CHLORIDE PERMEABILITY OF MEMBRANE VESICLES ISOLATED FROM *TORPEDO CALIFORNICA* ELECTROPLAX

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ABSTRACT The Cl^- permeability of membrane vesicles prepared from the electric organ of the marine ray *Torpedo californica* was studied by means of radioactive tracer exchange and by measuring the changes in the scattered-light intensity caused by osmotically induced volume changes. Both types of experiments indicate that a substantial fraction of the vesicles is extremely permeable to Cl^-. Furthermore, this permeability pathway is inhibited by 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene, a well-known inhibitor of anion transport in a variety of systems. The properties of this permeability pathway are consistent with its identification as the voltage-gated Cl^- channel studied in planar bilayers.

INTRODUCTION

Recent work on the fusion of membrane vesicles with planar phospholipid bilayers has led to the unexpected conclusion that the electroplax membrane of the marine ray *Torpedo californica* carries a voltage-gated Cl^- channel (White and Miller, 1979, 1981; Miller and White, 1980). The channel is unusually selective for Cl^-; of all other anions tested, only Br^- is appreciably permeant. In addition, the channel is inhibited by the stilbene disulfonates 4-acetamino-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS) and 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS), two well-known inhibitors of anion transport in a variety of systems. The SITS- and DIDS-sensitive site is accessible from only one side (the *cis* side) of the membrane; the *trans* side of the channel is completely insensitive to these agents. Finally, each vesicle that fuses with the bilayer contains, on the average, 200–300 channels.

The above information was obtained exclusively from experiments using the planar bilayer system. Although this system is ideal for studying the gating and conduction process of the channel, it does not allow one to determine if the channels are contained in a significant fraction of the total vesicle population.

In a recent publication, Taguchi and Kasai (1980) studied the anion permeability of vesicles prepared from the electric organ of a related ray, *Narke japonica*, by means of radioactive tracer efflux and by light-scattering changes caused by osmotically induced volume changes. Their data indicated that there was a DIDS-sensitive anion permeability pathway in these vesicles and that [^{3}H]dihydroDIDS bound specifically to a protein of a 180,000 molecular weight.

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However, there are several aspects of this work that make it difficult to determine if this anion permeability pathway is related in any way to a channel similar to that found in *Torpedo* electric organ. The bulk of their data were obtained using $SO_4^=$ as the permeant anion. We have shown that the *Torpedo* Cl^- channel studied in bilayers has no measureable $SO_4^=$ permeability (Miller and White, 1980). Although there may be a low $SO_4^=$ permeability similar to that found in the red cell band 3 anion transporter ($PCl^-/P_{SO_4^=} \sim 10^4$; Schnell et al., 1973), making it possible to measure $SO_4^=$ fluxes in the vesicles but not in the bilayer, this still does not address the question as to whether there exists in the electric organ of *Narke* a Cl^- channel similar to the one found in *Torpedo*. In addition, the light-scattering experiments do not unambiguously measure anion permeability, since the rate of reswelling is governed by the rate of permeation of the cation-anion pair, and the authors did not provide evidence that Cl^- flux is rate limiting.

The experiments reported here demonstrate that there is a Cl^- permeability pathway in a substantial fraction of the vesicles prepared from *Torpedo* electroplax and that this permeation is rapid enough to be attributed to a channel-type transporter such as the voltage-gated Cl^- channel.

MATERIALS AND METHODS

Preparation of Membrane Vesicles

All operations were carried out at 0–4°C. *Torpedo californica* were obtained from Pacific Bio-marine Laboratories Inc. (Venice, Calif.) and were used immediately. Fish were killed and the electric organs were removed and minced in a food processor (La Machine, Moulinex) and then homogenized using a Waring blendor in one volume distilled water containing 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 3 mM NaN_3. The homogenate were centrigued at 7,000 *g* for 10 min and the supernate was strained through cheesecloth and saved. The pellets were rehomogenized and centrifuged as before and then the combined supernates were centrifuged at 12,000 *g* for 2 h. The pellets were resuspended in a small volume of 0.4 M sucrose, 2 mM Hepes-KOH, pH 7.5 and sonicated in 5-ml aliquots for 30 s in a bath-type sonicator (Laboratory Supplies Co., Hicksville, N.Y.). The sonicated vesicles (5 ml/tube) were layered onto 15 ml 35% (wt/wt) sucrose and centrifuged overnight in a Beckman type 30 rotor at 25,000 rpm (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The Cl^- channel-enriched vesicles band at the sample-35% sucrose interface, whereas the acetylcholine receptor-enriched vesicles are found in the pellet. The channel-enriched vesicles were diluted with two volumes of distilled water and centrifuged at 100,000 *g* for 40 min and the pellets were taken up in a minimal volume of 0.4 M sucrose, 2 mM Hepes-KOH, pH 7.5 and stored in small aliquots at −70°C. The Cl^- channel activity, as measured in the planar bilayer system (White and Miller, 1979), is stable for at least 6 mo.

