J. Membrane Biol. 124, 199–206 (1991)

The Journal of
Membrane Biology

Solubilization and Functional Reconstitution of a Chloride Channel from *Torpedo californica* Electroplax

Andrew F.X. Goldberg and Christopher Miller
Howard Hughes Medical Institute, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts

Summary. Chloride channels were detergent-extracted from *Torpedo* electroplax plasma membrane vesicles and reconstituted into liposomes by rapid detergent removal and a freeze-thaw-sonication procedure. Concentrative uptake of $^{36}Cl^-$, driven by a Cl^- gradient was used to determine conductance properties of reconstituted channels. Chloride flux assayed by this method is strongly selective for Cl^- over cations, is blocked by SCN^-, inactivated by treatment with DIDS, and exhibits an anion selectivity sequence $Cl^- > Br^- > F^- > SO_4^{2-}$, as does the voltage-gated Cl^- channel from *Torpedo* observed in planar lipid bilayers. The channels are localized to the noninnervated face of the electrocyte, and a novel trapped-volume method is used to estimate a channel density on the order of 500 pmol/mg protein. An initial fractionation of the membrane extract by anion exchange chromatography yields fivefold enrichment of the channel activity.

Key Words ion channel · chloride · electroplax · reconstitution · liposomes · flux assay

Introduction

Marine electric rays generate high-power currents through the interplay of nicotinic acetylcholine receptor cation channels and voltage-gated Cl^- channels operating in the electroplax organ. The acetylcholine receptor channel functions as a discharge switch, while the Cl^- channel is thought to set a high potential across the electrocyte's noninnervated membrane and thus maintain the electric organ as a high-voltage, low-resistance battery (Miller & Richard, 1990). The Cl^- channel has been extensively characterized at the single-channel level in planar lipid bilyers (Miller & Richard, 1990), and more recently its cloning and functional expression have been reported (Jentsch, Steinmeyer & Schwarz, 1990). The lack of a specific high-affinity ligand for the channel, however, has hindered its isolation and precluded protein-level biochemical studies. Our intention here is to apply classical liposome reconstitution methods to the *Torpedo* Cl^- channel and develop a quantitative assay for its eventual purification. Several lines of evidence suggest that *Torpedo* electric organ ought to be a good biochemical source for this Cl^- channel protein. Previous estimates of 200–1000 channels/μm^2 of plasma membrane, based upon planar lipid bilayer reconstitution, have suggested an abundance of up to 250 pmol/mg membrane protein in electroplax plasma membrane vesicles (Miller & White, 1980; White & Miller, 1981*a*; Woodbury & Miller, 1990). Although encouraging, these estimates are inherently uncertain, and there is as yet no unequivocal indication of whether the Cl^- channel studied in planar lipid bilayers is in fact a major protein constituent in the electric organ plasma membrane.

In this paper we describe the solubilization, reconstitution, and functional assay of the Cl^- channel from *Torpedo* electroplax. We have developed two types of tracer flux techniques for quantitative assay of Cl^- channels reconstituted into liposomes. First, a trapped volume assay allows an independent estimate of the absolute density of Cl^- channels present in the membrane preparation. Second, we employ a concentrative uptake assay to characterize the reconstituted channel's transport properties and follow its activity through an initial purification procedure.

Materials and Methods

Materials

CHAPS,[1] polystyrene mini-columns, DIDS, and BCA reagents were purchased from Pierce. Bovine brain PE, PC, and PS are

[1] *Abbreviations used are:* DIDS, 4,4′-Diisothiocyanostilbene-2,2′-disulfonate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; BCA, bicinchoninic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; HEPES, N-[2-hydroxyethyl]piperazine N′-[2-ethanesulfonic acid]; FPLC, fast protein liquid chromatography.

products of Avanti Polar Lipids. Cholesterol, recrystallized from ethanol prior to use, and Dowex 1 × 4–100 (Cl^- form) were purchased from Sigma. Radioisotopes $^{36}Cl^-$ (18.5 mCi/g, 0.1 N HCl), L-[3,4-^{3}H]-glutamic acid (54.7 Ci/mmol), and $^{86}Rb^+$ (5.7 mCi/mg, 0.5 N HCl) were obtained from NEN/Dupont. *Torpedo californica* were purchased live from Pacific Biomarine, Venice, CA. All procedures were performed at room temperature unless otherwise indicated.

