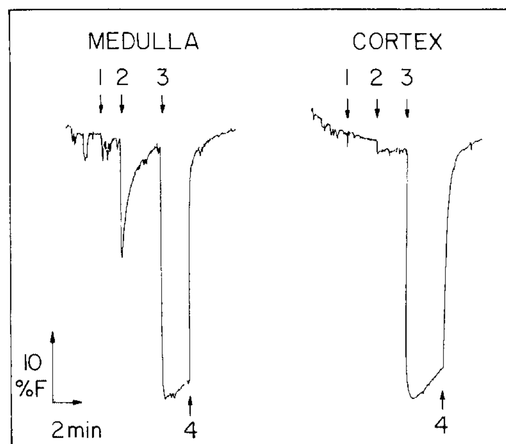

Fig. 4. Effect of Cl^- and valinomycin on the membrane potential of vesicles from the porcine kidney medulla and cortex. Sealed membrane vesicles from the porcine kidney cortex were prepared in the same manner as described for sealed medullary vesicles. A 30-μl aliquot of vesicles prepared in 100 mM K-gluconate, 150 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM MOPS-Tris at pH 7.3 was added to a stirred cuvette at room temperature containing 1.5 ml of 100 mM Na-gluconate, 150 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM MOPS-Tris at pH 7.3, followed by the addition of 2 μM $diSC_3(5)$. The dye was allowed to equilibrate and the fluorescence signal was adjusted to 70%. The sequential additions are indicated by the arrows: *1*: 30 μl of 2-M Na-gluconate, 40 mM final; *2*: 30 μl of 2 M Na-Cl, 40 mM final; *3*: 3 μl of 0.5 mM valinomycin, 1 μM final; *4*: 15 μl of 1 M K-gluconate, 10 mM final. The trace on the left was obtained with sealed vesicles from the red outer medulla, 80 μg protein, and on the right with sealed vesicles from the cortex, 120 μg protein

tential were measured directly using the potential-sensitive carbocyanine dye $diSC_3(5)$ (Fig. 3). This positively charged dye distributes across membranes according to the membrane potential and undergoes quenching when accumulated inside vesicles. Thus an inward Cl^- gradient should result in the induction of a diffusion potential negative inside detectable as a decrease in dye fluorescence. The traces in Fig. 3*A* and *B* show that the addition of Cl^-, but not gluconate, to low chloride vesicles (Cl_i = Cl_o = 4 mM; K-gluconate conc. inside = outside; zero membrane potential) produced a marked transient signal. This effect was dependent on the Cl^- concentration. Identical responses were obtained using NMG.Cl instead of NaCl (*not shown*). The change in fluorescence due to Na-gluconate addition was the result of dilution of the cuvette contents, as a similar response was obtained in the absence of vesicles. Similarly, the addition of NaCl in the absence of vesicles had the same result as Na-gluconate. The calculated Cl^- equilibrium potential (E_{Cl}) at external Cl^- concentrations of 16.5, 29, 54 and 104 mM was −37, −51, −67 and −83 mV, respectively (Cl_i^- = 4 mM). The plot of maximal change in dye fluorescence versus calculated Cl^- equilibrium potential intersects at 0 and is linear to about −70 mV (Fig. 3*C*), indicating that in this range the dye reports the Cl^- potential.

As shown in Fig. 4, addition of 40 mM Cl^- induced a hyperpolarization also in the presence of an outward K^+ gradient (100 mM K-gluconate inside; 100 mM Na-gluconate and 2 mM K-gluconate outside; Cl_i = Cl_o = 4 mM). In comparison, little Cl^--induced hyperpolarization was detectable in membrane vesicles derived from pig kidney cortex. Valinomycin hyperpolarized both types of membrane vesicles. The calculated K^+ equilibrium potential after addition of valinomycin was −100 mV. The ensuing addition of 10 mM K^+ produced a depo-

