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Reconstitution, identification, and purification of the *Torpedo californica* electroplax chloride channel complex

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Abstract

A voltage-gated chloride channel was identified in the electric organ of the marine ray *Torpedo californica* by White and Miller (J. Biol. Chem. 254, 10161–10166 (1979)). The experiments reported here concern the purification and identification of this channel which was accomplished by solubilization of electric organ plasma membranes and reconstitution of the channel into vesicles made of phosphatidylethanolamine, phosphatidylserine, and cholesterol. Channel activity was measured in these vesicles by assaying $^{36}Cl^-$ uptake against an outwardly directed chloride chemical gradient as described by Garty et al. (J. Biol. Chem. 258, 13094–13099 (1983)). Maximal uptake occurred by 15 s. Addition of valinomycin after 10 min released intravesicular $^{36}Cl^-$ suggestin that chloride is moving through a channel. Channel activity was inhibited by DIDS ($K_{0.5}$ of 56 mM) and NBD chloride ($K_{0.5}$ of 176 mM). In a 40 lipid/1 protein (w/w) reconstitution, approx. 30% of the vesicles contained a functional chloride channel, based upon uptake done in the presence of chlorotriphenyltin (an anion ionophore), indicating that the *Torpedo* electric organ is an enriched source as shown by White and Miller (Biophys. J. 35, 455–462 (1981)). The chloride channel was purified approx. 40-fold by sedimentation velocity. In this purified preparation, four polypeptides (210, 95, 55, and 40 kDa) were visible by silver-staining after nonreducing SDS-PAGE. Of the four polypeptides, the largest (210 kDa) is not sufficient for Cl^- channel activity by itself, but it is labeled by DIDS, an inhibitor of channel activity. Channel activity was approx. 20-fold greater in material that bound to concanavalin A compared to the concanavalin A flow-through; all four polypeptides were present in the bound materia. It is possible that some of these polypeptides are subunits of the chloride channel.

Key words: Chloride channel; Chloride uptake; DIDS inhibition; Electroplax; Reconstitution; (*T. californica*)

1. Introduction

Voltage-gated and ligand-gated cation channels have been isolated and cloned from a variety of organisms and tissues [1,2]. Several plasma membrane chloride channels have also been cloned: ligand-gated channels [3,4]; the cystic fibrosis transmembrane regulator [5] and the P-glycoprotein [6,7], two membrane proteins with ATP-binding cassettes; and a family of voltage-gated channels [8–10]. These were cloned by homology to the structure of a chloride channel [11] identified by White and Miller [12] in the electric organ, electroplax, of the marine ray *Torpedo californica*. They demonstrated that this channel was specific for chloride, slightly permeable to bromide, and impermeable to other anions and to several cations [12,13]. And further, chloride conductance in planar bilayers could be inhibited by two stilbene disulfonates: SITS and DIDS [12]. In addition, experiments in which only a single Cl^- channel was inserted into the planar bilayer led Miller and White [14] to propose that the active form

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Abbreviations: SITS, 4-acetamino-4′-isothiocyano-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid; ^3H-H_2DIDS, 1,2-ditritio-4,4′-diisothiocyano-2,2′-disulfonic acid; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; PE, phosphatidylethanolamine; PS, phosphatidylserine; $C_{12}E_9$, polyoxyethylene 9 lauryl ether; DE 52, diethylaminoethyl cellulose; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate; con A, concanavalin A.

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of the channel is a homodimer. These are also the properties of the chloride channel cloned by Bauer et al. from the *Torpedo* electroplax [15].

While considerable progress has been made on the cloning and electrophysiological characterization of this family of voltage-gated chloride channels, their biochemical properties are relatively unknown. Kanemasa et al. [16] characterized an anion-specific channel from the electric organ of a related ray, *Narke japonica*, and demonstrated that the *Narke* channel is inhibited by DIDS and that the ion-selectivity of the *Narke* and *Torpedo* channels is very similar. Tank et al. [17] were able to solubilize and reconstitute the *Torpedo* chloride channel into artificial liposomes; the conductance properties of the reconstituted and native channels were identical. In addition, planar bilayer experiments with native *Torpedo* and *Narke* vesicles indicated that the channel was fairly abundant (approx. 50 to 300 copies per vesicle) [16,18]. Taguchi and Kasai [19] showed that tritiated-DIDS bound specifically to a polypeptide of 180 kDa present in *Narke* electroplax membranes. Under reducing conditions, this DIDS-binding polypeptide migrated with an apparent molecular mass of 90 kDa.

Landry et al. [20] purified a chloride channel from bovine kidney and trachea membranes by affinity chromatography on Sepharose 4B coupled to a derivative of indanyloxyacetic acid; four polypeptides with apparent molecular masses of 97, 64, 40, and 27 kDa were found in the fraction with chloride transport activity. Subsequent work suggested that the 64 kDa polypeptide was the most likely component of the chloride channel [21]. Anion channel activity has also been associated with proteins isolated from *Necturus* gallbladder epithelium ($M_r = 219\,000$) [22], bovine trachea and renal cortex ($M_r = 200\,000$ and 30 000–40 000) [23], and B lymphocytes ($M_r = 31\,000$) [24]. Ran et al. [25] isolated a protein of $M_r = 140\,000$ with anion conducting properties from bovine tracheal apical membranes; the protein appeared to be composed of four subunits of $M_r = 38\,000$ held together by disulfide bonds. Recently, Weber-Schurholz et al. [26] used the channel blocker indanyloxyacetic acid to enrich for chloride channels from rabbit skeletal muscle sarcolemma; a fraction with chloride channel activity isolated by affinity chromatography contained two specifically bound polypeptides ($M_r = 110\,000$–$120\,000$ and 60 000 by reducing SDS-PAGE). The 110–120 kDa polypeptide has the size of the chloride channel subunit deduced from the rat Clc-1 cDNA [8].

The ability to reconstitute Cl^- channel activity and the likelihood that the electroplax is an enriched source led us to attempt to purify this channel. The experiments described here concern the reconstitution, isolation, and identification of the *Torpedo* electroplax Cl^- channel. Chloride channel activity was monitored in reconstituted vesicles by measuring isotope fluxes. Several polypeptides are present in the fractions with the highest channel activity.

Goldberg and Miller [27] have also reconstituted the *Torpedo* channel in liposomes by similar methods.