90° Light-scattering Experiments

The overall procedure closely followed that reported previously for studying volume changes induced by osmotic imbalances (Kometani and Kasai, 1978). All solutions used were filtered through Millipore type HA 0.45-μm filters (Millipore Corp., Bedford, Mass.). Vesicles were thawed on ice, diluted to 0.4 mg protein/ml with 10 mM KCl, 5 mM Hepes-KOH, pH 7.35, and incubated overnight on ice to equilibrate. All subsequent operations were carried out at 20°C. The equilibrated vesicles were brought to 20°C and then degassed along with any other solutions to be used. The vesicles were mixed with an equal volume of 150 mM KCl, 5 mM Hepes-KOH, pH 7.35 in a stopped-flow light-scattering apparatus (mixing time 2 ms, Durrum Instrument Corp., Sunnyvale, Calif.) and the 90° light scattering at 420 nm was followed on a chart recorder or oscilloscope as the vesicles transiently shrunk and then reswelled. Valinomycin, if present, was added to both the vesicle solution and to the 150-mM KCl solution 5 min

before mixing. All values are reported as the scattering change, ΔI, relative to the initial (and equilibrium) scattering, I_0. The data were analyzed by means of a least-squares program that decomposed the data into the sum of two exponential decays by first fitting the data points at late (>1 min) times to a single exponential decay and then subtracting this fitted curve from the data points at early times to give another single exponential decay.

Radioactive Tracer Exchange Experiments

All operations were carried out at 0–4°C. Vesicles were thawed on ice, washed twice with 100 mM NaCl, 100 mM Na-gluconate, 50 mM sucrose, 5 mM Hepes-NaOH, pH 7.35, and then resuspended in the same buffer at a protein concentration of 15–20 mg/ml. Na^{36} Cl and Na[^{14}C]gluconate (ICN Pharmaceuticals, Inc., Irvine, Calif.) were added to a specific activity of 3–5 μCi/ml and the vesicles were incubated overnight. After equilibration, the vesicles were diluted 100-fold into the same medium without radioactive labels and 1-ml aliquots were taken at various times, filtered through Millipore type HA 0.45-μm filters, and washed with 5 ml buffer. The filters were placed in scintillation vials, and after drying, 10 ml ACS scintillant (Amersham/Searle Corp., Arlington Heights, Ill.) as added. After the filters had completely dissolved (~1 h) the samples were counted in a scintillation counter and the amount of $^{36}Cl^-$ and [^{14}C]gluconate remaining in the vesicles as determined. Values are reported as apparent isotope space microliters per milligrams of protein.

RESULTS

Fig. 1 shows the light-scattering changes resulting from the volume changes that occur after vesicles loaded with 10 mM KCl are diluted into a medium containing 150 mM KCl. In all cases the vesicle first transiently shrink as water moves out, and then reswell as KCl moves in. The rate of reswelling is determined by the ion-pair permeability (Kometani and Kasai, 1978; Kometani and Kasai, 1978). Fig. 1 *A* shows the effect of the K^+ ionophore valinomycin on the time-course of reswelling. In the absence of valinomycin, the relaxation can be described as the sum of two exponential decays: a fast component with a half-time of 8.0 s that accounts for approximately 35% of the relaxation, and a slow phase with a half-time of 230 s. The extrapolated zero-time scattering amplitude $(\Delta I/I_0)_0$, is 0.130.

When iso-osmotic sucrose or sorbitol is used instead of the 150 mM KCl medium, $(\Delta I/I_0)_0$ is 0.127 and 0.132, respectively, indicating that the initial shrinkage caused by the jump into KCl is due to all of the vesicles shrinking; this eliminates the possibility that a significant fraction of the vesicles are highly permeable to both K^+ and Cl^-.

