Biochimica et Biophysica Acta, 1022 (1990) 229–236
Elsevier

BBAMEM 74749

Reconstitution of a kidney chloride channel and its identification by covalent labeling

William Breuer

Department of Membrane Research, Weizmann Institute of Science, Rehovot (Israel)

(Received 16 August 1989)

Key words: Anion transport; DIDS; Liposome; (Pig kidney); (TALH)

The basolateral membrane of the thick ascending loop of Henle (TALH) of the mammalian kidney is characterized by its high content of Na^+/K^+-ATPase and a Cl^- conductance, which function in parallel in salt reabsorption. In order to reconstitute the Cl^- channels, TALH membrane vesicles were solubilized in 1% sodium cholate in buffer containing 200 mM KCl, followed by dilution with soybean lipids (final ratio of protein/detergent/lipid of 1:3:15 in mg) and removal of the detergent by gel filtration on Sephadex G-50. Cl^- channel activity in the liposomes was determined by a $^{36}Cl^-$ uptake assay where the accumulation of the radioactive tracer against its chemical gradient is driven by the membrane potential (positive inside) generated by an outward Cl^- gradient. The $^{36}Cl^-$ uptake by the KCl-loaded liposomes was dependent on the inclusion of membrane protein and was abolished by valinomycin, indicating the involvement of a conductive pathway. It was also inhibited by 36% by 100 μM 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). Solubilization of the Cl^- channels in cholate was optimal in the presence of 200 mm KCl, but was found to decrease markedly at low ionic strength. SDS-PAGE analysis of the proteins extracted by cholate at high and low salt concentrations showed that the Cl^- channel-containing high KCl extract was enriched in the 96 and 55 kDa α- and β-subunits of the Na^+/K^+-ATPase (the major proteins in the membrane preparation) and several minor protein bands. Treatment of the membrane vesicles with the radioactive analogue of DIDS, $[^3H]_2$DIDS, labeled primarily a 65 and a 31 kDa protein. The solubilization of the 31 kDa protein by cholate depended markedly on the ionic strength and thus paralleled the solubilization pattern of Cl^- channel activity. Furthermore, the labeling of the 31 kDa protein was prevented by nonradioactive DIDS and by NPPB but not by other compounds, indicating that it may be a Cl^- channel component.

Introduction

The thick ascending loop of Henle (TALH) of the mammalian nephron, is a specialized epithelium with a high capacity for salt reabsorption. The net movement of NaCl from the lumen of the nephron to the blood side is thought to be accomplished by the concerted action of five transport systems; an $Na^+/K^+/2Cl^-$ co-transporter and a Ca^{2+}-activated K^+ channel in the luminal membrane, and the Na^+/K^+-ATPase, a KCl co-transporter and Cl^- channel in the basolateral membrane (for review, see Ref. 9). The coupled entry of Na^+, K^+ and Cl^- via the luminal co-transporter is driven by the inward Na^+ gradient generated by the basolateral Na^+/K^+-ATPase, while K^+ and Cl^- exit via the specific channels located at opposite poles of the cells as well as via a basolateral KCl co-transporter. The basolateral Cl^- channel in the mouse TALH has been found to be activated by cAMP, presumably by protein kinase phosphorylation [19]. This is analogous to the apical Cl^- channels in the cells of Cl^- secreting epithelia which include the airways [5,22], colon [15] and elasmobranch rectal gland [10]. These channels have been well characterized by single channel techniques (recently reviewed in Ref. 8).

Recently several candidate Cl^- channel proteins have been purified by affinity chromatography from mem-

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; $[^3H]_2$DIDS, 4,4′-diisothiocyanoditritiostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DPC, diphenylamine-2-carboxylate; IAA-94/5, (±)-[(2-cyclopentyl-6,7-dichloro-2-methyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxyl]acetic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; TALH, thick ascending loop of Henle; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMSO, dimethylsulfoxide; kDa, kilodaltons.

Correspondence: W. Breuer, Department of Membrane Research, Weizmann Institute of Science, Rehovot, 76100 Israel.

branes derived from bovine kidney cortex and trachea [24]. The purpose of the present work was to initiate the identification of the TALH Cl^- channel proteins using a different approach. Previously, membrane vesicles from the TALH of porcine kidney were shown to be enriched in Cl^- channels [2]. These channels have now been solubilized in sodium cholate and reconstituted in liposomes with preservation of their function. Selective detergent solubilization under conditions of high and low ionic strength as used in conjunction with reconstitution and covalent labeling with $[^3H]_2$DIDS to identify a potential candidate protein as a component of the TALH Cl^- 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 sealed membrane vesicles were prepared as described by Jørgensen (1988) [12] with slight modifications. Minced red outer medullas were hand-homogenized at 10 ml/g wet tissue in 250 mM sucrose, 1 mM EDTA, 10 mM Na-MOPS (pH 7.2) (homogenization buffer) in a teflon-glass motor driven homogenizer at 4°C. The homogenate was centrifuged at $6000 \times g$ for 15 min at 5°C, the pellet containing tissue and cell debris was discarded and crude microsomes were collected from the supernatant by centrifugation at $39000 \times g$ for 40 min at 5°C. In order to separate sealed vesicles from membrane fragments 1.5 ml of the crude microsomes was layered on top of 9 ml of 15% metrizamide (from Sigma Chem. Co.) in homogenization buffer and centrifuged for 1 h at 35000 rpm in an SW 41 rotor. The sealed vesicles were recovered from the top of the metrizamide cushion (leaky membranes sediment to the bottom), washed once in homogenization buffer, suspended at 15 mg protein/ml and stored frozen at −80°C. Recovery was 1–2 mg sealed vesicle protein per gram of wet tissue. All protein determinations were done by the method of Bradford [1], using bovine serum albumin as standard. Protein concentration in cholate containing samples was estimated from protein standards containing an equivalent amount of cholate.