2. Experimental procedures

Materials. All chemicals used were reagent grade or better. DIDS was purchased from Pierce; valinomycin was obtained from Sigma; NBD-Cl and chlorotriphenyltin were obtained from Aldrich. $^{36}Cl^-$ (13–17 mCi/g) was obtained from NEN as $H^{36}Cl$. ^3H-H_2DIDS (202 mCi/mmol) was purchased from Research Development Corporation (Toronto, Canada). 7-Chloro-4-nitro[U-^{14}C]benz-2-oxa-1,3-diazole (^{14}C-NBD-Cl) (130 mCi/mmol) was acquired from Research Products International. Phosphatidylethanolamine (PE), isolated from bovine brain, and cholesterol were purchased from Avanti Polar Lipids. Phosphatidylserine (PS), isolated from bovine brain, was obtained from Sigma. Na cholate and polyoxyethylene 9 lauryl ether ($C_{12}E_9$) were purchased from Sigma. Wheat germ agglutinin (WGA) (agarose-bound, 7 mg protein/ml) was purchased from Vector Laboratories and concanavalin A (con A) (Sepharose 4B-bound, 11 mg protein/ml) was obtained from Sigma. Diethylaminoethyl cellulose (DE 52) was obtained from Whatman. Anion exchange resin AG-1X8 (acetate form, 100–200 mesh) was purchased from Bio-Rad, as were poly-prep columns.

Preparation of plasma membrane. Plasma membranes were isolated from either fresh or frozen electric organs of *Torpedo californica* (obtained from Pacific Biomarine, Venice, CA) as described by White and Miller [19]. Membranes were stored in 0.4 M sucrose, 2 mM Hepes-KOH (pH 7.5) at −70°C; channel activity was stable for at least one year.

Reconstitution. Lipids were prepared as follows. PE, PS, and cholesterol (w/w ratio 6.3:2.4:1) were dried under nitrogen then lyophilized briefly. Lipids were solubilized (at a concentration of 10 mg/ml), in reconstitution buffer (150 mM KCl, 10 mM Hepes, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.4 adjusted with Tris base) containing 2% Na cholate, by heating at 60–70°C with vigorous stiring under nitrogen. Lipids were cooled on ice, membrane proteins, solubilized as described below, were added, the mixture was then transferred to Spectra/Por 4 dialysis tubing (12 000–14 000 molecular weight cut-off, 25 mm width) and dialyzed, in a cold room, against 1.6 liters of reconstitution buffer for 3–4 days with two or three changes of buffer.

$^{36}Cl^-$ uptake. All uptake experiments were performed on ice. Artificial liposomes, prepared as described above in reconstitution buffer were centrifuged

at 100 000 × *g* for 20 min. The pellet was resuspended in 300 μl 150 mM K gluconate, 10 mM Hepes, pH adjusted to 7.4 with Tris base. Vesicles were then added to 15 μl 150 mM K gluconate, 10 mM Hepes, 0.5 mCi $^{36}Cl^-$, pH adjusted to approx. 7 with Tris base. Final $^{36}Cl^-$ concentration was 3–4 mM. At various times after mixing, 50-μl aliquots were assayed for channel activity as follows. Extravesicular chloride was removed by passage of the 50 ml aliquot on an ice-cold 0.5 ml disposable anion exchange (AG-1X8) column, followed by 0.7 ml ice-cold isoosmolar glycerol. Aquasol (4 ml) was added to the eluate and the sample was counted in a Beckman LS 1801 scintillation counter. To demonstrate that uptake had occurred and that vesicles were sealed, two ionophores were used. After vesicles were incubated with $^{36}Cl^-$ for 10 min as described above, a 50 μl aliquot was added to either 5 μl valinomycin (0.025 mg/ml in DMSO) or to 5 μl chlorotriphenyltin (0.1 mM in DMSO). These samples were incubated on ice for an additional 5 min then passed on AG-1X8 as described above. Background counts were determined by passage of a 50 μl aliquot of $^{36}Cl^-$ in 150 mM K gluconate, 10 mM Hepes (pH 7.4). Typically, background counts were 90–120 cpm and were subtracted from all uptake values.

Ion selectivity. Experiments to test the permeability of other anions were performed as follows. Radiolabeled Cl^- uptake was carried out as described above except that the extravesicular salt was 120 mM K_2SO_4 or 145 mM KI or 160 mM KBr or 160 mM KNO_3 instead of 150 mM K gluconate; the salts were used at concentrations which provide constant osmolality. The concentration of $^{36}Cl^-$ was as described above. In some cases, liposomes were formed in the presence of either 145 mM KI or 160 mM KNO_3. Chloride uptake in these vesicles was as described above with K gluconate as the extravesicluar salt.

Purification of channel activity. Membranes (6 mg protein), solubilized in $C_{12}E_9$ as described below, were fractionated sequentially by ion-exchange chromatography, lectin affinity chromatography, and velocity sedimentation. PS (2.5 mg/ml in solubilization buffer containing 1% $C_{12}E_9$) was added to solubilized proteins to a final concentration of 0.25 mg/ml.

Solubilization of electroplax membranes. Native membranes, prepared as described above, in 0.4 M sucrose, 2 mM Hepes-KOH (pH 7.5), were thawed on ice, diluted 5–10 fold with solubilization buffer (150 mM KCl, 10 mM Hepes, 1 mM EDTA, pH adjusted to 7.4 with Tris base), then centrifuged at 100 000 × *g* for 15 min. The pellet was resuspended in 100–200 μl solubilization buffer and alkali extracted as follows. Ten volumes of 10 mM EDTA (pH 10.0) were added and the mixture was incubated at room temperature for 15 min. Membranes were centrifuged at 100 000 × *g* for 15 min and the pellet was solubilized in either Na cholate or $C_{12}E_9$ as follows. Membranes were resuspended in solubilization buffer at a concentration of approx. 4.5 mg starting protein/ml, then solubilized with either Na cholate at a concentration of 15 mg/ml or $C_{12}E_9$ at a concentration of 10 mg/ml, for 1 h on ice. Non-solubilized material was removed by centrifugation at 100 000 × *g* for 15 min.

Ion exchange chromatography. Solubilized membranes (4.5 mg of protein in 1.3 ml with 0.3 mg of added PS) prepared as described above were layered on a 1.2 ml (packed bed volume) DE 52 column (equilibrated with approx. 20 volumes of column buffer (solubilization buffer containing 0.25 mg/ml PS, 0.1% $C_{12}E_9$)) packed in a 11 ml poly-prep column. The first 1 ml was discarded, the remainder was saved and then pooled with material eluted by the addition of 1 ml column buffer. The final volume of this material was 1.5 ml and was termed the DE 52 flow-through. An aliquot of this material was saved for sedimentation velocity as described below.