Preparation of *Torpedo* Electroplax Membranes

The procedure of White and Miller (1981*a*) was used with the following modifications. *Torpedo* electroplax (typically 400 g) was coarsely diced with a butcher knife, and NaN_3 was omitted from the homogenization step. The 12,000 × *g* pellets were resuspended in 10% (wt/wt) sucrose, 10 mM HEPES-KOH, pH 7.4, (1 ml/10 g starting material), and passed ten times through a Wheaton glass-glass homogenizer (pestle A). Vesicles (4 ml aliquots) were sonicated in a bath-type sonicator (Laboratory Supplies, Hicksville, NY) for 30 sec, then centrifuged at 5000 × *g*, 4°C, for 10 min. Collected supernatants were diluted with 10% (wt/wt) sucrose, 10 mM HEPES-KOH, pH 7.4, to concentrations of 3 mg/ml protein. Samples (10 ml) were layered into tubes containing sucrose steps of 35% (16 ml) and 39% (11 ml) containing 10 mM HEPES-KOH, pH 7.4, and centrifuged in a Beckman SW-28 rotor, 16 hr, 4°C at 24,000 rpm. Material from the upper interface was collected, diluted with two volumes of water and centrifuged 60,000 × *g*, 4°C, for 70 min. The pellets, enriched for noninnervated face membranes (Sobel, Weber & Changeux, 1977), were resuspended in 10 mM HEPES-KOH, 0.4 M sucrose, pH 7.4, at 10–15 mg/ml. Aliquots were frozen on dry ice and are referred to as "light-fraction membranes." Material collected from the lower interface and treated in an identical manner consists predominately of innervated-face membranes (Sobel et al., 1977). Frozen samples stored in liquid nitrogen retained activity for at least six months.

Rapid Gel Filtration and Ion-Exchange Methods

We used the "spin-column" method (Penefsky, 1977) for rapid external medium exchange and for removal of detergent to form reconstituted liposomes (Radian & Kanner, 1985). Polystyrene mini-columns fitted with polyethylene frits were filled with 1.5 ml of eluant-equilibrated Sephadex G-50 (50–80 μm) and centrifuged (approx. 1000 × *g*, 13 sec), to remove excess solution. Samples of 50–100 μl were applied to the top of the compacted gel bed and eluted by centrifugation (approx. 700 × *g*, 45 sec). This method results in at least 90% recovery of reconstituted protein and complete exchange of small molecules in the medium.

Measurements of $^{36}Cl^-$ concentrative uptake utilized the Dowex mini-column method (Garty, Rudy & Karlish, 1983) to terminate influx and separate liposomes from external tracers. Dowex 1 × 4–100 was prepared in the glutamate form after converting to the hydroxide form by overnight column washing with 0.5 M NaOH, followed by addition of a twofold excess of glutamic acid. Glutamate-form resin was extensively washed with water before storage. Liposome samples were applied to polystyrene mini-columns containing 1.5 ml Dowex-1 × 4–100 (glutamate form), which had been washed with 2 ml of 5 mg/ml BSA in 175 mM sucrose and 2 ml of 175 mM sucrose prior to use. Liposomes were eluted from the columns into scintillation vials with 1.5 ml of 175 mM sucrose, and entrapped $^{36}Cl^-$ was determined by scintillation counting.

Solubilization and Reconstitution of *Torpedo* Membranes

Torpedo membranes were extracted for 30 min on ice at 2.5 mg/ml protein in "Buffer 1"; 125 mM NaCl, 25 mM KCl, 10 mM glutamic acid, 0.5 mM EGTA, 20 mM Tris-HCl, pH 7.6, also containing 16 mM CHAPS and 100 mM sucrose. The extract was centrifuged at either 120,000 × *g* for 10 min in a Beckman Airfuge, or for 30 min, 4°C at 100,000 × *g* in a Beckman Ti 70.1 rotor to sediment insoluble material, and typically yielded a soluble fraction at about 1.2 mg/ml protein. Aliquots of this soluble fraction (0–50 μg protein) were added to 50 μl of a solubilized 20 mg/ml lipid mixture (5 : 1 : 2 : 2 molar ratio of PE : PC : PS : cholesterol in Buffer 1 containing 37 mM CHAPS), and the final volume was adjusted to 100 μl with additional Buffer 1. CHAPS was removed by centrifuging 80 μl (0.8 mg lipid) of the sample through a Sephadex G-50 spin-column equilibrated in Buffer 1. The effluent was quick-frozen in a dry ice/acetone bath and stored overnight at −20°C. Samples were thawed at room temperature, bath sonicated (5–10 sec), and used for assay within a few minutes. Activity solubilized and assayed by either method (described below) is stable in 16 mM CHAPS for at least 12 hr at 4°C, although significant loss of activity occurs within a few hours at 25°C. Protein determinations were made with the BCA assay kit obtained from Pierce.

Trapped Volume Measurement

Immediately following removal of CHAPS from solubilized lipid/protein mixtures, $^{36}Cl^-$ and ^{3}H-glutamate were added to approximately 5 μCi/ml each tracer. Samples were frozen in a dry ice/acetone bath, then thawed at room temperature and briefly bath sonicated to break up multilamellar liposomes. This procedure produces predominately unilamellar liposomes with diameters in the range of 50–500 nm whose internal volumes are fully equilibrated with tracers added prior to the freeze-thaw step (Pick, 1981). Efflux of tracer was initiated by a 10-fold dilution of the tracer-loaded liposomes into Buffer 1. At indicated time points, efflux was terminated and external tracers removed by centrifugation of 100 μl aliquots (0.1 mg lipid) through Sephadex G-50 spin-columns equilibrated with Buffer 1. Trapped volumes (μl/mg lipid) were determined by scintillation counting of effluents and reference to radioisotope concentrations (cpm/μl) at which liposomes were equilibrated. Liposomes typically gave trapped volumes of 1.2–1.8 μl/mg lipid. Similar results were obtained if gluconate or glycine is used instead of glutamate as the impermeant marker. Measurements of $^{86}Rb^+/^3$H-glutamate trapped volume ratios were made in an identical manner, with $^{86}Rb^+$ substituted for $^{36}Cl^-$; Buffer 1 contained 5 mM RbCl for these experiments. All tracers were detergent-releasable, hence sequestered inside the liposomes.