Solubilization and reconstitution of Cl^--channels. Sealed membrane vesicles were suspended at 4 mg/ml in 200 mM KCl, 0.5 mM EGTA, 5 mM Na-Mops (pH 7.2) and sodium cholate was added to a final concentration of 1%. After 10 min on ice with intermittent mixing the samples were centrifuged in a Beckman airfuge (Beckman Instruments, Geneva, CH) at $100000 \times g$ for 20 min. The clear supernatant was carefully removed and mixed immediately with an equal volume of 50 mg/ml L-α-phosphatidylcholine (type II-S from soy benas, Sigma Chem. Co.) in 200 mM KCl, 0.5 mM EGTA, 5 mM Na-Mops (pH 7.2), which had been previously sonicated to clarity in a bath sonicator (Laboratory Supplies Co., Hicksville, NY). The mixture was vortexed vigorously, incubated for 20 min at room temperature and thereafter stored on ice. The liposomes were used for $^{36}Cl^-$ uptake determinations within 3 h.

Preparation of anion exchange Dowex-1 columns. Dowex-1 × 10, 50–100 mesh (Fluka, CH) was washed in distilled water, the slurry was titrated with NaOH to pH 12.5 and then washed extensively with distilled H_2O until the pH of the eluate was 8–9. The slurry was then titrated with 50% gluconic acid (from Merck, München, F.R.G., decolorized with charcoal) to pH 2.8 and left overnight. The excess gluconic acid was washed out with distilled water until the pH of the eluate was stable at 4.5. For use in $^{36}Cl^-$ fluxes pasteur pipettes plugged with Dacron wool were packed to a height of 3 cm. 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 1.5 ml 350 mM sucrose.

Preparation of Sephadex G-50 spin-columns. Hypodermic syringes (3 ml) pluged with Dacron wool were packed with approx. 2.75 ml of Sephadex G-50 (coarse, Pharmacia, Sweden), and washed with 10 ml of 350 mMsucrose, 2 mM $MgSO_4$, 10 mM Na-Mops (pH 7.2). The minocolumns were dried before use by centrifugation in 13 × 100 mm disposable glass tubes for 30 s at 1200 rpm in an IEC HN-SII centrifuge equipped with a manual brake (International Equipment Co., Needham, MA).

Assay of $^{36}Cl^-$ uptake by liposomes. In initial experiments the liposomes were passed through two consecutive gel filtration spin-columns, the first for removal of excess cholate, the second for generating an outward Cl^- gradient to drive $^{36}Cl^-$ uptake. In this procedure the first Sephadex G-50 spin-column was equilibrated with 200 mM KCl, 0.5 mM EGTA, 5 mM Na-Mops (pH 7.2), and the second with 350 mM sucrose, 2 mM $MgSO_4$, 10 mM Na-Mops (pH 7.2). Subsequently it was found that the first gel filtration step was unnecessary, and the procedure using only one Sephadex G-50 column equilibrated with 350 mM sucrose, 2 mM $MgSO_4$, 10 mM Na-Mops (pH 7.2) was adopted for routine use. The assay was initiated by loading the liposomes on Sephadex G-50 spin-columns (150 μl/column) and immediately centrifuging them for 24 s at 1200 rpm. This step served to remove > 98% of the free Cl^- (determined using $^{36}Cl^-$ as tracer). Also a partial removal of cholate was assumed to have occurred at this stage. The eluted liposomes were rapidly mixed with an equal volume of 350 mM sucrose, 2 mM $MgSO_4$, 10 mM Na-Mops (pH 7.2) and 5.0 mM $Na^{36}Cl$ (Amersham, U.K., 0.64 mCi/mmol) with or without inhibitors. At the times indicated, 100 μl of the reaction mixture were loaded on chilled Dowex-1 columns and the liposomes were eluted immediately into scintillation vials with 0.8

ml ice-cold 350 mM sucrose (time of vesicle elution was 25–35 s). The eluted $^{36}Cl^-$, representing liposome-trapped isotope was determined by scintillation counting in 10 ml Lumax scintillation fluid (Lumac, Longraaf, The Netherlands). All experiments were performed at least three times separately.

Solutions and chemicals. Cholic acid, 99% pure (Fluka, CH) was recrystallized once from 70% ethanol and kept as 10% stock solution neutralized to pH 7.2 with NaOH. Valinomycin (from Sigma Chem. Co.) was kept as a 0.5 mM stock solution in DMSO. DIDS, DNDS and $[^3H]_2$DIDS ($1.5 \cdot 10^9$ cpm/μmol), kept as 10 mM solutions in 10 mM sodium phosphate (pH 7.4) were generous gifts from Dr. Z.I. Cabantchik (Hebrew University of Jerusalem, Israel). NPPB, kept as a 10 mM solution in DMSO was kindly provided by Dr. R. Greger (Albert-Ludwigs Universität, Freiburg, F.R.G.). IAA-94/5 [14,24] was obtained from Biological and Chemical transport Systems (New York).

Gel electrophoresis. SDS-PAGE was carried out according to the method of Laemmli [25] using 10% polyacrylamide gels. Proteins were visualized by staining with Coomassie brilliant blue. Radioactivity was visualized by fluorography using Amplify (Amersham, U.K.) and preflashed Agfa-Curix X ray film exposed for 4–8 days at −80 °C.