WGA affinity chromatography. A 1.2 ml (packed bed volume) WGA column was equilibrated as described above for DE 52. The DE 52 flow-through was applied to the WGA column and, as for ion-exchange, the first 1 ml was not saved. The remainder of the flow-through plus the material eluted by the addition of 1 ml column buffer was saved (final volume 1.5 ml). An aliquot of this material was also saved for sedimentation velocity as described below.

Sedimentation velocity. Continuous sucrose gradients (5–30% or 5–37.5%) were made in solubilization buffer containing 0.2% Na cholate. The $C_{12}E_9$-solubilized material that was fractionated sequentially by DE 52 and WGA-agarose was layered on two 4.1 ml 5–37.5% sucrose gradients; the higher sucrose concentration was used to move the channel activity from the bottom of the tube. Aliquots saved from each fractionation step were layered on 4.7 ml 5–30% sucrose gradients. Gradients were centrifuged at 35 000 rpm in a SW 50.1 rotor for 15.5 h; 120-μl fractions were collected from each gradient. Fractions 1–25 from each 5–30% sucrose gradient (i.e., the region of each 5–30% gradient that contained all of the chloride channel activity, data not shown) were pooled, added to PE/PS/cholesterol, and reconstituted as described above. Fractions from the 5–37.5% sucrose gradients were treated as follows. Fractions 1 + 2, 3 + 4, etc. from each of the two gradients were pooled (final volume was 480 μl). 200 μl of each combined fraction were saved and used for protein determination, while 90 μl of each were reconstituted with 3 mg (total lipid) PE/PS/cholesterol as previously described.

Con A affinity chromatography. Material to be purified on con A was solubilized in 1.5% Na cholate as previously described. Solubilized material was applied to a con A column (equilibrated in solubilization buffer

containing 1% Na cholate) with a bed volume equal to the sample volume. The flow-through was collected, the column was washed with 2–3 column volumes of solubilization buffer/1% Na cholate, and bound material eluted with three column volumes of solubilization buffer containing 1% Na cholate, 0.5 M α-methyl-mannoside.

Inhibition of channel activity. Native membranes (1.8 mg protein) were alkali extracted and solubilized in Na cholate as previously described. Solubilized protein (70–80 μg) was treated with various amounts of either DIDS or NBD-Cl for 30 min at 0°C, added to 5 mg (total lipid) PE/PS/cholesterol and reconstituted as described above. Control samples were treated with an equivalent amount of H_2O (solvent for DIDS) or DMSO (solvent for NBD-Cl) for 30 min, added to 5 mg PE/PS/cholesterol and reconstituted as described above.

Polyacrylamide gel electrophoresis. Polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed, under non-reducing conditions, as described by Laemmli [28]. Bands were visualized by staining with silver as described by Wray et al. [29]. Gels were transferred to nitrocellulose by the procedure of Towbin et al. [30]. The position of the α-subunit of the Na^+,K^+-ATPase was determined using a 1:1000 dilution of antibody 620 (Lytton, J., Harvard University).

^{3}H-H_2DIDS labeling. Electroplax membranes (46 μg protein) were incubated in all cases with 0.4 mCi (100 mM) ^{3}H-H_2DIDS for 1 h at 0°C. A total of three reactions were done with increasing amounts (0, 100, and 200 μM) of non-radioactively labeled DIDS. Samples were fractionated on a 7.5% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250, destained, and then treated with EN^{3}HANCE (NEN) as described by the manufacturer. The gel was dried under vacuum and exposed at −70°C to Kodak XAR-5 film.

Protein determination. Protein determinations were performed utilizing the method of Lowry et al. [31] as modified by Peterson [32]; bovine serum albumin was used as the standard.

Statistical analysis. Nonlinear least-squares analyses were done on an AT&T AT computer using the RS/1 package from BBN research Systems (Cambridge, MA). The data were fit to the equation:

$$\text{uptake} = (\text{uptake}_{max} \times K_{0.5})/(I + K_{0.5})$$

where $K_{0.5}$ is the concentration of inhibitor for inhibition of half the uptake and I is the concentration of inhibitor.

3. Results

Reconstitution of chloride channel activity

Tank et al. [17] demonstrated that membranes from *Torpedo* electroplax could be solubilized in sodium cholate and reconstituted into artificial liposomes. Channel activity was measured by patch-clamping freeze-thawed reconstituted liposomes. This solubilization and reconstitution procedure was repeated (except for freeze-thawing) with slight modifications as described in Experimental procedures; channel activity was measured in the following way. Vesicles were formed, in the presence of 150 mM KCl, by dialysis of the Na cholate-solubilized lipid/protein mix. Typically, a lipid to protein ratio of 40:1 (w/w) was used for cholate-solubilized material. External KCl was replaced, by centrifugation, with isoosmolar K gluconate containing 3–4 mM $^{36}Cl^-$ to generate a chloride gradient from inside to outside. The assumption is that the concentration gradient for chloride would cause an inside positive membrane potential if there is a chloride channel; under these circumstances, as described by Garty et al. [33], radioactive chloride will be driven into the vesicle by the electrical gradient. As shown in Table 1, chloride uptake was very rapid in protein-containing vesicles; maximal uptake had occurred by the first time point (15 s). The amount of uptake did not change over a 15 min period indicating that vesicles of this composition (PE/PS/cholesterol) are not leaky to either chloride or potassium. If these vesicles were permeable to potassium, then, as described below, equilibration and not accumulation of the isotope would occur. The fact that non-protein-containing vesicles did not accumulate $^{36}Cl^-$ indicated that these liposomes are also not leaky to chloride (Table 1).

To demonstrate that the uptake observed was due to $^{36}Cl^-$ entering the vesicle and not to non-specific sticking of the isotope to protein-containing vesicles,

Table 1
Chloride uptake in reconstituted vesicles

Time (min)	^{36}Cl (cpm) [a]	
	lipid vesicles	proteoliposomes
0.25	35 ± 15	905 ± 320
1	40 ± 20	825 ± 155
15	30 ± 10	710 ± 50
10 (+valinomycin)	80 ± 25	320 ± 130
10 (+triphenyltin)	950 ± 120	2580 ± 310

Electroplax membranes were solubilized in Na cholate and reconstituted into vesicles made of PE/PS/cholesterol at a final lipid/protein ratio (w/w) of 40:1. Vesicles were also formed without added electroplax proteins. Uptake was performed as described under Experimental procedures. Ionophores were added to a final concentration of 2.5 μg/ml for valinomycin and 10 μM for chlorotriphenyltin. Vesicles were incubated for 5 min with either ionophore before removal of extravesicular $^{36}Cl^-$ with AG-1X8.