The addition of valinomycin alters the light-scattering time-course. Fig. 1 *A* shows that in the presence of valinomycin, the fast phase can no longer be detected within the time resolution of the chart recorder (0.3 s), while the slow phase is unaffected. When an oscilloscope is used to increase the time resolution, a fast phase with a half-time of 125 ms can be detected (Fig. 1 *B*) and the extrapolated zero-time scattering change is 0.127, as in the control. We interpret these data as indicating that there are two populations of vesicles: one fraction (~40% of the total) very permeable to Cl^- but not K^+ (the fast relaxation) and the other (~60%) quite impermeable to Cl^- (the slow relaxation). Without valinomycin present, K^+ permeation is the rate-limiting step in the reswelling of the fast-phase vesicles; addition of valinomycin releases this constraint on KCl permeability, allowing salt to equilibrate rapidly.

Finally, Fig. 1 *C* shows the effect of preincubating the vesicles for 3 h with 0.1 mM DIDS before mixing with the 150 mM KCl-25 μg/ml valinomycin solution. The rate of the fast phase is now decreased 20-fold (half-time 4.0 s), while the slow phase remains unaffected. Use

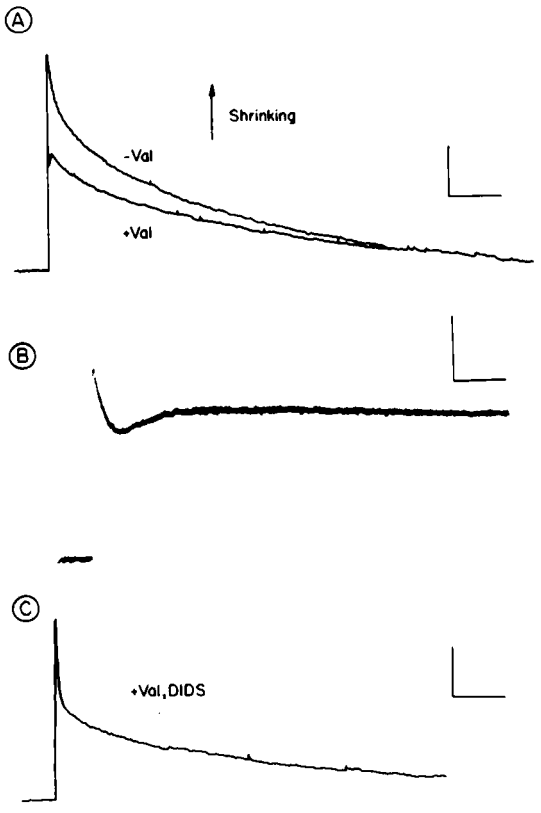

Figure 1 Change in scattered-light intensity due to volume changes. Light-scattering changes after an osmotic jump were measured as described in Methods. The vertical calibration marks correspond to $(\Delta I/I_0) = 0.04$, and the horizontal marks correspond to either 1 min (*A*,*C*) or 1 s (*B*). (*A*) Effect of valinomycin on the reswelling rates. The upper trace shows the biphasic decay in scattering intensity as the vesicles reswell due to the movement of KCl into the vesicles. The lower trace shows the effect of 25 μg/ml valinomycin on the reswelling rates. Note the disappearance of the early part of the relaxation. The half-times for the slow phase of the relaxation are 230 and 195 s in the absence and presence of valinomycin, respectively. The half-time for the fast-phase relaxation was 8.0 s in the absence of valinomycin. (*B*) The same experiment as the lower trace of *A* at higher time resolution. The early fast phase is clearly visible and has a half-time of 0.125 s. (*C*) Effect of DIDS on the reswelling rates. The vesicles were preincubated for 3 h with 0.1 mM DIDS before mixing with the 150-mM KCl buffer in the presence of 25 μg/ml valinomycin. The fast phase now has a half-time of 4.0 s, a 20-fold reduction of the rate, while the slow phase has a half-time of 185 s.

of higher concentrations of DIDS or longer incubation times did not result in additional inhibition.