Concentrative Uptake Assays

Liposome samples (80 μl, 0.8 mg lipid) prepared the previous day were thawed and centrifuged through Sephadex G-50 columns equilibrated with "Buffer 2"; 125 mM Na-glutamate, 25 mM K-

glutamate, 10 mM glutamic acid, 20 mM Tris-glutamate, pH 7.6, into an additional 360 μl Buffer 2 to replace external Cl^- with glutamate. Uptake was initiated by addition of 0.2 μCi of $^{36}Cl^-$ (which brings along 1.3 mM nonradioactive Cl^-), and 100 μl samples (0.18 mg lipid) were assayed at various times by the Dowex mini-column procedure. Cl^- channel activity measured by this method was quantified as nmol Cl^- uptake/mg lipid.

FPLC Procedures

Soluble *Torpedo* light-fraction membrane extracts (prepared as described above) containing 0.1–1.2 mg protein were loaded onto a Buffer A-equilibrated Mono Q HR 5/5 anion exchange column at 0.5 ml/min, then washed with three column volumes of Buffer A. The column was eluted with a 20-ml linear gradient from 0 to 45% Buffer B at a flow rate of 1 ml/min. Buffer A: 20 mM Tris-HCl, 200 mM NaCl, 8 mM CHAPS, 0.1 mg/ml PC, pH 7.6. Buffer B: 20 mM Tris-HCl, 1 M NaCl, 8 mM CHAPS, 0.1 mg/ml PC, pH 7.6. Fractions of 1 ml were collected and 15–50 μl of selected fractions were reconstituted and assayed for Cl^- channel activity as described. Protein determinations were made with the BCA assay kit as supplied by Pierce. Total protein and activity recoveries were routinely greater than 90%.

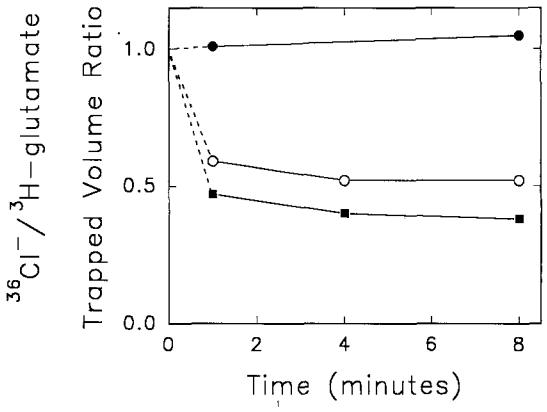

Fig. 1. Reconstitution of a Cl^- permeability from *Torpedo* membrane extracts. The ratio of trapped volume measured with $^{36}Cl^-$ to trapped volume measured with ^3H-glutamate is plotted as a function of time. Liposomes (1 mg) reconstituted with varying amounts of light-fraction membrane extract were assayed for trapped volume of $^{36}Cl^-$ and ^3H-glutamate, at the indicated times after beginning the efflux reaction, as described in Methods. (●) no protein, (○) 23 μg, (■) 46 μg.

Results

Measurement of Trapped Volumes

Planar bilayer studies have suggested that the Cl^- channel is abundant in electroplax. However, these estimates may misrepresent the average channel density in the membrane preparation, since they assume both uniform channel distribution, and uniform probability of fusion for the entire vesicle population (Miller & Richard, 1990; Woodbury & Miller, 1990). These may be falacious assumptions since the fusion of membrane vesicles into planar bilayers is a highly selective process (Miller, 1983) and may be affected by channel distribution. We introduce here a tracer-efflux method which allows calculation of the absolute Cl^- channel density in the membrane extract. The analysis is based on the high turnover rates of ion channels ($>10^7$/sec); a liposome containing just a single open channel should equilibrate its permeant ions very rapidly, on the millisecond timescale (Miller & Racker, 1979); even if the channel opens infrequently, tracer equilibration should still occur in a few seconds. Therefore, if we "dope" Cl^- channels into liposomes at low ratios (<0.5 channels/liposome), the liposomes will form two distinguishable populations: those containing no channels and remaining Cl^- impermeable, and those containing one or more channels, and hence extremely leaky to Cl^-. By measuring the fraction of liposomes devoid of channels as a function of protein/lipid ratio, we can estimate the channel's abundance in extracts of native membranes.

We reconstituted CHAPS-solubilized *Torpedo* membranes into liposomes at low protein/lipid ratios, and loaded the liposomes to equilibrium with $^{36}Cl^-$ and an impermeant internal-volume marker, ^3H-glutamate. Efflux was initiated by dilution of external tracers and terminated at later times by collection of liposomes via rapid gel filtration. $^{36}Cl^-$ and ^3H-glutamate remaining inside the liposomes were measured and trapped volume for each tracer was determined by reference to concentrations at which liposome loading took place.