Results

In order to study the Cl^- channels in liposomes an adaptation of the potential-driven isotope uptake method was used. This method has been previously applied to various channels in membrane vesicles and liposomes, such as Na^+ channels from toad bladder and kidney papilla [6,7], K^+ channels form the TALH [3,13] as well as Cl^- channels from the TALH [2] and kidney cortex [14]. The assay makes use of the principle that the creation of an outward concentration gradient for Cl^- will generate an inside-positive potential in those vesicles or liposomes 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 potential and on the number of vesicles or liposomes containing Cl^- channels. As shown in Fig. 1, liposomes prepared with cholate in the absence of protein show little $^{36}Cl^-$ uptake. However, the inclusion of increasing amounts of cholate-solubilized membrane protein resulted in substantial $^{36}Cl^-$ accumulation. The optimal ratio of protein/cholate/lipid was 0.32 : 1.0 : 5.0 in mg, where the lipid concentration is based on the dry weight of soybean phospholipids added initially. An additional increase in the amount of input protein did not significantly enhance the influx. In separate experiments (not shown) the optimal sodium cholate concentration for reconstitution was found to be 1%, corresponding to 22 mM.

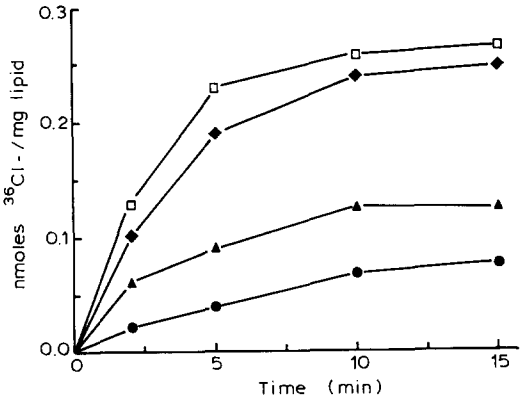

Fig. 1. $^{36}Cl^-$ uptake by liposomes: dependence on membrane protein. TALH membrane vesicles were incubated in 200 mM KCl, 0.5 mM EDTA, 1% sodium cholate, 5 mM Mops (pH 7.2) at 8 mg protein/ml for 10 min at 4 °C, and the insoluble proteins were removed by centrifugation at 100000 × *g* for 20 min in an airfuge. The supernatants were combined and aliquots were diluted with the solubilization buffer to give final protein concentrations of 6.4, 3.2 and 1.6 mg/ml. These were then mixed with an equal volume of 50 mg/ml sonicated soybean lipids giving final concentrations of cholate and lipids of 5 and 25 mg/ml, respectively. The $^{36}Cl^-$ uptake for each preparation was determined after gel filtration as described in Materials and Methods. The various ratios of input protein/cholate/lipid (in mg) were 0.64 : 1.0 : 5.0 (□); 0.32 : 1.0 : 5.0 (◆); 0.16 : 1.0 : 5.0 (▲); 0 : 1.0 : 5.0 (●). Results are expressed as nmoles $^{36}Cl^-$ taken up per mg of input lipid.

The $^{36}Cl^-$ accumulation by the liposomes was significantly reduced in the presence of 5 μM valinomycin (Fig. 2). Since the liposomes contained 200 mM KCl and > 98% of the KCl was removed by gel filtration, valinomycin induced an inside-negative diffusion poten-

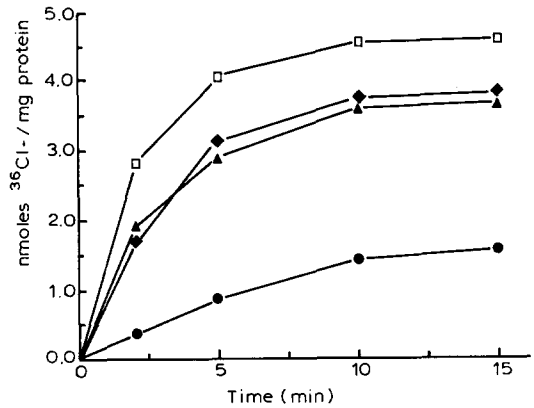

Fig. 2. Effect of inhibitors on $^{36}Cl^-$ uptake by liposomes. TALH membrane vesicles were incubated in 200 mM KCl, 0.5 mM EDTA, 1% sodium cholate, 5 mM Mops (pH 7.2) at 4 mg/ml protein for 10 min at 4 °C, and the insoluble protein was removed by centrifugation in an airfuge. The combined soluble supernatants were then mixed with an equal volume of sonicated soybean lipids, free Cl^- was removed by gel filtration and $^{36}Cl^-$ uptake was determined as described in Materials and Methods. The various inhibitors were added to the liposomes simultaneously with $^{36}Cl^-$. Uptake was measured in the presence of: 1% DMSO (vehicle solvent), control (□); 100 μM DIDS (◆); 100 μM NPPB (▲) and 5 μM valinomycin (●). Results are expressed as nmoles $^{36}Cl^-$ taken up per mg of input protein.

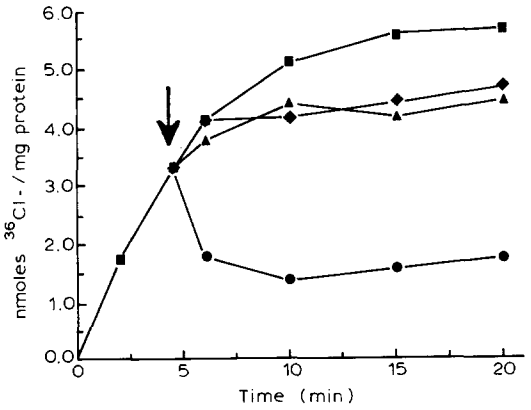

Fig. 3. Effect of inhibitors on $^{36}Cl^-$ retention by liposomes. TALH membrane vesicles were solubilized in sodium cholate, centrifuged, and the soluble fraction was reconstituted with soybean lipids as described for Fig. 2. Uptake of $^{36}Cl^-$ was followed with time for 4.5 min, then the sample was divided into four equal portions and the following additions were made as indicated by the arrow: 1% DMSO (vehicle solvent), control (■); 100 μM DIDS (◆); 100 μM NPPB (▲); 5 μM valinomycin (●). The determination of $^{36}Cl^-$ uptake was continued for another 15 min. Results are expressed as nmols of $^{36}Cl^-$ taken up per mg of input protein.