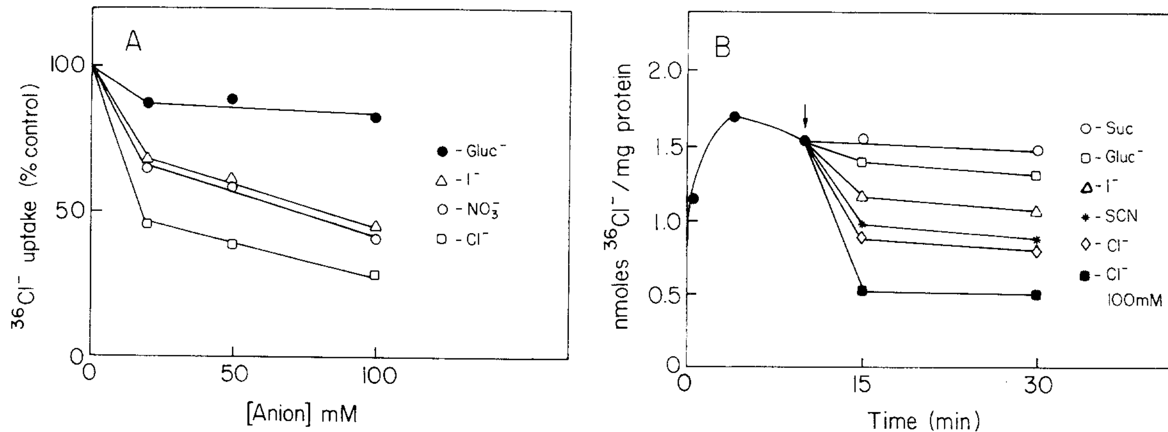

Fig. 5. Effect of various anions on $^{36}Cl^-$ uptake and retention by the vesicles. Crude medullary vesicles were prepared in medium containing 100 mM KCl as the major salt. (*A*): uptake of $^{36}Cl^-$ was allowed to proceed for 1 min in the presence of increasing concentrations of salts in the uptake medium. The salts added were: Na-gluconate (●), NaI (△), $NaNO_3$ (○) and NaCl (□). Results of $^{36}Cl^-$ uptake are expressed as a percentage of control with no additions. (*B*) Uptake of $^{36}Cl^-$ was followed with time for 10 min, then the vesicles were divided into six equal portions and the following additions were made, in mM, as indicated by the arrow: 40 sucrose (○), 20 Na-gluconate (□), 20 NaI (△), 20 NaSCN (*), 20 NaCl (◇), 100 NaCl (■). The determination of intravesicular $^{36}Cl^-$ content was continued for 10 more min. Results are expressed as nmol $^{36}Cl^-$ taken up per mg vesicle protein

larization as expected in vesicles with high K^+ conductivity due to the ionophore. The interpretation and quantitation of these measurements requires caution because of the heterogeneity of the vesicle populations used. Thus if only a minor subpopulation of vesicles contains the channels of interest, it might not be detectable by this assay. Conversely, a substantial response such as that of the medullary vesicles to Cl^- does not necessarily indicate, although it strongly suggests, that a major population of the vesicles contains the channels. A further limitation of the dye technique is the susceptibility of the fluorescent signal to interference by a variety of organic compounds. For this reason, it was not possible to assess the effects of inhibitors using this assay.

The specificity of the $^{36}Cl^-$ uptake by the medullary vesicles was assessed by the relative ability of I^-, NO_3^-, Cl^- and gluconate to inhibit it (Fig. 5*A*). Their respective IC_{50} for inhibition were 83, 75, 18 and ≫100 mM, giving an order of selectivity of $Cl^- > NO_3^- \gtrsim I^- \gg$ gluconate. In addition, the ability of various anions to induce the efflux of accumulated $^{36}Cl^-$ from the vesicles was tested (Fig. 5*B*). At 20 mM concentrations, gluconate, I^-, thiocyanate (SCN^-) and Cl^- released 12, 29, 41 and 45% of the accumulated isotope giving a selectivity sequence of $Cl^- > I^- \gg$ gluconate. Since SCN^- can dissipate the membrane potential by permeating through the lipid, the selectivity for this ion cannot be assessed. Nevertheless, the data are indicative of anion discrimination by the channels.