[a] Mean ± S.D. ($n = 4$). This experiment is representative of four similar experiments.

the potassium ionophore, valinomycin, was used. Addition of valinomycin for 5 min after a 10 min period of incubation with $^{36}Cl^-$ resulted in a loss of label from the vesicles (Table 1). Valinomycin conducts potassium across the membrane and, in the absence of a potassium concentration gradient, reduces the membrane potential to zero decreasing the influx of chloride and increasing its efflux. This result strongly suggests that chloride is moving through a channel.

Addition of a chloride ionophore, chlorotriphenyltin [34], allowed for an estimate of channel abundance (i.e., percentage of vesicles containing a functional channel). As is shown in Table 1, the amount of chloride taken up by the protein-containing vesicles was 30% of that taken up by the same vesicles in the presence of chlorotriphenyltin (710 cpm $^{36}Cl^-$ for 15 min time point versus 2580 cpm $^{36}Cl^-$ in the same vesicles treated with chlorotriphenyltin). Since the equilibrium value was reached in 15 s and did not change with time, this result suggests that 30% of the vesicles have an active chloride channel. Chlorotriphenyltin-induced uptake in vesicles made without protein was about half that as for protein-containing vesicles (950 cpm versus 2580 cpm, Table 1). A difference in vesicle size between protein and non-protein-containing vesicles is not uncommon in the reconstitution of membrane proteins; the size of the liposomes depends on the detergent, the lipids and the membrane used for the reconstitution [35,36].

Taken together, these results indicate that the electroplax Cl^- channel can be incorporated into artificial vesicles as described by Tank et al. [17] and that activity can be measured by isotope fluxes. The ability to follow channel activity by isotope fluxes, which is directly related to channel abundance, indicates that a chloride channel is enriched in the electroplax of marine rays, as first described by White and Miller [18] and Kanemasa et al. [16]. A question remained, however, as to whether the channel reconstituted as described above is the same channel originally studied by White and Miller in the planar bilayer system.

DIDS inhibition

Stilbene disulfonates have been used to inhibit the activity of the chloride/bicarbonate exchanger of the red cell, Band III [37]. The voltage-gated chloride channel present in *Torpedo* electroplax is also inhibited by DIDS with an apparent $K_{0.5}$ of approx. 10 μM [12]. It was also shown that inhibition in the planar bilayer system, employed by White and Miller, is orientation-specific; DIDS only affected channel activity when added to the *cis* side (the same side to which native vesicles were added) [12].

With the reconstituted vesicles described above, addition of DIDS did not affect chloride uptake (data not shown). A possible, and the most likely, explanation for this lack of DIDS inhibition is that channel orientation in these vesicles was not random. The arrangement of reconstituted proteins in lipid vesicles, whether random or oriented, has been shown to be variable depending on the procedure for reconstitution and the lipid mixture [38–41]. To demonstrate that channel activity could be inhibited by DIDS, cholate solubilized electroplax membrane proteins were treated with various concentrations of the stilbene for 30 min at 0°C then reconstituted into liposomes. DIDS inhibited channel activity with a $K_{0.5}$ of 56 μM (Fig. 1). It should also be noted that DIDS-treated cholate-solubilized proteins were dialyzed (during reconstitution with lipid) for several days with several changes of buffer. The fact that DIDS still inhibited channel activity indicated that, as observed by White and Miller [12], DIDS inhibition was not readily reversible. The ability of DIDS to inhibit $^{36}Cl^-$ uptake suggests that the channel being studied here is the same or very similar to the channel described by White and Miller. The larger value for $K_{0.5}$ for the recostituted channel compared to the native one is a result of the exposure of the protein to the detergent [42].

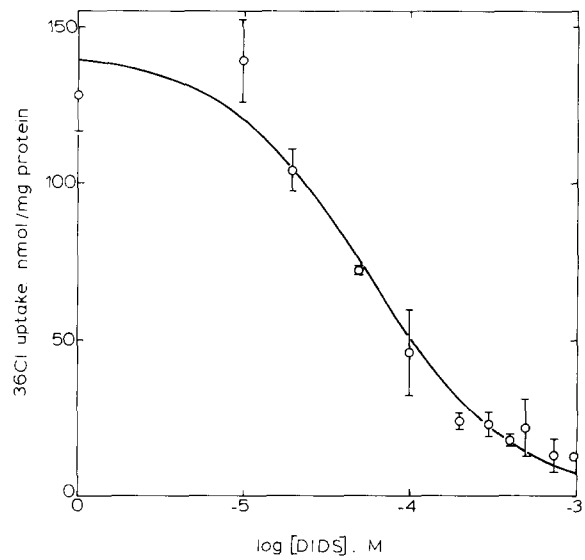

Fig. 1. Inhibition of $^{36}Cl^-$ uptake by DIDS. DIDS inhibition of $36Cl^-$ uptake was demonstrated by treating Na cholate solubilized electroplax membranes with the indicated concentrations of DIDS for 30 min on ice. Reconstitution was carried out and $^{36}Cl^-$ uptake was measured as described in Experimental procedures. The figure is the average of the 15 s, 1 min and 15 min time points at each concentration $\pm$S.D.; $n = 3$. The data were fit according to a single-component inhibition curve; $K_{0.5} = (56.5 \pm 9.7) \cdot 10^{-6}$ M, $r^2 = 0.988$.

When tritiated H_2DIDS was allowed to react with native electroplax membranes for 30 min and the proteins were separated by SDS PAGE (Fig. 2A, lane 1), several polypeptides were observed by staining with Coomassie blue, including prominent ones of 210 and 95 kDa. There was a strongly-labeled polypeptide of 210 kDa under nonreducing conditions (Fig. 2B, lane