Valinomycin was used in some of the experiments shown in Fig. 1 to make the vesicles so permeable to K^+ that its permeation would not be rate limiting and thus the rate or reswelling would reflect the Cl^- permeability of the vesicles. Fig. 2 shows the concentration dependence of the valinomycin effect. Note that the slow phase is unaffected by valinomycin whereas the rate of the fast phase is progressively increased with increasing valinomycin concentration, reaching a maximum rate at concentrations >20 μg/ml. Assuming that in the absence of valinomycin K^+ flux is limiting the rate of reswelling and that at saturating valinomycin Cl^-

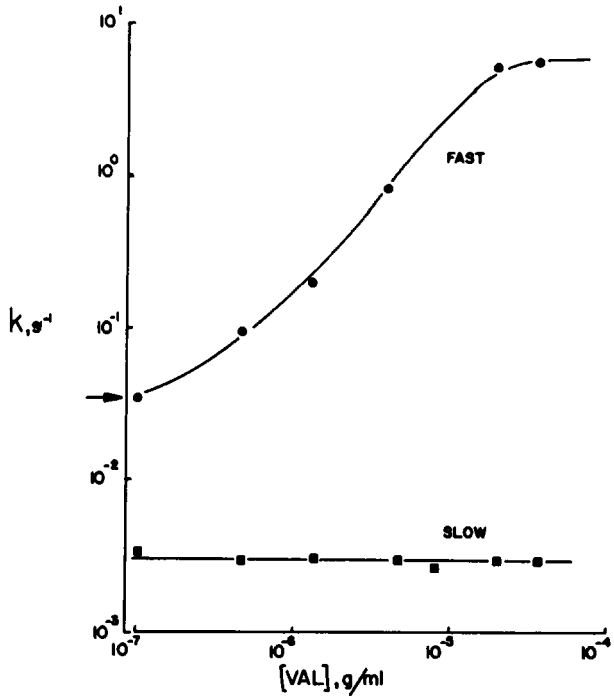

Figure 2 Effect of valinomycin on reswelling rates. Stopped-flow light-scattering experiments were carried out as described in the legend to Fig. 1. The rate constants were determined from the measured half-times of reswelling for both the fast and slow phases. The arrow on the ordinate indicates the rate constant of the fast-phase reswelling in the absence of valinomycin.

is limiting, we can place a lower limit on the permeability ratio, (P_{Cl^-}/P_{K^+}), for the fast-phase population of vesicles. From these data, $(P_{Cl^-}/P_{K^+}) \geq 200$.

An alternative method to demonstrate this Cl^- permeability is to examine the exchange of internal-labeled anions with unlabeled external ones. Fig. 3 shows the exchange of intravesicular $^{36}Cl^-$ and [^{14}C]gluconate with external Cl^- and gluconate. note that although gluconate slowly exchanges with a half-time of 500–600 s, a substantial fraction (50%) of the Cl^- exchanges before the first time point is taken (15 s), and the rest then exchanges slowly like gluconate.

Both sets of experiments are consistent with there being two populations of vesicles present in the *Torpedo* electroplax vesicle preparation: one fraction (40–50%) very permeable to Cl^-, but not K^+ $(P_{Cl^-}/P_{K^+} \geq 200)$, and the other very impermeable to Cl^-. In addition, this Cl^- permeability pathway is inhibited at least 20-fold by DIDS. All of these results are consistent with the known properties of the voltage-gated Cl^- channel studied in planar bilayers (White and Miller, 1979, 1981; Miller and White, 1980).

DISCUSSION

In order to biochemically analyze, solublize and purify a membrane protein, the protein in question should be present in reasonably large amounts. Although we have examined the

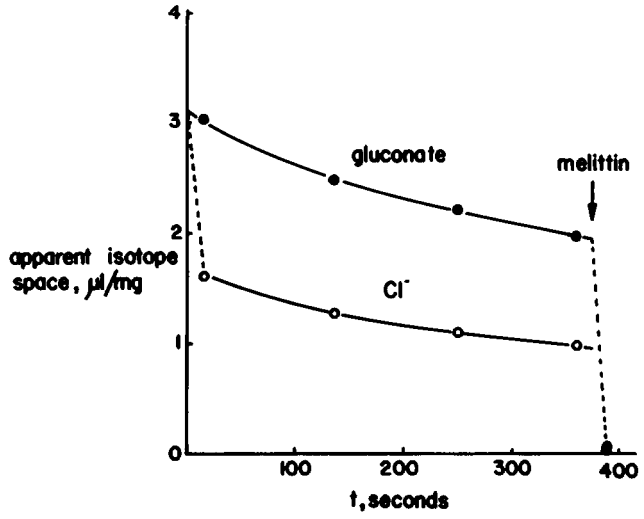

Figure 3 Comparison of Cl^- and gluconate exchange. Vesicles (22 mg/ml) were incubated overnight with 100 mM NaCl, 100 mM Na-gluconate, 50 mM sucrose, 5 mM Hepes-NaOH, pH 7.35, containing 5 μCi/ml $^{36}Cl^-$ and 3 μCi/ml [^{14}C]gluconate. After equilibration, the vesicles were diluted 100-fold into the same buffer without radioactive tracers and aliquots were sampled at various times to determine the intravesicular $^{36}Cl^-$ and [^{14}C]gluconate contents. At t = 360 s, melittin (15 μg/ml) was added to release the intravesicular contents.