Several Cl^- transport inhibitors were tested for their effects on initial $^{36}Cl^-$ uptake (Fig. 6). The concentrations of DIDS, NPPAB, and NPBAB required for 50% inhibition (IC_{50}) were 200, 115 and 80 mM, respectively. Bumetanide, DPC and PPAB inhibited uptake by 22, 23 and 37%, respectively, at 200 μM. In a separate experiment, the IC_{50} for DPC was found to be 550 μM. The nonreactive analogue of DIDS, DNDS, was 25% inhibitory at 200 μM. Furosemide was ineffective up to 500 μM (*data not shown*). The relative potency of the inhibitors was

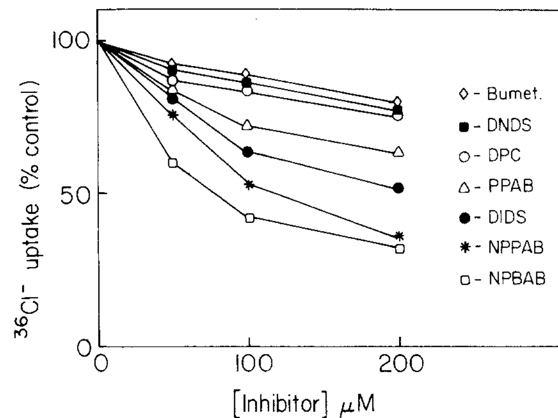

Fig. 6. Effect of inhibitors on $^{36}Cl^-$ uptake by the vesicles. Sealed medullary membrane vesicles were prepared in medium containing 100 mM KCl as the major salt. Uptake of $^{36}Cl^-$ was allowed to proceed for 1 min in the presence of increasing concentrations of inhibitors in the uptake medium. The inhibitors were: bumetanide (◇), DPC (○), PPAB (△), DNDS (■), DIDS (●), NPPAB (*), NPBAB (□). Results of $^{36}Cl^-$ uptake are expressed as a percentage of control with no additions

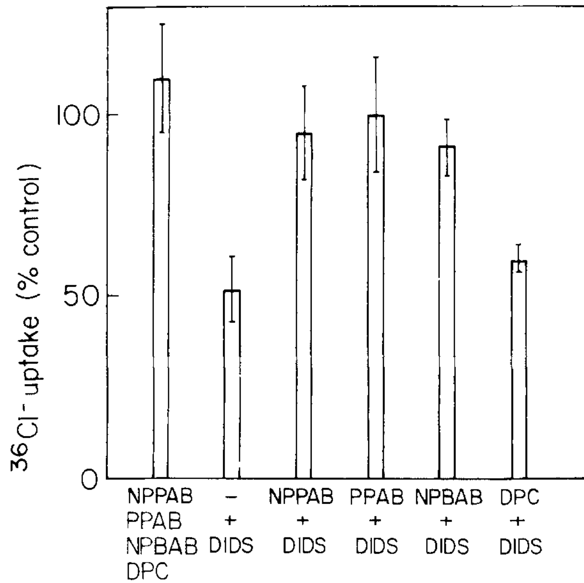

Fig. 7. Reversibility of inhibitor action. Sealed medullary vesicles (1 mg protein/ml) in pH 7.3 medium containing 100 mM KCl as the major salt were incubated with the various inhibitors at room temperature for 15 min. The addition of DIDS was made 1 min after each competing inhibitor. Then the vesicles were passed through an anion exchange minicolumn (Dowex-1, gluconate form), which removed the inhibitors as well as the extravesicular Cl^-, and $^{36}Cl^-$ uptake over 1 min was determined. Each inhibitor or combination of inhibitors was tested individually. The concentration of DPC, PPAB, NPPAB and NPBAB was 250 μM and of DIDS was 200 μM in each case. Error bars indicate SD, n = 2 to 6

NPBAB > NPPAB > DIDS > PPAB > DPC = bumetanide = DNDS > furosemide.

It was of importance to test whether the various compounds inhibit $^{36}Cl^-$ flux by dissipating the membrane potential or by some nonspecific membrane-permeabilizing effect. NPPAB has been found to depolarize TALH cells in the rat at concentrations above 100 μM (Wangemann et al., 1986). In a separate experiment (*not shown*), the various inhibitors were added to vesicles, which had accumulated $^{36}Cl^-$ for 10 min. DIDS, DPC and PPAB did not induce an appreciable Cl^- efflux at 250 μM, whereas NPPAB and NPBAB, respectively, induced an 11% and 14% loss of $^{36}Cl^-$ in 1 min. Thus, apparently inhibition by NPBAB and NPPAB at concentrations above 100 μM is caused in small part by nonspecific effects.