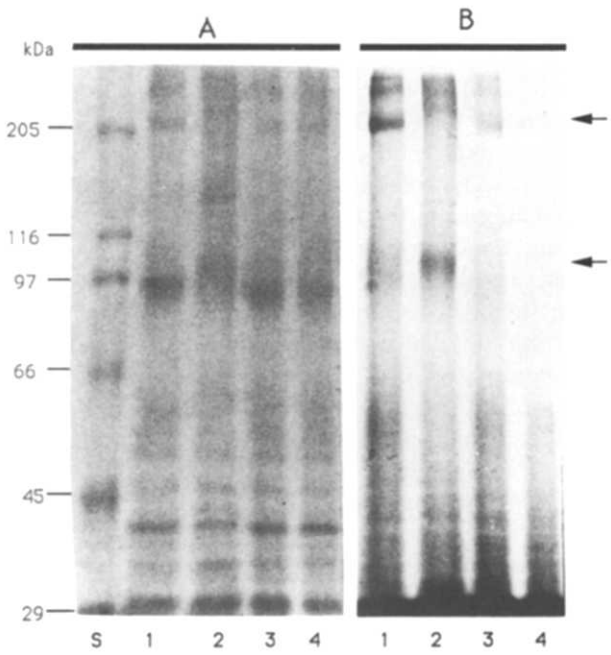

Fig. 2. ^{3}H-H$_2$DIDS binding polypeptides. Native electroplax membranes (46 μg protein) were incubated with 100 μM ^{3}H-H$_2$DIDS for 1 h at 0°C. Samples were fractionated on a 7.5% SDS-polyacrylamide gel, stained with Coomassie blue, and treated with EN^{3}HANCE. (A) Coomassie stained gel; lane S molecular mass markers: 205 kDa, 116 kDa, 97 kDa, 66 kDa, and 45 kDa; lane 1 sample treated with ^{3}H-H$_2$DIDS; lane 2 same as lane 1 except that the sample was treated with 2-mercaptoethanol before loading; lane 3 same as lane 1 except that the sample was treated with 100 mM ^{3}H-H$_2$DIDS and 100 μM DIDS for 1 h at 0°C; lane 4 same as lane 1 except that the sample was treated with 100 μM ^{3}H-H$_2$DIDS and 200 mM DIDS for 1 h at 0°C. (B) Autoradiograph of gel shown in A. Gel was exposed to Kodak XAR-5 film at −70°C for 2 days with an intensifying screen. Arrows point to the principal labeled polypeptides before and after reduction.

Table 2
Anion selectivity of the reconstituted channel

Intravesicular anion [a]	Extravesicular anion [a]	$^{36}Cl^-$ uptake
Cl^-	gluconate	yes
Cl^-	Br^-	no
Cl^-	NO_3^-	no
NO_3^-	gluconate	yes
Cl^-	I^-	uptake reduced by ~70%
I^-	gluconate	no
Cl^-	SO_4^{2-}	yes

[a] In all cases the potassium salt was used. Concentration of each was such that the osmolarity was equal to that of 150 mM KCl.

1); upon reduction with mercaptoethanol, the label was principally associated with a 105 kDa polypeptide derived from the 210 kDa peptide (Fig. 2B, lane 2). This 105 kDa polypeptide is distinct from the 95 kDa polypeptide, which is weakly labeled. Similar results were obtained by Taguchi and Kasai [19] with the membranes from the *Narke* electroplax and by Jentsch et al. [43] who cloned the cDNA for this polypeptide and showed that it does not itself promote channel activity.

Ion selectivity

Miller and White [13] demonstrated that the electroplax channel was very specific for chloride. Ion selectivity of the reconstituted channel was examined by $^{36}Cl^-$ uptake experiments as described except that the intravesicular and extravesicular anions were varied. Experiments in which Br^- was the extravesicular anion demonstrated that $^{36}Cl^-$ uptake was abolished

Table 3
Purification of chloride channel activity

Step	Total protein (mg)	Specific activity [b] (nmol $^{36}Cl^-$/mg protein)	Purification (fold)	Peak position
Native vesicles	6.0	n.d. [a]		
Alkali extracted	3.9	n.d.		
C12E9 solubilized	2.2	89.0 ± 7.0	1	
DEAE flow through	1.2	68.1 ± 1.7	0.77	
WGA flow through	0.45	72.3 ± 9.8	0.81	
Sucrose gradient fractions				
3 + 4	0.0045	no uptake		
5 + 6	0.0081	1279.5 ± 291.4	14	β-galactosidase (15.9 S)
7 + 8	0.013	3538.3 ± 232.1	40	
9 + 10	0.018	3496.1 ± 207.9	39	
11 + 12	0.021	2150.1 ± 303.2	24	
13 + 14	0.026	1025.1 ± 212.2	11	
15 + 16	0.032	no uptake		catalase (11 S)
25 + 26	0.032–0.050	no uptake		

Cholate solubilized 20% of the membrane proteins; the specific activity of this material was 16.8 ± 0.4 nmol $^{36}Cl^-$/mg protein. Chloride channel activity was measured as described in Experimental procedures.
[a] n.d., not determined; [b] Mean ± S.D.

(Table 2). The most likely explanation is that, as observed by Miller and White [13], the *Torpedo* electroplax channel is also selective to this anion. In contrast to the planar bilayer system, extravesicular NO_3^- was also capable of eliminating $^{36}Cl^-$ uptake. Either the Cl^- channel under these experimental conditions is also selective for NO_3^- or NO_3^- may block the channel and prevent chloride movement. To distinguish between these two possibilities, artificial vesicles were formed with 160 mM KNO_3 inside instead of 150 mM KCl. Chloride channel activity was also observed in these vesicles (Table 2, experiment 4), indicating that this channel is capable of exchanging intravesicular NO_3^- for extravesicular $^{36}Cl^-$.

Of all the other anions tested by Miller and White [13], only I^- and SCN^- had an effect on channel activity. Both of these anions blocked chloride movement. As shown in Table 2 (experiment 5), extravesicular I^- did in fact reduce $^{36}Cl^-$ uptake. And unlike NO_3^-, intravesicular I^- eliminated $^{36}Cl^-$ uptake (experiment 6) suggesting that I^- does block the channel. External sulfate did not affect chloride movement (experiment 7), indicating that the channel is not selective for this ion.

Purification

Channel activity was monitored by reconstitution of solubilized membrane proteins and assayed by radioactive chloride uptake as described. Electroplax membranes, prepared as described by White and Miller [18], were, as a first step, subjected to alkali extraction to remove membrane associated proteins. Alkali extraction removed approx. one-third of the total protein (Table 3). Membranes, after alkali extraction, were resuspended in a KCl buffer and solubilized in the non-ionic detergent polyoxyethylene 9 lauryl ether ($C_{12}E_9$). $C_{12}E_9$-solubilized proteins were reconstituted by switching from $C_{12}E_9$ (which has a very low critical micellar concentration (CMC)) to sodium cholate (which has a higher CMC) via a sucrose gradient. This switch served two purposes. One was to allow vesicle formation by dialysis. A second purpose was as a purification step (see below). As shown in Table 3, $C_{12}E_9$-solubilized material contained a fair amount of chloride channel activity (89.0 nmol $^{36}Cl^-$ uptake/mg protein) which was about 5-fold greater than cholate-solubilized material (16.8 nmol $^{36}Cl^-$ uptake/mg protein).