tial due to the outward K^+ gradient. This result indicates that the pathway mediating the $^{36}Cl^-$ uptake is electroconductive as expected from a channel. DIDS and NPPB which have been found to block various anion transport systems including Cl^- channels [8,14,21,23,27], inhibited the $^{36}Cl^-$ uptake. Both compounds at 100 μM produced 36% inhibition at 2 min. This is comparable to the 48% and 36% inhibition by 100 μM NPPB and DIDS, respectively, in TALH membrane vesicles [2]. However, the possibility cannot be excluded that additional inhibitor-insensitive pathways for Cl^- are also present in the liposomes, which would account for the apparently low activity of the inhibitors. As shown in Fig. 3, inhibition by NPPB and DIDS was not due to dissipation of the membrane potential since neither inhibitor caused the release of accumulated $^{36}Cl^-$. On the other hand, 5 μM valinomycin did cause a rapid efflux of $^{36}Cl^-$, presumably by reversing the inside-positive potential.

The solubilization of the Cl^- channels with cholate was found to be highly dependent on the ionic strength of the solubilizing solution. This was shown by sequential cholate solubilization. First, the membrane vesicles were exposed to 1% sodium cholate in low ionic strength solution (250 mM sucrose, 1 mM EDTA, 10 mM Na-Mops (pH 7.2)), centrifuged for 20 min at $100000 \times g$ and the soluble supernatant was recovered. Although 45% of the membrane protein was solubilized, reconstitution of this soluble fraction yielded $^{36}Cl^-$ uptake at 10 min of 2.55 ± 0.4 nmol $^{36}Cl^-$/mg protein (Fig. 4a) compared to 4.7 to 5.2 nmol $^{36}Cl^-$/mg protein obtained in high ionic strength (see Figs. 2 and 3). Extraction of the remaining membrane pellet with 1% sodium cholate in high ionic strength solution containing 200 mM KCl resulted in the solubilization of 55% of the pellet protein and substantial Cl^- channel activity (Fig. 4c). On the other hand, a similar extraction of the same pellet material with 1% sodium cholate in low ionic strength solution containing no added KCl solubilized only 20% of the pellet protein and negligible Cl^- channel activity (Fig. 4b).

While pre-extraction of the membrane vesicles with cholate at low ionic strength did not increase the specific activity in terms of $^{36}Cl^-$ uptake/mg protein, it enhanced the inhibitor-sensitive portion of the flux. This is shown in Fig. 5A. $^{36}Cl^-$ accumulation at 2 min was inhibited by 50% in the presence of 100 μM DIDS and NPPB, compared to 36% inhibition obtained without the pre-extraction step (see Fig. 2). Thus, the relative contribution of the inhibitor-sensitive pathways to the total $^{36}Cl^-$ uptake was enhanced.

In order to demonstrate that cumulative uptake of $^{36}Cl^-$ into the liposomes took place, the experiment in Fig. 5B was carried out. Preincubation of the liposomes

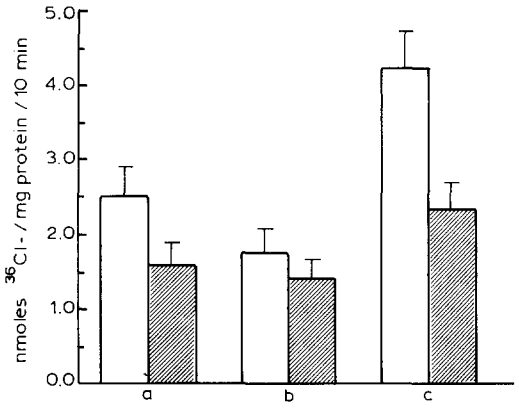

Fig. 4. Sequential solubilization of Cl^- channels with cholate at low and high ionic strength. TALH membrane vesicles were incubated in low ionic strength buffer: 250 mM sucrose, 1 mM EDTA, 1% sodium cholate, 10 mM MOPS (pH 7.2), (1.6 mg protein/330 μl), for 10 min at 4° C and then centrifuged at $100000 \times g$ for 20 min in an airfuge. The soluble supernatants containing a total of 0.72 mg protein were saved (fraction 'a'). The remaining pellets were suspended in a total of 300 μl of 250 mM sucrose, 1 mM EDTA, 1% sodium cholate, 10 mM Mops (pH 7.2), combined together and divided into two equal portions to which was added either H_2O or 4 M KCl to give a final concentration of 200 mM. After 10 min at 4° C both samples were centrifuged again and the soluble supernatants were collected (respectively designated as fractions 'b' and 'c' for the low and high KCl extract). The respective protein content of fractions 'b' and 'c' was 0.09 and 0.24 mg. Prior to reconstitution, fractions 'a' and 'b' were supplemented with 200 mM KCl and fraction 'c' was diluted 2-fold in order to obtain a protein concentration comparable with fraction 'b'. Fractions a, b and c were reconstituted by mixing a 75 μl aliquot with an equal volume of soybean lipids, followed by gel filtration and 36 Cl^- uptake determination as described in Materials and Methods. Each pair of bars represents the $^{36}Cl^-$ taken up per mg input protein in 10 min for each fraction (a, b and c); plain bars: control, no addition; stippled bars: in the presence of 100 μM NPPB; ($n = 4$, error bars ± S.D.).