Pretreatment of vesicles with 250 μM NPPAB, NPBAB, PPAB and DPC was fully reversible, whereas with DIDS it was not (Fig. 7). Since all of these components carry at least one negative charge at pH 7.3, they were quantitatively separated from the vesicles on the Dowex-1 anion-exchange columns. The retention of NPPAB, NPBAB and DIDS on the columns was readily visible as they are strongly colored compounds. Preincubation of the vesicles with 200 μM DIDS in the presence of the reversible inhibitors showed that while 250 μM NPPAB, NPBAB and PPAB fully prevented the irreversible inhibition by DIDS, DPC did not. In additional experiments, vesicles, which had been pretreated with 200 μM DIDS in the presence of 250 μM NPPAB followed by removal of the inhibitors, showed $^{36}Cl^-$ uptake, which was fully inhibitable by readdition of NPPAB or DIDS.

Discussion

The membrane vesicle preparation used in this study has been previously shown to be highly enriched in the basolateral marker Na^+/K^+ ATPase (Jorgensen, 1974), as well as luminal TALH markers such as the $Na^+/K^+/2Cl^-$ cotransporter and K^+ channel (Burnham et al., 1985; Burnham, Braw & Karlish, 1986; Klaerke et al., 1987). Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that the 95 kilodalton α-chain of the Na^+/K^+ ATPase was by far the predominant protein band in the membrane preparation (*not shown*). Also it has been previously shown that the majority of the vesicles are in the right side out configuration based on determinations of ouabain-sensitive Na^+/K^+ ATPase activity (Burnham et al., 1985; C. Burnham, *unpublished data*), and Ca^{2+} activation of K^+ channels (Klaerke et al., 1987).

Two lines of evidence indicate that the Cl^- uptake pathway described here is conductive rather than electroneutral, i.e., involving Cl^-/Cl^- or Cl^-/OH^- exchange: (i) valinomycin abolished $^{36}Cl^-$ uptake and caused efflux of accumulated $^{36}Cl^-$ from the vesicles by clamping the membrane potential, and (ii) addition of Cl^- to low-Cl^- vesicles produced a marked polarization as determined by the potential-sensitive dye diSC_3(5). The reason for the apparently transient character of the potential response is unclear since the vesicles retain $^{36}Cl^-$ for many minutes, which indicates that the inside positive potential does not dissipate rapidly. However, the rapid reversal of the fluorescent signal could be due to the rapid equilibration of Cl^- within the vesicles since their internal volume is small, or alternatively, due to the dissipation of the potential by the dye itself.

Comparison of the Cl^- diffusion potential response of membrane vesicles from the outer medulla and the cortex indicates that the former contain considerably higher Cl^- channel activity. Although this does not necessarily mean that these channels are absent from cortical vesicles, it indi-

cates that the proportion of channel-containing vesicles is low relative to medullary vesicles. This is likely due to the greater heterogeneity of the membranes from the cortex, reflecting the variety of different cell types found there. Conversely, these results are consistent with the assumption that the medullary vesicles are enriched in basolateral TALH membranes, which have a high Cl^- conductance.

Recently, Cl^- channels were demonstrated in membrane vesicles from bovine kidney cortex by a $^{36}Cl^-$ uptake assay similar to the one used in this report (Landry et al., 1987). Several differences are notable between the properties of these two systems. First, the time required for half maximal uptake of $^{36}Cl^-$ was 0.5 to 1 min in the present study and approximately 10 min in the cortical vesicles. This 10-fold difference could be explained either by a larger number of channels per vesicle, or greater conductance per channel in the porcine medullary vesicles. These two possibilities cannot be distinguished on the basis of the present data. Second, the reported inhibitory potency of bumetanide, NPPAB and DIDS was 10- to 20-fold higher in vesicles from the bovine cortex, compared to those from the porcine medulla. The difference is not likely due to technical reasons as the assay systems used were similar. However, it may reflect a difference in the intrinsic properties of the membrane vesicles. Several factors could contribute to the variability: (i) the relatively hydrophobic inhibitors (in particular NPPAB, NPBAB and PPAB) could accumulate in the lipid matrix of the vesicles and thus affect the free-inhibitor concentration, (ii) the orientation of the vesicles used here is mainly right side out, whereas the opposite orientation is thought to predominate in the preparation of Landry et al. (1987). This could affect the relative affinity of the inhibitors for the channels. It also means that the membrane potential across the channel is opposite in the two situations, which could affect the conductance properties as well. (iii) Landry et al. (1987) preincubated the vesicles with the inhibitors for 9 min before the $^{36}Cl^-$ uptake was measured in order to obtain maximal inhibition, whereas in the present work the inhibitors were added together with the $^{36}Cl^-$. Hence a variety of factors could affect the apparent affinities of the channel inhibitors in different preparations.