Because chloride channel activity was greater in $C_{12}E_9$-solubilized membranes than in Na cholate-solubilized material, $C_{12}E_9$ was chosen for subsequent purification steps. Ion exchange chromatography (DE 52 cellulose) and lectin affinity chromatography (WGA) did not result in an increase in specific activity (Table 3). The flow-through from the WGA column was fractionated on a 5–37.5% sucrose gradient containing Na cholate. Channel activity migrated close to the bottom of the gradient (fractions 7–10 out of 31 total fractions). An estimate of channel size was achieved using β-galactosidase and catalase as markers; the peaks of these proteins eluted at fractions 5 + 6 and 15 + 16, respectively. Channel activity peaked slightly after the

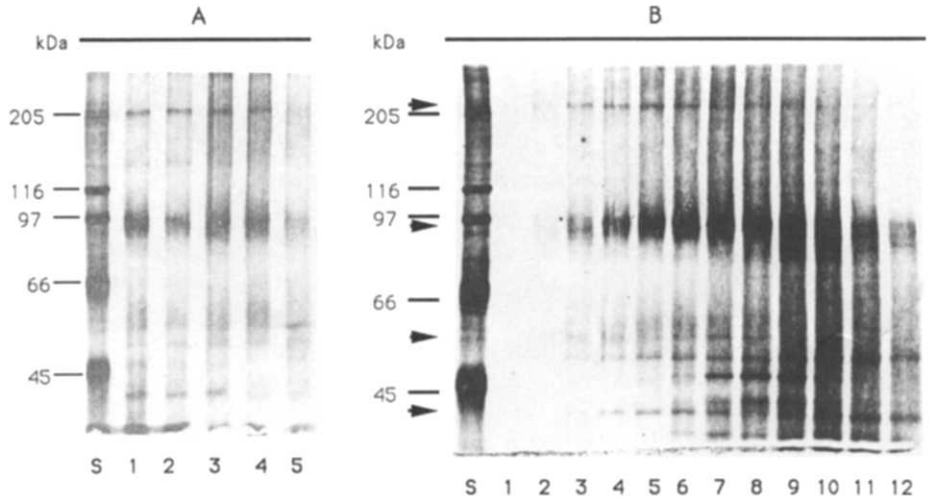

Fig. 3. Electrophoretic pattern of proteins present in each purification step. (A) Approximately 0.6–0.8 μg of each fraction was subjected to SDS-PAGE (7.5% gel). Lane S, molecular mass markers: 205 kDa, 116 kDa, 97 kDa, 66 kDa, and 45 kDa; lane 1, native electroplax membranes; lane 2, membranes after alkali extraction; lane 3, $C_{12}E_9$ solubilized material; lane 4, DEAE 52 flow-through; lane 5, WGA flow-through. (B) An aliquot (15 μl) of each fraction from the 5–37.5% sucrose gradient was subjected to nonreducing SDS-PAGE on a 7.5% polyacrylamide gel. Arrows point to the four polypetides that correlate with chloride channel activity. Lane S molecular mass markers: 205 kDa, 116 kDa, 97 kDa, 66 kDa, and 45 kDa; lane 1 fractions 3 and 4; lane 2 fractions 5 and 6; lane 3 fractions 7 and 8; lane 4 fractions 9 and 10; lane 5 fractions 11 and 12; lane 6 fractions 13 and 14; lane 7 fractions 15 and 16; lane 8 fractions 17 and 18; lane 9 fractions 19 and 20; lane 10 fractions 21 and 22; lane 11 fractions 23 and 24; lane 12 fractions 25 and 26.

position as β-galactosidase, suggesting that the channel or channel complex has a sedimentation coefficient in the Na cholate micelle of 14 S, corresponding to an apparent M_r of 400000. A 40-fold increase in specific activity was obtained in the most active fractions [7–10] (3500 nmol $^{36}Cl^-$ uptake/mg protein, Table 3) of the sucrose gradient compared to $C_{12}E_9$-solubilized material (89.0 nmol $^{36}Cl^-$ uptake/mg protein, Table 3).

Polyacrylamide gel analysis of the proteins present in each stage of the procedure (Figs. 3A and 3B) shows that ion exchange and lectin affinity chromatography produce little change in the composition of the fractions (Fig. 3A); after sucrose density centrifugation several polypeptides, visible by silver staining, are present in the fractions with channel activity (Fig. 3B, lanes 2–6 in this experiment). The four polypetides indicated by the arrows, with apparent masses of 210, 95, 55, and 40 kDa, are suggested as components of the channel for the following reasons. (i) Although present in gradient fractions without channel activity, most of the 210 kDa, the 55 kDa and the 40 kDa polypeptides are present in fractions 2 to 8, the amount in lane 8 less than that in lane 7. (ii). The material with a mass of 95 kDa is present in all fractions because the major part of this band is the α-chain of the Na^+,K^+-ATPase. However, the material in lanes 3 and 4 appears as a doublet, suggesting heterogeniety. (iii). The peaks with masses of 50 kDa, 47 kDa, 42 kDa and 30 kDa are unlikely candidates for channel components because their amounts are largest in peaks 8, 9 and 10.

As mentioned previously, the 210 kDa polypeptide has been shown not to be necessary for channel activity in oocyte injection experiments [43]. The question, therefore, was whether any, all or a subset of these four polypeptides, which appear to copurify, are part of the channel.

As a means of identifying the channel protein, reagents capable of covalently modifying amino acid side chains were tested for their ability to inhibit $^{36}Cl^-$ uptake. Diethyl pyrocarbonate (at 1 mM) completely inhibited channel activity (data not shown); however, radiolabeled diethyl pyrocarbonate was not readily available. Hence, other compounds were tested. NBD-Cl inhibited chloride uptake with a $K_{0.5}$ of 176 μM (Fig. 4). Incubation of ^{14}C-NBD-Cl with native membranes followed by fractionation on SDS-PAGE revealed a major labeled band which had an apparent molecular mass of approx. 95 kDa (data not shown). However, these membranes are enriched in the Na^+,K^+-ATPase whose catalytic (α-chain) subunit migrates with an apparent molecular mass of 93 kDa [43]. And, further, Cantley et al. [44] have shown that NBD-Cl binds to the α-chain of the Na^+,K^+-ATPase. It seemed possible, therefore, that much if not all of the NBD-Cl binding in the region of 95 kDa could be due to the ATPase. In an attempt to separate chloride