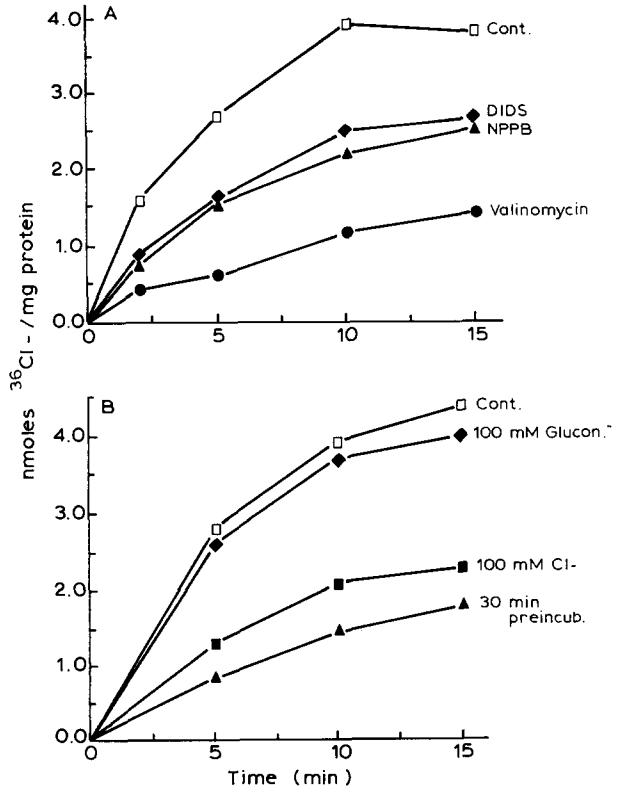

Fig. 5. Solubilization and reconstitution of Cl^- channels after pre-extraction of membranes with cholate at low ionic strength. TALH membrane vesicles were incubated in 250 mM sucrose, 1 mM EDTA, 1% sodium cholate, 10 mM Mops (pH 7.2) (2.0 mg protein/400 μl) for 10 min at 4°C, then centrifuged at 100000 × g for 20 min in an airfuge. The soluble supernatants were discarded and the remaining pellets resuspended in 300 μl of 200 mM KCl, 0.5 mM EDTA, 1% sodium cholate, 5 mM Mops (pH 7.2). After 10 min at 4°C the samples were centrifuged again, and the solute supernatants (corresponding to fraction 'c' in Fig. 4) were collected. The combined supernatants were reconstituted by mixing with an equal volume of soybean lipids, followed by gel filtration and $^{36}Cl^-$ uptake determination as before. (A) Effect of inhibitors. Uptake was measured in the presence of: 1% DMSO (vehicle solvent), control (□); 100 μM DIDS (◆); 100 μM NPPB (▲); 5 μM valinomycin (●). (B) Effect of potassium gluconate, KCl and Cl^--free preincubation. Uptake was measured in the presence of: 100 mM potassium gluconate (◆) and 100 mM KCl (■), both added from 2 M solutions, or equivalent volume of H_2O (□). A separate aliquot of the liposomes eluted from the Sephadex G-50 spin-columns was incubated for 30 min at room temperature prior to addition of $^{36}Cl^-$ and determination of $^{36}Cl^-$ uptake (▲). Results are expressed as nmol $^{36}Cl^-$ taken up per mg input protein.

in Cl^--free medium for 30 min prior to addition of $^{36}Cl^-$ resulted in markedly decreased $^{36}Cl^-$ uptake, presumably due to the dissipation of the outward Cl^- gradient. Addition of 100 mM KCl but not of 100 mM potassium gluconate (gluconate is a non-permeant anion) had a similar effect.

Previously it was shown that DIDS is an irreversible inhibitor of the Cl^- channels in intact TALH vesicles [2]. Therefore, $[^3H]_2$DIDS should be a potentially useful covalent affinity probe for the Cl^- channel protein. As shown in Fig. 6 (lane 1), $[^3H]_2$DIDS labeled principally two proteins in intact membrane vesicles, with molecular mass of 65 and 31 kDa. Two additional proteins, 95 kDa and 55 kDa which probably correspond to the α- and β-subunits of the Na^+/K^+-ATPase were labeled weakly. Treatment of the $[^3H]_2$DIDS-labeled vesicles with 1% cholate in 250 mM sucrose, 1 mm EDTA, 10 mM Na-Mops (pH 7.2) (low ionic strength) solubilized the 65 kDa but not the 31 kDa protein (Fig. 6, lane a). The latter protein was found in the insoluble pellet (Fig. 6, lane 2). Extraction of the insoluble pellet with 1% cholate at low ionic strength again failed to solubilize the 31 kDa protein (Fig. 6, lane b), although protein staining showed that a number of other protein bands were solubilized. On the other hand, extraction of the insoluble pellet with 1% cholate at high ionic strength (200 mM KCl) solubilized the $[^3H]_2$DIDS-labeled 31 kDa protein (Fig. 6, lane c). The respective insoluble pellets showed corresponding retention (Fig. 6, lane d) and depletion (Fig. 6, lane e) of the 31 kDa protein. Therefore, the pattern of solubility of the 31 kDa protein in cholate was parallel to the pattern of solubilization of reconstitutable Cl^- channel activity (see Fig. 4).