Figure 1 illustrates the $^{36}Cl^-/^3H$-glutamate trapped volume ratio of reconstituted liposomes as a function of time after dilution of external radioactivity. Protein-free liposomes yield identical trapped volumes for the two tracers, i.e., $^{36}Cl^-/^3H$-glutamate trapped volume ratio of unity, as expected for liposomes that have been fully equilibrated with both tracers. Moreover, since the absolute trapped volume for each tracer does not change over the experimental time course (data not shown), these liposomes are well-sealed for both Cl^- and glutamate. In contrast, liposomes reconstituted with *Torpedo* membrane extracts show decreased $^{36}Cl^-/^3H$-glutamate trapped volume ratios demonstrating the preferential loss of Cl^-. This protein-dependent loss of trapped Cl^- behaves as a rapid "burst," complete before our first time point (1 min), as expected for channel-mediated efflux from a discrete population of the liposomes. The lack of post-burst Cl^- leakage indicates a second population of liposomes which do not contain channels, and hence retain their Cl^-. Raising the protein concentration in the liposomes

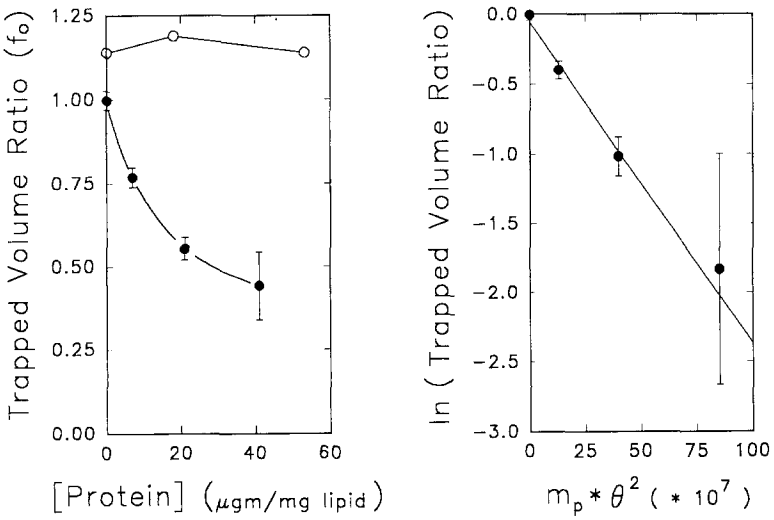

Fig. 2. Cl^- permeability induced by *Torpedo* membrane extracts. *Left panel:* $^{36}Cl^-/^3H$-glutamate trapped volume ratios (●) measured at 1 min are plotted for liposomes prepared with indicated amounts of light-fraction membrane extract. Each point represents the mean ± SE of four determinations. Single $^{86}Rb^+$ measurements (○) were performed in an analogous manner, substituting $^{86}Rb^+$ for $^{36}Cl^-$ at a similar concentration. *Right panel:* Variation of $^{36}Cl^-/^3H$-glutamate trapped volume ratio (f_0) with protein reconstituted (m_p) is plotted according to Eq. (3), after correction for 25% "nonreceptive" liposomes. The least-squares line represents a channel density of 625 pmol of channel/mg CHAPS-extracted protein, with $A = 1.5 \times 10^3$ cm^2/mg.

increases the fraction of Cl^- permeable liposomes (burst size), leaving a smaller population which retains Cl^-.

Figure 2 (*left panel*) illustrates the $^{36}Cl^-/^3H$-glutamate trapped volume ratio dependence upon protein concentration and suggests that the liposome population is being progressively titrated with Cl^- channels. The trapped volume ratio approaches a nonzero value at the higher protein concentrations, indicating that about 25% of the liposomes are unable to reconstitute protein, as seen in other systems (Carroll & Racker, 1977). Below, we quantitatively treat the data in Fig. 2 to estimate the abundance of Cl^- channels in the detergent extract.

To examine the possibility that a nonspecific small-ion leak, rather than Cl^- channel, provides the efflux pathway for Cl^-, we substituted $^{86}Rb^+$ for $^{36}Cl^-$ and measured $^{86}Rb^+$ efflux in an identical manner. In no case did we observe efflux of $^{86}Rb^+$; that is, data consistently showed $^{86}Rb^+/^3H$-glutamate trapped volume ratios near unity, regardless of protein concentration (Fig. 2). In fact, the Rb^+ trapped volume is about 10% higher than the glutamate trapped volume, as expected for a Donnan equilibrium generated by negatively charged phosphatidylserine lining the liposome inner face. We conclude that the Cl^- efflux pathway is strongly selective for Cl^- over both a large anion, glutamate, and a small cation, Rb^+.

Estimation of Absolute Channel Density

The time resolution of this manual assay is orders of magnitude too low to measure channel-mediated efflux rates (Miller & Racker, 1979); the assay's value lies in its capability of "counting" Cl^- channels in our detergent extract. We utilize the $^{36}Cl^-/^3H$-glutamate trapped volume ratio as a direct measure of the fraction of the liposome population which does not contain any Cl^- channels. Thus, if the solubilized and reconstituted channels are randomly distributed in a liposome population of uniform radii, a Poisson distribution will describe the channel-free fraction, f_0, in terms of the total number of channel molecules, n_C, and of liposomes, n_L

$$f_0 = \exp(-n_C/n_L). \tag{1}$$

The number of channels in a given sample containing m_p mg of membrane protein, is given in terms of the channel density s (mol channels/mg protein):