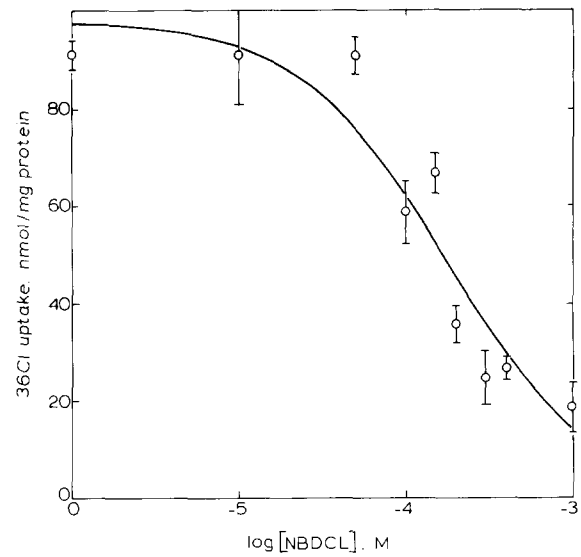

Fig. 4. Inhibition of $^{36}Cl^-$ uptake by NBD chloride. NBD chloride inhibition of $^{36}Cl^-$ uptake was demonstrated by treating Na cholate solubilized electroplax membranes with the indicated concentrations of NBD chloride for 30 min on ice. Reconstitution was carried out and $^{36}Cl^-$ uptake was measured as described in Experimental procedures. The figure is the average of the 15 s time points at each concentration ±S.D.; $n = 4$. The data were fit according to a single-component inhibition curve; $K_{0.5} = (176 \pm 44) \cdot 10^{-6}$ M, $r^2 = 0.980$.

channel activity from the Na^+,K^+-ATPase, native membranes were solubilized in Na cholate and fractionated on concanavalin A. Chloride channel activity is present almost exclusively in the α-methylmannoside eluted fraction (Table 4). The polypeptides in the flow-through (lanes 1, 2, and 3) and the bound fractions (lane 4) of the con A column were analyzed by polyacrylamide gel electrophoresis; the results are shown in Fig. 5A. Lanes 1, 2, and 3 were loaded with 0.5, 1, and 5 μg of protein, respectively; the principal band is present at a $M_r = 95000$, although other polypeptides are visible in lane 3. On the other hand, the polypeptides with masses of 210, 95, 55, and 40 kDa, associated with the chloride channel activity in the sucrose gradients, are clearly discernible in lane 4, loaded with 5 μg of protein from the bound fraction. The 210 and 40 kDa polypeptides are not present in the flow-through of the column. Immunoblotting of these fractions with antibody 620 (Lytton, J., Harvard University), specific for the α-chain of the Na^+,K^+-

Table 4
Purification of chloride channel activity on concanavalin A

	Protein (μg)	nmol $^{36}Cl^-$/mg protein [a]
Con A flow-through	396	2.5 ± 0.5
Con A bound	66	49.0 ± 1.7

Chloride channel activity was not recovered when $C_{12}E_9$-solubilized material was fractionated on the con A column.
[a] Mean ± standard deviation.

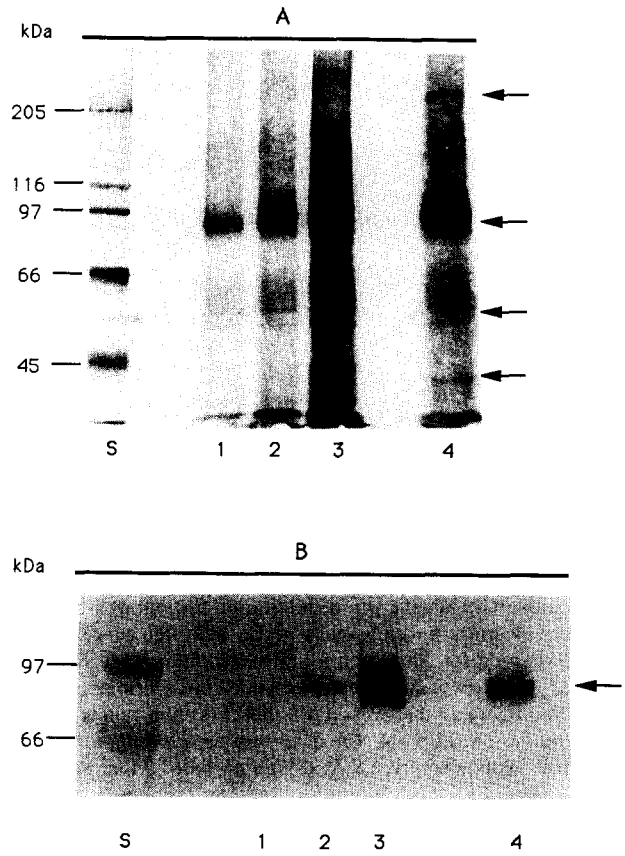

Fig. 5. Protein pattern and immunoblot of material purified on concanavalin A. Membranes were solubilized in Na cholate and fractionated on concanavalin A as described in Experimental procedures. (A) Material that did not bind to con A and con A bound material were electrophoresed on a nonreducing 7.5% polyacrylamide gel. Gel was silver stained by the method of Wray et al. (1981). Lane S, molecular mass markers: 205 kDa, 116 kDa, 97 kDa, 66 kDa, and 45 kDa; lanes 1, 2, and 3: 0.5, 1, and 5 μg, respectively, of con A flow-through; lane 4, approx. 5 mg of con A bound material. Arrows point to polypeptides present in the con A bound material which are also associated with chloride channel activity in the sucrose gradients. (B) A second gel run as in A was transferred to nitrocellulose and probed with a 1:1000 dilution of antibody 620 (J. Lytton, Harvard University). Lane S: molecular mass markers; lanes 1, 2, and 3: con A flow-through; lane 4 con A bound. The position of the α-subunit was determined using a horse radish peroxidase-conjugated second antibody.

ATPase, demonstrated that only a small amount of the α-chain was bound to con A (Fig. 5B).

To determine whether or not all of the NBD-Cl binding in the 95 kDa region was due to the α-chain of the Na pump, electroplax membranes were solubilized in Na cholate, reacted with 1 mM ^{14}C-NBD-Cl, and fractionated on con A agarose; the con A flow-through and α-methylmannoside eluted fractions were then analyzed by SDS-PAGE. There were similar amounts of labeled 95 kDa polypeptide in both con A fractions, although most of the α-chain of the Na^+,K^+-ATPase is in the flow-through. This result suggests that NBD-Cl preferentially labels a polypeptide that is different from the α-chain of the Na^+,K^+-ATPase. Since the polypeptide of approx. 95 kDa is present in the gradient fractions with channel activity and is labeled by an inhibitor (NBD-Cl) of $^{36}Cl^-$ uptake, it is likely that this polypeptide is part of the channel complex. The role of the other polypeptides in channel function is not known.