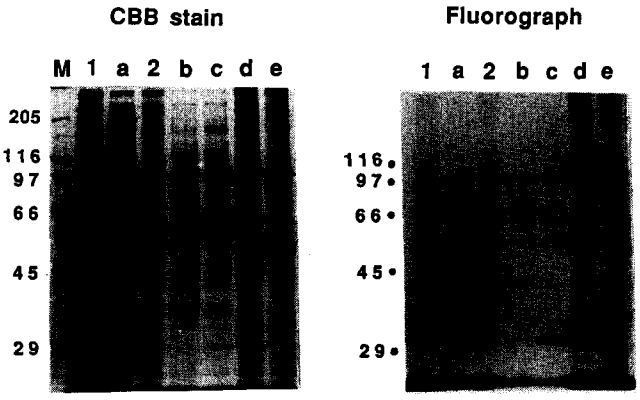

Fig. 6. Sequential solubilization of $[^3H]_2$DIDS-labeled TALH membranes with cholate at low and high ionic strength: SDS-PAGE analysis. TALH membrane vesicles were incubated in 250 mM sucrose, 1 mM EDTA, 10 mM Mops (pH 7.2) (at 3 mg protein/ml) and 100 μM $[^3H]_2$DIDS for 10 min at room temperature. The reaction was stopped by removal of the free reagent on Sephadex G-50 spin-columns as described for $^{36}Cl^-$ uptake experiments. The labeled membrane vesicles were then sequentially solubilized with cholate in low and high ionic strength solutions exactly as described in Fig. 4, and the various soluble and insoluble fractions were subjected to SDS-PAGE. Lane 1: original TALH membrane vesicles, 50 μg; lane a: fraction 'a', first low-ionic-strength soluble supernatant, 41 μg; lane 2: membrane pellet remaining after first low-ionic strength solubilization, 46 μg; lane b: second low-ionic-strength soluble supernatant, 22 μg; lane c: second soluble supernatant in high ionic strength (200 mM KCl), 31 μg; lane d: membrane pellet remaining after two low-ionic-strength extractions, 43 μg; lane e: membrane pellet remaining after a low- and high-ionic-strength extraction, 38 μg. Lane M: molecular weight markers in K, myosin, 205; β-galactosidase, 116; phosphorylase B, 97; bovine serum albumin, 66; egg albumin, 45; carbonic anhydrase, 29. The gel was 10% polyacrylamide. (Left panel) Coomassie brilliant blue stain; (right panel) corresponding fluorograph.

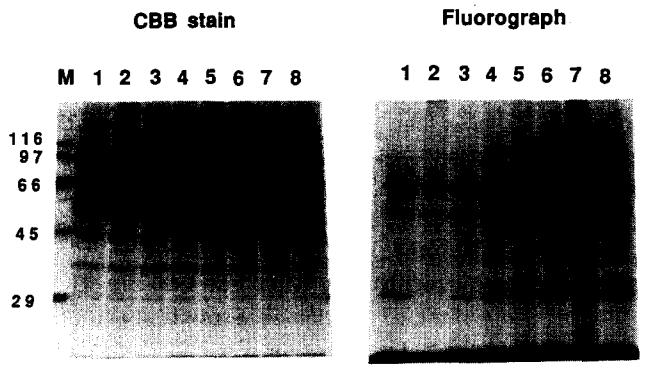

Fig. 7. Effect of Cl^- channel inhibitors on $[^3H]_2$DIDS labeling of membrane vesicles. TALH membrane vesicles were incubated in 250 mM sucrose, 1 mM EDTA, 10 mM Mops (pH 7.2) at 3 mg protein/ml and various Cl^- channel inhibitors at 200 μM. After 5 min at room temperature, 100 μM $[^3H]_2$DIDS was added to each system and incubation was continued for another 10 min at room temperature. The reaction was stopped by removal of the free reagents on Sephadex G-50 spin-columns as previously. Each lane contained 38 μg protein. Lane 1: no addition; lane 2: DIDS; lane 3: NPPB; lane 4: IAA-94/5; lane 5: DNDS; lane 6: DPC; lane 7: 1% sodium cholate; lane 8; no addition. (Left panel) Coomassie brilliant blue stain; (right panel) corresponding fluorograph.

The capacity of several Cl^- channel inhibitors to interfere with $[^3H]_2$DIDS labeling was examined (Fig. 7). Pre-exposure of the membrane vesicles to nonradioactive 200 μM DIDS completely prevented the labeling of the 31 kDa protein, and partially decreased the labelling of the 65 kDa protein. NPPB at 200 μM reduced the labeling of the 31 kDa but had little effect on the 65 kDa protein. In contrast, other compounds with inhibitory activity on Cl^- channels in other systems, DPC, IAA-94/5 and DNDS at 200 μM failed to reduce appreciably the labeling of any of the protein bands. Cholate produced an increase in the labeling of a number of bands, in particular the 95 kDa α-subunit of Na^+/K^+-ATPase, presumably due to the permeabilization of the vesicles. This result indicates that $[^3H]_2$DIDS labeling was restricted to the external surface of intact vesicles, as expected from an impermeant label. The α subunit of purified Na^+/K^+-ATPase has been shown to be stoichiometrically labeled with $[^3H]_2$DIDS, probably at a site facing the cytoplasmic compartment [26]. Therefore, the labeling of the α-subunit only after permeabilization of the vesicles is consistent with their known right-side-out configuration [3].

Discussion

The objective of this study was to identify the TALH Cl^- channel protein by a combination of approaches involving solubilization and reconstitution in conjunction with covalent labeling. A procedure for selective detergent solubilization of TALH membrane vesicles was developed in order to obtain an enrichment of the Cl^- channel proteins as determined by functional reconstitution. In parallel membrane vesicles were covalently labeled with $[^3H]_2$DIDS, subjected to the identical solubilization procedure and analyzed by SDS-PAGE and fluorography. The rationale for labeling of the Cl^- channel proteins with $[^3H]_2$ DIDS has been provided previously by the demonstration that DIDS is an irreversible inhibitor of Cl^- channels in TALH membrane vesicles and that DIDS and NPPB bind to the same or adjacent sites on the Cl^- channel [2]. This finding also provided the basis for another approach aimed at assessing the ability of NPPB and other compounds to prevent the $[^3H]_2$DIDS labeling of specific proteins.