There are considerable discrepancies between the effectiveness of the inhibitors in vesicles and intact cells. For example, the reported IC_{50} for inhibition by NPPAB in intact mouse TALH was 0.1 μM (Wangemann et al., 1986), whereas DIDS was virtually ineffective (DiStefano et al., 1985). This could be due to (i) species specific differences, (ii) alteration of the properties of the channels during vesicle preparation, or (iii) differences in the assay systems used. In the vesicles used here, Cl^- channel activity is measured with a strongly inside positive potential, which would correspond to a highly depolarized condition in the native TALH where the resting basolateral membrane potential is approximately -70 mV. This condition could conceivably alter the sensitivity of the channels to inhibitors. However, Cl^- channel inhibitors are known to differ in their effectiveness in various systems as illustrated by the variable inhibitory potency of disulfonic stilbenes. These compounds (DNDS, SITS, DIDS) are powerful blockers of anion exchangers (Cabantchik, Knauf & Rothstein, 1978), but have also been found to inhibit Cl^- channels in resting A6 toad kidney cells (Nelson et al., 1984) electric eel electroplax (White & Miller, 1979), bovine kidney cortex (Landry et al., 1987), rat colonic mucosa (Bridges, Benos & Frizzell, 1988) and pig TALH (present report). They have been found to be ineffective in mouse TALH (DiStefano et al., 1985), cAMP-stimulated A6 cells (Yanase & Handler, 1986), or human colonic T84 cells (Mandel et al., 1986). Conversely, DPC at 100 μM has been found to have no effect on the cAMP-stimulated apical Cl^- channel in *Necturus* gall bladder epithelium, but inhibited Cl^-–HCO_3^- exchange in the same membrane (Reuss, Constantin & Bazile, 1987). Similarly DPC and other anthranilic acid derivatives have been found to inhibit anion exchange in human red blood cells (Z.I. Cabantchik, *personal communication*). These differences may reflect subtle variations in the architecture of the ion-binding sites of anion channels and exchangers.

Inhibition by the covalent amino reagent DIDS was found to be irreversible in contrast to the nonreactive inhibitors NPBAB, NPPAB and PPAB (Fig. 7). Since the nonreactive analogue of DIDS, DNDS, was found to be a poor inhibitor ($IC_{50} \geqslant 200$ μM), it appears that inhibition by DIDS is to some extent due to its covalent binding. NPPAB, NPBAB and PPAB prevented the irreversible inhibition by DIDS, while the related compound DPC did not. The lack of effect of DPC is consistent with its relatively low potency in this system. The reaction of DIDS with any of the reversible inhibitors is unlikely since they are all secondary amines and the isothiocyano groups of DIDS react with unprotonated primary amines preferentially. It is proposed that DIDS reacts with an amino group on the channel, which has a low pK, perhaps due to the proximity of a cluster of positive charges. The inhibitors NPPAB, NPBAB and PPAB presumably prevent this reaction either directly by steric hindrance or by changing the conformation of the channel and thus reducing the availability of the DIDS reactive site.