4. Discussion

Biochemical purification of ion channels has proven to be difficult due to low protein abundance in many systems and the necessity to monitor activity by reconstitution. To circumvent these difficulties, tissues rich in the desired channel have been used, such as electric organs for Na channels and certain ligand-gated channels [45,46]. Studies by White and Miller [19] and Kanemasa et al. [16] demonstrated that the electroplax of certain marine rays is an enriched source for a voltage-gated chloride channel.

In this study, chloride channel activity was monitored by isotope fluxes. Radioactive Cl^- uptake, as a measure of channel activity, was rapid; maximal uptake had occurred by the first time point (15 s) (Table 1). This result was expected since White and Miller [18] showed that in light-scattering experiments with native electroplax vesicles, vesicle reswelling (indicative of net Cl^- or KCl movement) has a half-time of 125 ms. This result, however, is at odds with that obtained by Goldberg and Miller [27] using liposomes with the composition PE/PC/PS/cholesterol in the molar ratio 5:1:2:2; in that study the half time for the concentrative uptake was approx. 20 min. We attribute the difference in the time for uptake to the lipid composition which results in very small vesicles with the mixture of PE/PS/cholesterol in the weight ratio of 6.3:2.4:1, as compared to the large vesicles obtained by Goldberg and Miller [27].

Even though detailed kinetics for chloride movement could not be measured by isotope fluxes, it is very likely that the channel identified in this study is the same channel studied by White and Miller in the planar bilayer system. This statement comes from the fact that DIDS inhibits channel activity as measured by isotope fluxes (Fig. 1) and in planar bilayers [12]. A slight difference between these two types of experiments, however, was the magnitude of DIDS inhibition. White and Miller [12] using the planar bilayer system determined a $K_{0.5}$ for DIDS of 10 μM. This is in contrast to a $K_{0.5}$ of 56 μM for $^{36}Cl^-$ uptake as shown in Fig. 1. One contributing factor for this difference is the number of channels studied by the two methods. On the order of 3–5 channels, and occasionally only one, were studied and inactivated by DIDS in the bilayer system [14]. Whereas in the experiment

shown in Fig. 2, ~70 μg of solubilized protein was reacted with DIDS. This dramatic difference in the amount of total membrane protein present during incubation with DIDS may have contributed to the 5-fold difference in $K_{0.5}$ values, since other polypeptides that bind stilbenes, such as the Na^+,K^+-ATPase, were present in cholate solubilized-treated material. Moreover, in the planar bilayer system used by White and Miller, it is possible that only the Cl^- channel was incorporated; the cation channel associated with the acetylcholine receptor was not detected [12] and therefore other proteins such as the Na^+,K^+-ATPase may not have fused with the bilayer and were not present to react with the stilbene. A second contributing factor, which is related to the amount of protein, for this approx. 5-fold difference in $K_{0.5}$ values was the amount of phospholipid present during DIDS treatment. Interaction of DIDS with the lipid headgroups (most likely PE) could result in a greater underestimate in the experiments reported here than in the planar bilayer system.

Another indication that the channel studied by $^{36}Cl^-$ uptake is probably the same as that studied in the planar bilayer was its ion specificity. Experiments in which the extravesicular anion was varied demonstrated that, in addition to Cl^-, this channel was also selective for Br^- and NO_3^- and not for SO_4^{2-} (Table 2). In addition, I^- appeared to block the channel, possibly in an analogous manner to that of Cs^+ blocking certain K^+ channels [47]. These results are consistent, with the exception of NO_3^-, to those obtained by Miller and White [13].

Tank et al. [17] showed that chloride channel activity could be reconstituted from the electric organ of *T. californica*. This fact, coupled with an estimate of channel abundance as high as 10% in a related ray [16], raised the possibility that this channel could be purified by conventional biochemical techniques. In this report, channel activity was purified approx. 40-fold by sedimentation velocity. Channel activity was not enriched appreciably by either ion-exchange or WGA affinity chromatography.

Four polypeptides (210, 95, 55, and 40 kDa) are present in the fractions from the sucrose gradients with channel activity (Fig. 3B). It is likely that a component of the 95 kDa polypeptide is part of the channel. In the first place, the channel isolated by expression cloning from *Torpedo* marmorata by Jentsch et al. [11] is composed of 805 amino acids with a calculated $M_r = 89\,000$; glycosylation of the protein would be expected to increase the size to approx. 95 kDa. Secondly, NBD-Cl inhibits chloride channel activity and labels a band of 95 kDa which binds to con A. Channel activity is present exclusively in material that binds to con A (49 nmol ^{36}Cl uptake/mg protein in con A bound material compared to 2.5 nmol ^{36}Cl uptake/mg protein in con A flow-through, Table 4). Since the α-chain of the Na^+,K^+-ATPase is also labeled by NBD-Cl [44], it could account for the labeled 95 kDa polypeptide bound to con A. Immunoblotting of the fractions, however, revealed that 15–20% of the α-chain (Fig. 4B) and more than 50% of the NBD-Cl labeled polypeptides were bound to con A, suggesting that part of the labeled polypeptide bound to con A was a component of the channel. The largest polypeptide, 210 kDa, is by itself insufficient for chloride channel activity. This was shown by *Xenopus* oocyte injection experiments performed by Jentsch et al. [43] and by fractionation on sucrose gradients which demonstrated that channel activity does not completely correlate with this polypeptide (Fig. 3B). However, the 210 kDa polypeptide could be a channel subunit, which might not be detected by the hybrid deletion experiment [43] because present endogenously in the *Xenopus* oocyte. In this work, the 210 kDa polypeptide was present in all fractions that contained channel activity; the inability to separate channel activity from this polypeptide raises the possibility that the 210 kDa polypeptide may be a channel subunit.

This notion is supported by several observations. One is that channel activity is inhibited irreversibly by DIDS and that DIDS (or the related SITS) appears to bind to only two polypeptides in electroplax membranes, one of which is the 210 kDa (43 and these results). A second fact is the apparent mass of the channel. Sedimentation velocity experiments indicate that the channel migrates with a mass equivalent to approx. 14 S. However, as mentioned above, separation of chloride channel-containing fractions by SDS-PAGE, under non-reducing conditions, did not reveal a polypeptide of 400 kDa (approximate mass of a protein with a sedimentation coefficient of 14 S). Instead four different polypeptides were visible. It is tempting to speculate that the channel might be composed of one 210 kDa polypeptide, itself a disulfide linked dimer of 105 kDa subunits, and single or multiple copies of the 95 kDa, 55 kDa, and 40 kDa channel proteins with a total mass of approx. 400 kDa.

Acknowledgments

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