$$n_C = s \cdot m_p \cdot N_o \tag{2}$$

where N_o is Avagadro's number. For a mass, m_L, of spherical liposomes, n_L is readily calculated in terms of the internal volume, θ (ml/mg lipid), and the specific bilayer surface area per mg lipid, A (cm^2/mg lipid). Since θ is measured directly in these experiments as the glutamate trapped volume, we obtain the $^{36}Cl^-/^3H$-glutamate trapped volume ratio as

$$f_0 = \exp(-36\pi N_o m_p \theta^2 s/m_L A^3). \tag{3}$$

Accordingly, for a fixed amount of lipid, we expect the trapped volume ratio of uniformly sized liposomes to decrease exponentially as the amount of protein reconstituted, m_p, is increased. Since liposome preparations vary in absolute trapped volumes, θ, it is necessary to plot trapped volume ratio, f_0, against $m_p\theta^2$ to quantify the density of channels, s, in the detergent extract, according to Eq. (3). Analysis of such a plot (*right panel*, Fig. 2) yields an estimate of 630 pmol channel/mg protein. Assuming full solubilization of all of the channels present in

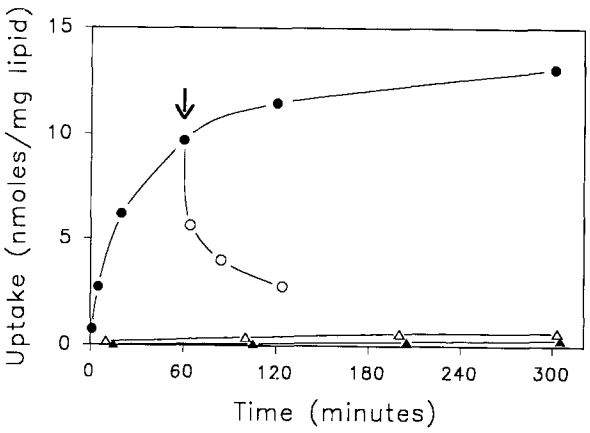

Fig. 3. Concentrative uptake of $^{36}Cl^-$ driven by a Cl^- potential. Uptake was initiated by addition of $^{36}Cl^-$, and measured at indicated times as described in Methods. Liposomes were recontituted with 0 μg (△) or 7 μg (●,▲) of *Torpedo* light-fraction membrane extract/mg lipid. The arrow indicates addition of nonactin to liposomes at a concentration of 10 μg/ml (○). Liposomes lacking an outward Cl^- gradient (▲) were reconstituted with glutamate replacing internal Cl^-.

the native membranes, this translates into a channel density of about 2000 channels/μm^2, somewhat higher than estimates of 200–1000 channels/μm^2 based on planar bilayer reconstitution work (Miller & White, 1980; White & Miller, 1981*a*; Miller & Richard, 1990). This estimate is inherently rough, since it assumes random distribution of channels in a spherical liposome population of uniform radii; nevertheless, it represents a very high channel abundance and argues that the *Torpedo* electroplax should be a rich biochemical source of Cl^- channel protein.

Concentrative Cl^- Uptake

Although the trapped volume method allows an estimation of absolute channel density, its poor time resolution and low signal strength make it unsuitable for characterizing the Cl^- fluxes or as a routine assay for channel purification. For this purpose, we have adapted the concentrative uptake method (Garty et al., 1983), which has been used to assay Cl^- channels from epithelial tissues (Landry et al., 1990). With this technique, a large outward Cl^- gradient is established across the membrane of Cl^--loaded liposomes by replacing external Cl^- with the impermeant anion glutamate. A high membrane potential (positive inside) is generated only in those liposomes containing a Cl^--specific conductance and drives the accumulation of externally added $^{36}Cl^-$.

Liposomes formed without protein do not support concentrative uptake, since they are impermeable to Cl^- (Fig. 3). However, when a small amount of *Torpedo* membrane extract is reconstituted into liposomes, we observe a large uptake of $^{36}Cl^-$ into the liposomes, which proceeds on a time scale of tens of minutes, as expected (Garty et al., 1983; Landry et al., 1990). The extent to which tracer is concentrated inside the liposomes indicates an effective Cl^- concentration gradient of at least 20-fold over the entire population; since under these conditions only about 30% of the liposomes contain Cl^- channels, the tracer is actually concentrated about 60-fold in these liposomes, a value equivalent to a membrane potential of 104 mV. This requires the presence of a permeability selective for Cl^- over the cations present, Na^+ and K^+. Figure 3 also shows that concentrative uptake of radioactive Cl^- is abolished if the outwardly directed Cl^- gradient is removed by replacing internal Cl^- with glutamate. Finally, concentrative uptake is reversed by the cation ionophore nonactin; this result argues that the Cl^- permeability pathway operates by a conductive rather than electroneutral mechanism. We conclude that reconstitution of CHAPS-extracted *Torpedo* membranes into liposomes provides a conductive permeability pathway specific for Cl^- over both Na^+ and K^+.