Detergent solubilization and reconstitution in liposomes or lipid bilayers has been achieved with a number of ion channels such as the voltage-dependent Na^+ and K^+ channels, the acetylcholine receptor-channel (reviewed in Ref. 16), the kidney epithelial amiloride-sensitive Na^+ channel [18] and the Ca^{2+} activated K^+ channel in the TALH luminal membrane [3,13]. Liposome reconstitution of epithelial Cl^- channels from membranes of bovine trachea cells [4] and kidney cortex [24] have also been reported.

In this study a modification of the cholate dilution procedure was used, which is similar to that for reconstitution of the γ-aminobutyrate transporter from rat brain [17]. The detergent most suitable for membrane solubilization using this method was found to be sodium cholate. Use of other detergents such as CHAPS, Triton X-100 and octyl glucoside in place cholate was found to result in very low $^{36}Cl^-$ accumulation. Presumably these detergents were not as efficiently removed by the Sephadex G-50 spin-columns, resulting in leaky liposomes, due to residual detergent. However, membrane solubilization in 20 mM CHAPS followed by addition of lipid and overnight dialysis for more thorough removal of the detergent, yielded liposomes with $^{36}Cl^-$ uptake efficiency equal to that obtained with cholate and gel filtration.

The assay of Cl^- channel activity involving $^{36}Cl^-$ uptake against its chemical gradient has been shown previously to be applicable to Cl^- channels in membrane vesicles [2,14]. However, this assay will also detect electroneutral anion exchange as has been shown for rat intestinal brush-border vesicles where $^{36}Cl^-$ uptake was insensitive to valinomycin-induced changes in membrane potential [20]. The uptake of $^{36}Cl^-$ by the liposomes was cumulative since it could be inhibited by excess Cl^- (100 mM) and was abolished by preincubation of the liposomes in Cl^--free medium (Fig. 5B). The finding that $^{36}Cl^-$ accumulation in the proteoliposomes was abolished (Fig. 2) and reversed (Fig. 3) by valinomycin indicates that the reconstituted Cl^- transport pathway was conductive. Also inhibition by 100 μM NPPB and DIDS, albeit partial (Figs. 2 and 4), indicates that the pathways are similar to or the same as previously characterized in the TALH vesicles.

Although DIDS has been found not to affect Cl^- channels in intact TALH tubules where other inhibitors such as NPPB were highly effective [27], it has been shown to inhibit epithelial Cl^- channels in a variety of other studies involving membrane vesicles or planar lipid bilayers [2,14,21,23,28]. One explanation for this discrepancy is the possible problem of accessibility of this probe to the channel site in the intact tissue, due to its relatively large size and double negative charge (M_r 452, two sulfonate groups), compared to NPPB (M_r 300, one carboxyl group). In addition, depletion of the reagent might occur in the tubules due to the abundance of free amino groups. However, the possibility can not be excluded at present that the DIDS-sensitive Cl^- pathways described here are distinct from the Cl^- channels observed by electrophysiological methods in intact TALH tubules.

Cl^- channels in other systems show higher sensitivity to DIDS than observed here. Planar-lipid-bilayer reconstituted Cl^- channels from bovine trachea [21], *Torpedo* electroplax [23] or rat colonic enterocytes [28] were fully inhibited by DIDS at concentrations below 100 μM. One explanation for the apparently low inhibitor sensitivity in both the original TALH vesicles and proteoliposomes could be the presence of additional inhibitor-insensitive Cl^- conductive pathways. An observation consistent with this possibility is that removal of irrelevant protein by pre-extraction of the TALH membrane vesicles with cholate at low ionic strength did not increase the total specific $^{36}Cl^-$ uptake, but enhanced the DIDS and NPPB-sensitive portion of the uptake (Fig. 5A), presumably as a result of the partial removal of the inhibitor-insensitive pathways.

An attempt was made to correlate the reconstitutable Cl^- channel activity with specific proteins identified by SDS-PAGE. Initially it was found that 45% of the membrane protein but little activity were solubilized by 1% cholate at low ionic strength (Fig. 4a), whereas most of the reconstitutable activity was solubilized by a second extraction in 200 mM KCl (Fig. 4c). In comparison, a second extraction with 1% cholate in low ionic strength failed to solubilize significant Cl^- channel activity (Fig. 4b). The critical micellar concentration of cholate is highly dependent on the ionic strength, such that its capacity to solubilize membranes increases with increasing salt concentration [11]. Therefore, the membrane proteins likely to be solubilized by cholate in low ionic strength buffer will be of the peripheral rather than membrane-spanning type, whereas both types will be solubilized in high ionic strength buffer. Accordingly, a greater amount of the 95 kDa subunit of the Na^+/K^+-ATPase, an integral membrane protein [12], was solubilized by cholate in high than in low ionic strength buffer (Fig. 6, compare lanes b and c). A similar solubilization pattern would be expected for a presumably membrane-spanning Cl^- channel protein. Comparison of the Coomassie blue stained proteins solubilized by cholate at low and high ionic strength (Fig. 6A, lanes b and c) indicates that several proteins with molecular masses of 27, 31, 95 and 147 kDa were enriched in the latter fraction. A similar comparison of the $[^3H]_2$DIDS-labeled proteins solubilized by cholate at low and high ionic strength (Fig. 6, lanes b and c) reveals that only the 31 kDa protein is enriched in the high-ionic-strength soluble fraction. The specific enrichment of the 31 kDa protein in the fraction containing reconstitutable Cl^- channel activity together with its preferential labeling by $[^3H]_2$DIDS provide circumstantial evidence for its possible association with the Cl^- channel.