gating and conduction process of the *Torpedo* Cl^- channel in detail in the planar bilayer system (White and Miller, 1979, 1981; Miller and White, 1980), the nature of this system does not allow one to determine whether the channel-containing vesicles constitute a significant fraction of the total population. This is due to the extremely low probability of vesicle-bilayer fusion and to the fact that the bilayer system detects only channel-containing vesicles. Other methods must be used to examine the whole population.

Both 90° light-scattering and radioactive tracer exchange are ideally suited for such a task. Signals arising from several populations can be observed and analyzed. However, these two methods have certain drawbacks. Although tracer exchange can directly examine Cl^- fluxes from the entire vesicle population, the low time resolution (10–15 s to sample and wash the filters) makes quantitative analysis of processes faster than this impossible. One can calculate (Miller and Racker, 1979) that for a vesicle of 2,500-Å diameter containing just one channel of conductance 20 pmho (the size of the *Torpedo* vesicles and Cl^- channel, respectively), the half-time for equilibration is on the order of 300 ms, well beyond the time resolution of this technique. The light-scattering experiments approach the required time resolution, but are complicated by the fact that the reswelling rates depend on the permeability of the cation-anion pair. The use of valinomycin to increase the K^+ permeability to such a degree that Cl^- flux becomes rate-limiting simplifies matters somewhat, but at these high rates the rate of water permeation may become important. Thus both of these methods allow one to assign only lower limits to the permeability coefficients.

These difficulties notwithstanding, both types of experiments have yielded useful information concerning the anion permeability of membrane vesicles prepared from *Torpedo* electroplax. The data are consistent with the behavior expected from the vesicles that contain

the voltage-gated Cl^- channel studied in planar bilayers: the vesicles are highly permeable to Cl^- but not K^+ or gluconate, and this permeability can be inhibited by DIDS. In addition, we now can say that these vesicles constitute a significant (35–50%) fraction of the total population. Preliminary experiments indicate that the vesicles containing the Cl^- channels co-purify with vesicles containing the Na^+/K^+ ATPase, a marker enzyme for the noninnervated face of the electroplaque, and clearly separate from the vesicles containing the acetylcholine receptor, a marker for the innervated face (White, 1981). Based on these results, we suggest that the Cl^- channel is found in the nononnervated face of the cell.

Although these findings agree qualitatively with the findings of Taguchi and Kasai (1980), quantitative comparison between their findings and ours in both bilayers and vesicles is difficult. The light-scattering experiments carried out by those authors are difficult to interpret because no evidence is given to demonstrate that the reswelling half-times are related to the anion permeability of the *Narke* vesicles. The concentration of valinomycin used (1 μg/ml) is much lower than the concentration we needed to eliminate the contribution of the K^+ permeability to the half-time of reswelling; in the absence of any information about the valinomycin sensitivity of the *Narke* vesicles we have no way of knowing if this is a concentration that enables one to unambiguously measure anion permeation rates. In addition, DIDS does not appear to affect the rate of reswelling in the *Narke* vesicles; rather, it is the amplitude of the signal that changes (cf., Fig. 1 of Taguchi and Kasai [1980]). Although the radioactive flux work convincingly demonstrates that there is indeed a DIDS-sensitive $SO_4^=$ permeability pathway in *Narke* vesicles, interpretation of these data in terms of a channel similar to the one in *Torpedo* is difficult because Taguchi and Kasai used $SO_4^=$, not Cl^-, as the permeant anion and the immeasurably low $SO_4^=$ permeability of the *Torpedo* Cl^- channel makes it impossible to compare the properties observed in intact vesicles with those in bilayers.

However, both the results presented here and those of Taguchi and Kasai demonstrate that there exists a significant anion permeability in membrane vesicles prepared from the electric organ of marine rays, and more importantly, it exists in amounts that may make it amenable to biochemical manipulation and purification.

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