Probes of the Conduction Process of a Voltage-gated Cl^- Channel from *Torpedo* Electroplax

MICHAEL M. WHITE and CHRISTOPHER MILLER

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. Dr. White's present address is Department of Physiology, University of California Medical School, Los Angeles, California 90024.

ABSTRACT The open-channel conductance properties of a voltage-gated Cl^- channel derived from *Torpedo californica* electroplax and incorporated into planar bilayers were studied by several approaches. In neutral bilayers the channel conductance saturates with Cl^- activity according to a rectangular hyperbolic relation with a half-saturation activity of 75 mM and a maximum conductance of 32 pmho. The observation of identical behavior in charged membranes implies that ions permeating the channel do not sense the surface potential of the bulk membrane. The Cl^-:Br^- permeability ratio, measured under biionic conditions, is independent of salt concentration. SCN^- ion reversibly blocks the channel. The voltage dependence of the block implies the existence of two separate blocking sites within the channel: one accessible from the *cis* side only (the side to which vesicles are added) and the other accessible from the *trans* side only. The block at each site is competitive with Cl^-. The results are consistent with a single-ion Eyring model of the conduction process in which the ion must traverse three kinetic barriers as it permeates the channel and in which the channel can accommodate at most one ion at a time.

INTRODUCTION

In recent years our understanding of the nature of channel-mediated ionic conduction across biological membranes has increased substantially through the study of model systems, such as gramicidin A (Eisenman et al., 1978) and hemocyanin,[1] as well as of channels from animal cell membranes, such as the Na^+ (Hille, 1975 *a*; Cahalan and Begenisich, 1976) and K^+ (Armstrong, 1975) channels of nerve, the acetylcholine-gated channel (Lewis and Stevens, 1979; Horn and Brodwick, 1980), and the sarcoplasmic reticulum K^+ channel (Coronado et al., 1980). In all cases, it was possible to represent the conduction of cations through these channels as an Eyring-type mechanism in which the ion must cross one or more kinetic barriers as it permeates the channel.

Although it is reasonable to assume that the principles involved in channel-mediated conduction of cations would hold for anion channels as well, the lack of a simple, well-behaved system has prevented any detailed studies of

[1] Cecchi, X., O. Alvarez, and R. Latorre. A three-barrier model for the hemocyanin channel. Submitted for publication.

conduction in anion-selective channels. We have been characterizing a voltage-gated, Cl^--selective channel in the electroplax membrane of the marine ray, *Torpedo californica* (White and Miller, 1979 and 1981; Miller and White, 1980). The channel is studied by incorporating electroplax membrane vesicles into a phospholipid planar bilayer by a process resembling membrane fusion. The channels are incorporated with at least 98% orientation, allowing the definition of the *cis* and *trans* sides of the membrane (*cis* being the side to which membrane vesicles are added). Membranes containing many channels display voltage-dependent Cl^- conductance. The steady-state conductance is low at positive voltages (*trans* side defined as ground), increases *e*-fold per 11 mV as the voltage becomes more negative, and then saturates at highly negative voltages. Each individual channel, on the other hand, operates by a mechanism involving a single conducting state in which the probabilities of being in the conducting (open) and nonconducting (closed) states are voltage dependent; the open-state conductance is nearly voltage independent and has a value of 16 pmho in 0.1 M KCl. Furthermore, the channel is inhibited by SITS (4-acetamino-4′-isothiocyano-2, 2′-disulfonic acid stilbene) and DIDS (4,4′-diisothiocyano-2,2′-disulfonic acid stilbene), two well-known inhibitors of anion transport in a variety of systems (see White and Miller [1979]). This inhibition is seen only when these compounds are added to the *cis* side; *trans* additions have no effect.

We have found that the channel is unusually Cl^--selective; of all other ions tested, only Br^- is appreciably permeant ($P_{Br^-}/P_{Cl^-} = 0.7$). In our investigations of the ionic selectivity of the Cl^- channel we found that SCN^- and I^- reversibly block the channel at millimolar concentrations (Miller and White, 1980). Furthermore, the block showed asymmetry, with stronger inhibition from the *cis* side than from the *trans* side.