Channel-Mediated Flux Properties

To determine whether the reconstituted conductance is selective among anions, we compared the inhibitory effect of external anions upon $^{36}Cl^-$ concentrative uptake. If it is permeant, an external anion will reduce liposome transmembrane voltage, and thereby inhibit concentrative uptake. The degree of inhibition will increase with the anion's permeability and concentration. In Fig. 4 we test the effectiveness of external anions (added at 1 mM) to inhibit $^{36}Cl^-$ uptake. Cl^- itself is the most potent inhibitor of uptake, as expected from the selectivity observed at the single-channel level. The selectivity among anions seen by the present method, $Cl^- > Br^- > F^- > SO_4^{2-}$, is in qualitative agreement with selectivity data from bilayer studies (White & Miller, 1979; Miller & White, 1980). These anions inhibit uptake by virtue of their permeability, not by blockade of the channel, since they do not inhibit from inside the liposomes (data not shown). Our observation of significant permeability to F^- in the reconstituted liposomes, has not been observed in planar lipid bilayers and may be due to the different ionic conditions used in these experiments.

The pseudohalide SCN^- is known to block the *Torpedo* Cl^- channel, as studied in planar lipid bilayers (Miller & White, 1980; White & Miller,

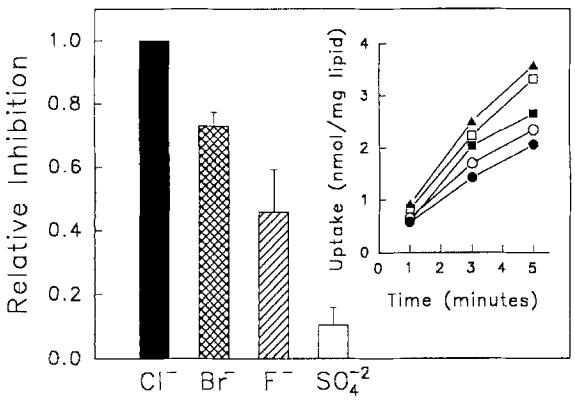

Fig. 4. Inhibition of concentrative uptake by external anions. Concentrative uptake for liposomes reconstituted at 8 μg protein/mg lipid was measured as in Fig. 3, except that Na^+ salts of the indicated anions were added, to final external concentrations of 1 mM, simultaneously with $^{36}Cl^-$. The bar graph plots the fractional decrease in uptake for a test anion relative to that observed for Cl^- at 3 min ($n = 3 \pm$ SE). The inset shows the time dependence of $^{36}Cl^-$ uptake for the various anions tested: (●) Cl^-, (○) Br^-, (■) F^-, (□) SO_4^{2-}, control (▲).

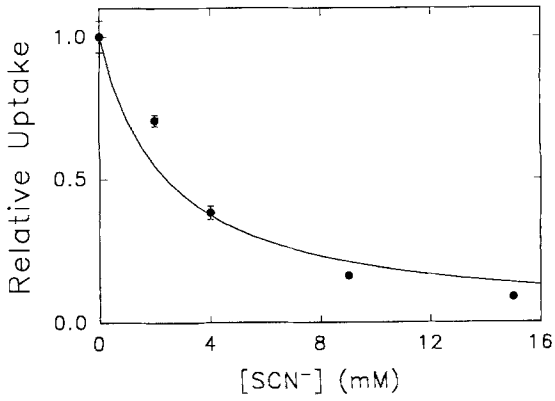

Fig. 5. Blockade of concentrative uptake by SCN^-. Liposomes reconstituted at 7 μg protein/mg lipid were loaded with buffer 1 also containing the indicated concentration of SCN^-, and were passed through rapid gel filtration columns to replace external Cl^- with glutamate and to remove external SCN^-. Isotonicity was maintained with external Na-glutamate. Uptake was measured at 3 min (mean ± SE, $n = 4$) and is reported relative to the value without SCN^- (2.1 nmol Cl^-/mg lipid). Solid curve represents simple inhibition with $K_i = 2.4$ mM.

1981*b*), and so we studied the effect of SCN^- on concentrative $^{36}Cl^-$ uptake. To preclude possible inhibition by permeation of external SCN^- (i.e., as above), SCN^- was added solely inside the liposomes. Internal SCN^- blocks Cl^- uptake (Fig. 5) half-maximally at 2–3 mM, in agreement with the 3.3 mM K_i (200 mM KCl, −30 mV) measured for *cis* blockade directly in planar lipid bilayers (Miller & White, 1980). Control experiments show that addition of the nonblocking Br^- anion (at concentrations up to 15 mM) does not reduce $^{36}Cl^-$ uptake. We have also observed inhibition by internal I^- in the millimolar range, as reported for the channel in bilayers (Miller & White, 1980). Finally, we observe block of Cl^- uptake by millimolar amounts of internal NO_3^-, a result not reported previously.

Irreversible inactivation of the *Torpedo* Cl^- channel by the stilbene disulfonate DIDS has been demonstrated for Cl^- channels in planar lipid bilayers and for Cl^- fluxes in native *Torpedo* membrane vesicles (Miller & White, 1980; White & Miller, 1981*a*; Miller & White, 1984). We applied DIDS to our soluble extracts to assess whether it inhibits Cl^- fluxes in the present assay as well. We find 50% inhibition of concentrative uptake after treating the CHAPS extract for 15 min with 250 μM DIDS, 4°C. Although this treatment is harsher than that used in planar bilayers, the observed inhibition is consistent with that reported for *Torpedo* native membrane vesicles (White & Miller, 1981*a*).