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References

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254

Bridges, R.J., Benos, D.J., Frizzell, R.A. 1988. Epithelial chloride channel blockade by DNDS. *Biophys. J.* **53:**525*a*

Burnham, C., Braw, R., Karlish, S.J.D. 1986. A Ca-dependent K channel in "luminal" membranes from the renal outer medulla. *J. Membrane Biol.* **93:**177–186

Burnham, C., Karlish, S.J.D., Jorgensen, P.L. 1985. Identification and reconstitution of $Na^+/K^+/2Cl^-$ cotransporter and K^+ channel from luminal membranes of renal red outer medulla. *Biochim. Biophys. Acta* **821:**461–469

Cabantchik, Z.I., Knauf, P.A., Rothstein, A. 1978. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of probes. *Biochim. Biophys. Acta* **515:**239–302

DiStefano, A., Wittner, M., Schlatter, E., Lang, H.J., Englert, H., Greger R. 1985. Diphenylamine-2-carboxylate, a blocker of the Cl^- conductive pathway in Cl^- transporting epithelia. *Pfluegers Arch.* **405:**S95–S100

Frizzell, R.A. 1987. Cystic fibrosis: A disease of ion channels? *Trends Neurosci.* **10:**190–193

Frizzell, R.A., Rechkemmer, G., Shoemaker, R.L. 1986. Altered regulation of airway epithelial cell chloride channels in cystic fibrosis. *Science* **233:**558–560

Garty, H., Asher, C., Yeger, O. 1987. Direct inhibition of epithelial Na^+ channels by a pH-dependent interaction with calcium and by other divalent ions. *J. Membrane Biol.* **95:**151–162

Garty, H., Rudy, B., Karlish, S.J.D. 1983. A simple and sensitive procedure for measuring isotope fluxes through ion-specific channels in heterogeneous populations of membrane vesicles. *J. Biol. Chem.* **258:**13054–13059

Greger, R. 1985. Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. *Physiol. Rev.* **65:**755–797

Greger, R., Schlatter, E. 1983*a*. Properties of the basolateral membrane of the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflueger's Arch.* **396:**325–334

Greger, R., Schlatter, E. 1983*b*. Properties of the lumen membrane of the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflueger's Arch.* **396:**315–324

Jorgensen, P.L. 1974. Purification and characterization of Na^+/K^+ ATPase: III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecyl sulfate. *Biochim. Biophys. Acta* **356:**36–52

Klaerke, D.A., Karlish, S.J.D., Jorgensen, P.L. 1987. Reconstitution in phospholipid vesicles of calcium-activated potassium channel from outer renal medulla. *J. Membrane Biol.* **95:**105–112

Kolb, H.A., Brown, C.D.A., Murer, H. 1985. Identification of a voltage-dependent anion channel in the apical membrane of a Cl^- secretory epithelium (MDCK). *Pfluegers Arch.* **403:**262–265

Landry, D.W., Reitman, M., Cragoe, E.J., Al-Awqati, Q. 1987. Epithelial chloride channel. Development of inhibitory ligands. *J. Gen. Physiol.* **90:**779–798

Mandel, K.G., Dharmsathaphorn, K., McRoberts, J.A. 1986. Characterization of a cAMP-activated Cl^- transport pathway in the apical membrane of a human colonic epithelial cell line. *J. Biol. Chem.* **261:**704–712

Nelson, D.J., Tang, J.M., Palmer, L.G. 1984. Single-channel recordings of apical membrane chloride conductance in A6 epithelial cells. *J. Membrane Biol.* **80:**81–89

Reuss, L., Constantin, J.L., Bazile, J.E. 1987. Diphenylamine-2-carboxylate blocks Cl^--HCO_3^- exchange in *Necturus* gall bladder epithelium. *Am. J. Physiol.* **253:**C79–C89

Sariban-Sohraby, S., Benos, D.J. 1986. Detergent solubilization, functional reconstitution and partial purification of epithelial amiloride-binding protein. *Biochemistry* **25:**4639–4646

Schlatter, E., Greger, R. 1985. cAMP increases the basolateral Cl^- conductance in the isolated perfused medullary thick ascending limb of Henle's loop of the mouse. *Pfluegers Arch.* **405:**367–376

Wangemann, P., Wittner, M., DiStefano, A., Englert, H.C., Lang, H.J., Schlatter, E., Greger, R. 1986. Cl^- channel blockers in the TALH. Structure activity relationship. *Pfluegers Arch.* **407:**S128–S141

White, M.M., Miller, C. 1979. A voltage-gated anion channel from the electric organ of *T. californica*. *J. Biol. Chem.* **254:**10161–10166

Yanase, M., Handler, J.S. 1986. cAMP stimulates chloride secretion in A6 epithelia. *J. Physiol. (London)* **251:**C810–C814

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