Further support for the above conclusion was provided by the finding that NPPB significantly reduced the labeling of the 31 kDa protein by $[^3H]_2$DIDS (Fig. 7). In contrast, DPC and DNDS which have been shown to be weak inhibitors of Cl^- channels in TALH vesicles (23% and 25% inhibition, respectively at 200 μM) [2] had no such effect. IAA-94/5 which inhibits the TALH Cl^- channel by 65% at 200 μM (Amir and Breuer, unpublished results) produced a barely detectable decrease in the labeling of the 31 kDa protein. However, the possibility that IAA-94/5 inhibits the Cl^- channels by binding to a site separate from the $[^3H]_2$DIDS binding site remains to be evaluated. Previously, both NPPB and DIDS have been shown to inhibit Cl^- channels in intact TALH membrane vesicles with ID_{50} of 100–200 μM, the former reversibly and the latter irreversibly. Moreover the irreversible inhibition by DIDS was prevented by NPPB but not by DPC [2]. Therefore the present finding that NPPB but not DPC protects the 31 kDa protein from labeling by $[^3H]_2$DIDS is consistent with previously observed effects on channel function.

In summary, two lines of evidence have been presented implicating a 31 kDa protein in TALH vesicles as a Cl^- channel component; (i) labeling of the 31 kDa protein by $[^3H]_2$DIDS and its specific protection by unlabeled DIDS and NPPB, and (ii) the presence of the $[^3H]_2$DIDS-labeled 31 kDa protein in detergent-soluble fractions containing reconstitutable Cl^- channel activity and its absence in fractions lacking this activity. However, the direct involvement of the 31 kDa protein in Cl^- conductance remains to be demonstrated.

Acknowledgement

The valuable technical assistance of Ms. Ada Zacharia is gratefully acknowledged. The author would like to thank Drs. Z.I. Cabantchik and R. Greger for generously providing $[^3H]_2$DIDS and NPPB. W.B. is a Jacob Pomereniec Renal Research Fellow and incumbent of the Roland Schaeffer Career Development Chair. This research was supported by the Fund for Basic Research

Administered by the Israel Academy of Sciences and Humanities.

References

1 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
2 Breuer, W. (1989) J. Membr. Biol. 107, 35–42.
3 Burnham, C., Karlish, S.J.D. and Jørgensen, P.L. (1985) Biochim. Biophys. Acta 821, 461–469.
4 Dubinsky, W.P. and Monti, L.B. (1986) Am. J. Physiol. 251, C713–C720.
5 Frizzell, R.A. (1987) Trends Neurosci. 10, 190–193.
6 Garty, H., Rudy, B. and Karlish, S.J.D. (1983) J. Biol. Chem. 258; 13054–13059.
7 Garty, H. and Benos, D.J. (1988) Physiol. Rev. 68, 309–373.
8 Gögelein, H. (1988) Biochim. Biophys. Acta 947, 521–547.
9 Greger, R. (1985) Physiol. Rev. 65, 755–797.
10 Greger, R., Schlatter, E. and Gogelein, H. (1985) Pflügers Arch. 403, 446–448.
11 Helenius, A., McCaslin, D.R., Fries, E. and Ranford, C. (1976) Methods Enzymol. 63, 734–749.
12 Jørgensen, P.L. (1988) Methods Enzymol. 156, 29–43.
13 Klaerke, D.A., Karlish, S. and Jørgensen, P.L. (1987) J. Membr. Biol. 95, 105–112.
14 Landry, D.W., Reitman, M., Cragoe, Jr., E.T. and Al-Awqati, Q. (1987) J. Gen. Physiol. 90, 779–798.
15 Mandel, K.G., Dharmsathaphorn, K. and McRoberts, J.A. (1986) J. Biol. Chem. 261, 704–712.
16 Miller, C. (ed.) (1986) Ion Channel Reconstitution, Plenum Press, New York.
17 Radian, R., Bendahan, A. and Kanner, B. (1986) J. Biol. Chem. 261, 15437–15441.
18 Sariban-Sohraby, S. and Benos, D.J. (1986) J. Biol. Chem. 261, 15437–15441.
19 Schlatter, E. and Greger, R. (1985) Pflügers Arch. 405, 367–376.
20 Vaandrager, A.B. and DeJonge, H.R. (1988) Biochim. Biophys. Acta 939, 305–314.
21 Valdivia, H., Dubinsky, W.P. and Coronado, R. (1988) Science 242, 1441–1444.
22 Welsh, M.J. (1987) Physiol. Rev. 67, 1143–1169.
23 White, M.M. and Miller, C. (1979) J. Biol. Chem. 254, 10161–10166.
24 Landry, D.W., Akabas, M.H., Redhead, C., Edelman, A., Cragoe, E.J. and AlAwqati, Q. (1989) Science 244, 1469–1472.
25 Laemmli, U.K. (1970) Nature 227, 680–685.
26 Pedemonte, C.H. and Kaplan, J.H. (1988) Biochemistry 27, 7966–7973.
27 Wangemann, P., Wittner, M., DiStefano, A., Englert, H.C., Lang, H.J., Schlatter, E. and Greger, R. (1986) Pflügers Arch. 407, S128–S141.
28 Bridges, R.J., Worrell, R.T., Frizzell, R.A. and Benos, D.J. (1989) Am. J. Physol. 256, C902–C912.