Channel Localization and Initial Purification

The *Torpedo* electrocyte is a polar cell, having an innervated-face membrane densely packed with nicotinic acetylcholine receptors (Cartaud et al., 1973). It has been suggested (White, 1981; White & Miller, 1981*a*) that the Cl^- channel resides in the opposing noninnervated-face membrane, but direct evidence to support this assertion is still lacking. To examine the localization of the Cl^- channel, we compared extracts from membrane fractions enriched for either innervated or noninnervated-face membranes for their ability to catalyze concentrative uptake. Uptake observed with either preparation is linear with protein concentration up to 15 μg protein/mg lipid (Fig. 6). Extracts from the noninnervated membrane fraction display fourfold higher Cl^- channel activity than do those from the innervated membrane fraction. This result is in keeping with the proposal (White, 1981; White & Miller, 1981*a*) that the Cl^- channel is located in the noninnervated face of the electrocyte.

We used the concentrative uptake assay to follow channel activity through an anion-exchange chromatography step. Application of solubilized light-fraction *Torpedo* vesicles to a Mono-Q column equilibrated with 200 mM NaCl resulted in the binding of about 80% of protein and virtually all Cl^- channel activity, and subsequent development of the

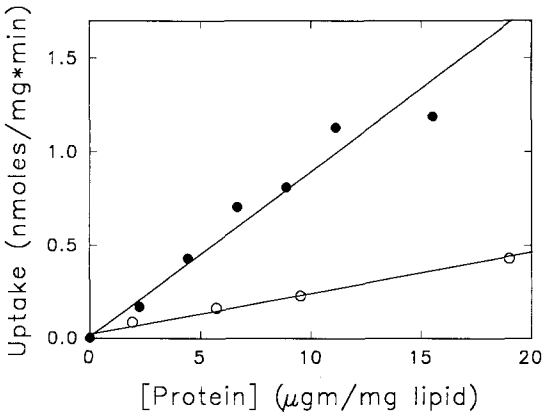

Fig. 6. Concentrative uptake as a function of protein concentration. Liposomes were reconstituted with CHAPS-extracted *Torpedo* plasma membranes at the indicated concentrations, and uptake of $^{36}Cl^-$ was measured at 20 min. (●) Noninnervated membrane fraction, (○) innervated membrane fraction.

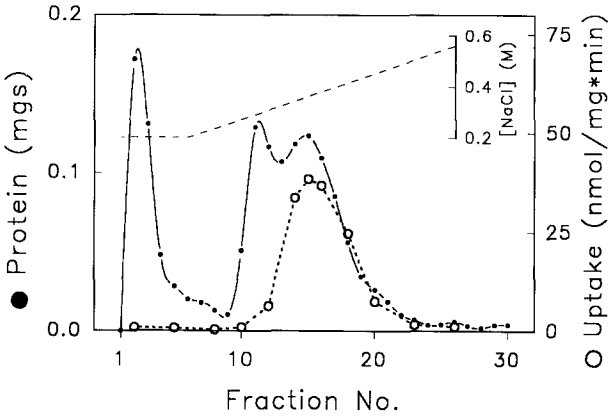

Fig. 7. Anion-exchange chromatography of CHAPS-solubilized extracts. Light-fraction membranes were solubilized in CHAPS, and the extract was chromatographed on an FPLC Mono-Q column, as described in Methods. Fractions were assayed for protein concentration (●), and concentrative uptake activity (○) after reconstitution into liposomes.

column with a linear NaCl gradient eluted protein in two partially resolved peaks. Activity, as measured by concentrative uptake, co-migrates with the second peak (Fig. 7) and represents a 90% recovery. Total protein recoveries are typically in the same range. This procedure routinely yields purification of about fivefold over the unfractionated solubilized membranes.

Discussion

We have developed procedures for solubilization, reconstitution, and functional assay in liposomes of Cl^- channels derived from membranes of *Torpedo* electroplax. Cl^- channel function is assayed by two radioisotope flux methods: a double-label trapped volume measurement which estimates Cl^- channel density, and a concentrative uptake assay which measures Cl^- influx driven by a membrane potential. We consider that the results presented here demonstrate that the Cl^- permeability pathway reconstituted into liposomes from CHAPS extracts of *Torpedo* membranes represents the voltage-gated Cl^- channel extensively characterized in planar lipid bilayers. Three lines of evidence support this claim. First, the Cl^- permeability studied in reconstituted liposomes is conductive, highly selective for Cl^-, and DIDS inhibitable. Second, the Cl^- fluxes are blocked by SCN^- and I^-; these are *not* common Cl^- channel blockers (Franciolini & Petris, 1990), but they do block the voltage-gated Cl^- channel from *Torpedo* electroplax (White & Miller, 1981*b*). Moreover, the fact that high concentrations of SCN^- block virtually all the Cl^- uptake suggests that we are studying a single type of Cl^- conductance pathway. Third, the Cl^- permeability is predominant in the light-fraction *Torpedo* membranes, which are derived mainly from the electrocyte noninnervated face (Sorbel et al., 1977); these are the membranes which give the highest activity of Cl^- channel fusion into planar bilayers as well (White, 1981). Although we have observed functionally "normal" Cl^- channels with electrical methods applied to reconstituted detergent extracts of *Torpedo* membranes (Tank, Miller, & Webb, 1982; Woodbury & Miller, 1990) these observations do not constitute convincing evidence for the proper reconstitution of Cl^- channels in a biochemical sense. Single-channel reconstitution methods are inherently selective, sampling only a few "successful" molecules in a population of reconstituted channel protein. It is for this reason that we have confined ourselves here to liposome flux experiments, which make a strong case for successful channel reconstitution based on whole-population assays.

The absolute density of channels estimated from liposome reconstitution, on the order of 500 pmol/mg protein, is 2 to 10-fold higher than that suggested upon the basis of channel reconstitution into planar lipid bilayers (Miller & Richard, 1990; Woodbury & Miller, 1990). Since both estimates rely upon simplified models, we consider that their agreement is reasonable. The present results illustrate that previous indications have *not* grossly overestimated channel abundance; indeed, they may have underestimated it. The abundance of Cl^- channel deduced here is impressive, only 5 to 10-fold lower than the very

high density of acetylcholine receptor channels in the innervated-face membranes (Cartaud et al., 1973). This means that the Cl^- channel is a major membrane protein in the *Torpedo* electric organ. Since we have shown an initial fractionation of the noninnervated membrane extracts, which leads to fivefold purification of channel activity, we are optimistic that we will be able to use liposome reconstitution as a practical assay for full purification of the Cl^- channel protein.

We are grateful to Dr. Dixon Woodbury for assistance in developing our magic lipid formula and for helpful suggestions throughout the course of this work. This research was supported by N.I.H. grant No. GM-31768.

References

Carroll, R.C., Racker, E. 1977. Preparation and characterization of cytochrome *c* oxidase vesicles with high respiratory control. *J. Biol. Chem.* **252:**6981–6990

Cartaud, J., Benedetti, E.L., Cohen, J.B., Mennier, J.C., Changeux, J.P. 1973. Presence of a lattice structure in membrane fragments rich in nicotinic receptor protein from the electric organ of *Torpedo marmorata*. *FEBS Lett.* **33:**109–113

Franciolini, F., Petris, A. 1990. Chloride channels of biological membranes. *Biochim. Biophys. Acta Rev. Biomembr.* **1031:**247–259

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:**13094–13099

Jentsch, T.J., Steinmeyer, K., Schwarz, G. 1990. Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes. *Nature* **348:**510–514

Landry, D.W., Akabas, M.A., Redhead, C., Al-Awqati, Q. 1990. Purification and reconstitution of epithelial chloride channels. *Methods Enzymol.* **191:**572–582

Miller, C. 1983. Integral membrane channels: Studies in model membranes. *Physiol. Rev.* **63:**1209–1242

Miller, C., Racker, E. 1979. Reconstitution of membrane transport functions. *In:* The Receptors, a Comprehensive Treatise. R.D. O'Brien, editor. pp. 1–32. Plenum, New York

Miller, C., Richard, E.A. 1990. The *Torpedo* chloride channel: Intimations of molecular structure from quirks of single-channel function. *In:* Chloride Transporters. F.J. Alvarez-Leefmans and J. Russell, editors. pp. 383–405. Plenum, New York

Miller, C., White, M.M. 1980. A voltage-dependent chloride channel from *Torpedo* electroplax membrane. *Ann. N.Y. Acad. Sci.* **341:**534–551

Miller, C., White, M.M. 1984. Dimeric structure of Cl^- channels from *Torpedo* electroplax. *Proc. Natl. Acad. Sci. USA* **81:**2772–2775

Penefsky, H.S. 1977. Reversible binding of P_i by beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **252:**2891–2899

Pick, U. 1981. Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. *Arch. Biochem. Biophys.* **212:**186–194

Radian, R., Kanner, B.I. 1985. Reconstitution and purification of the sodium- and chloride-coupled γ-aminobutyric acid transporter from rat brain. *J. Biol. Chem.* **260:**11859–11865

Sobel, A., Weber, M., Changeux, J.-P. 1977. Large-scale purification of the acetylcholine-receptor protein in its membrane-bound and detergent-extracted forms from *Torpedo marmorata*. *Eur. J. Biochem.* **80:**215–224

Tank, D.W., Miller, C., Webb, W.W. 1982. Isolated-patch recording from liposomes containing functionally reconstituted chloride channels from *Torpedo* electroplax. *Proc. Natl. Acad. Sci. USA* **79:**7749–7753

White, M.M. 1981. Characterization of a Voltage-Gated Chloride Ion Channel from *Torpedo* Electroplax. Ph.D. Thesis, Brandeis University, Waltham, MA

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

White, M.M., Miller, C. 1981*a*. Chloride permeability of membrane vesicles isolated from *Torpedo* electroplax. *Biophys. J.* **35:**455–462

White, M.M., Miller, C. 1981*b*. Probes of the conduction process of a voltage-gated chloride channel from *Torpedo* electroplax. *J. Gen. Physiol.* **78:**1–19

Woodbury, D.J., Miller, C. 1990. Nystatin-induced fusion of proteoliposomes: A versatile method for reconstitution in planar bilayers. *Biophys. J.* **58:**833–839

Received 11 June 1991