In this paper we extend our investigation of the properties of the open channel by examining the dependence of the single-channel conductance upon ion activity and voltage as well as the nature of the block by SCN^- ion. The integration of the data from these experiments will allow us to propose a free-energy profile for conduction through the channel.

MATERIALS AND METHODS

Biochemical

T. californica were obtained from Pacific Bio-Marine Laboratories, Inc., Venice, Calif., and were used immediately. Membrane vesicles were prepared from the electric organ as previously described (White and Miller, 1981). In all experiments the vesicles at the sample–35% sucrose interface of the density gradients were used, because they are significantly enriched in Cl^- channel activity and depleted in α-neurotoxin binding activity (M. M. White, unpublished observation). Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were prepared from egg yolk; cardiolipin (CL) from beef heart was obtained from Sigma Chemical Co., St. Louis, Mo. Phosphatidylglycerol (PG) was prepared from egg yolk PC by transphosphatidylation in the presence of glycerol, using phospholipase D from Savoy cabbage (Comfurius and Zwaal, 1977).

Electrical System

The planar bilayer system, previously described in detail (Miller, 1978), was of the Mueller-Rudin (1969) type. The system consists of two identical aqueous solutions separated by a phospholipid bilayer ~0.8 mm in diameter. Membranes were formed with solutions of 50–70 mM phospholipid in *n*-decane. The electrical conductance of the membrane was measured under voltage-clamp conditions, and the temperature was maintained at 25°C by a circulating water bath. The aqueous phase consisted of the desired concentration of KCl (or KBr), 5mM HEPES, and 0.1 mM EDTA, neutralized to pH 7.35 with Tris base. Before introduction of channels, bilayers had a voltage-independent conductance of $\sim 10^{-8}$ mho/cm^2. We refer to the two sides of the bilayer as *cis* and *trans*. Membrane vesicles were always added to the *cis* side, while the *trans* chamber is defined as zero voltage. A small volume of concentrated membrane suspension was added with stirring to the *cis* chamber to give a final protein concentration of 1–20 μg/ml, and fusion was allowed to proceed as previously described (White and Miller, 1979).

Instantaneous current-voltage (*I-V*) curves were obtained using a programmable four-step pulse generator designed and built by Mr. B. Bablouzian of our department. The time resolution of the current amplifier was 100 μs. The membrane voltage was clamped to 0 mV, stepped to −85 mV for 2 s to open the channels, and then pulsed to the test voltage for 1 s before returning to 0 mV. Channels did not close appreciably in the first 50 ms after application of the test voltage, and so current during this interval was taken as the "instantaneous" current. For the SCN^--blocking experiments, a control *I-V* curve was obtained, KSCN was added from a concentrated stock solution to the appropriate side of the bilayer, and the *I-V* curve was again obtained. The data were then analyzed as described in the Appendix. All data were corrected for leak conductance, measured at +50 mV under steady-state conditions (White and Miller, 1979).

Permeability ratios were determined under biionic conditions as described in Coronado et al. (1980). Membrane surface charge densities were determined by the nonactin method of McLaughlin et al. (1970).

RESULTS

Conductance-Activity Relation

Fig. 1 shows single-channel conductance fluctuations in symmetrical 0.1-M KCl solutions. As we have previously shown (Miller and White, 1980), the fluctuations occur among well-defined unitary conductance levels. Under these conditions, the single-channel conductance, γ, is 16 pmho. Fig. 2 shows the dependence of the single-channel conductance on Cl^- activity for two types of membranes: neutral (PE/PC) and charged (PE/CL). Neutral membranes were chosen to minimize changes in surface potential with changing ionic strength; charged membranes were chosen to investigate the effect of bulk membrane surface potential on the channel conductance. Each set of data can be described by a linear hyperbola with a surface potential term included:

$$\gamma = \frac{\gamma^m}{1 + \frac{K}{a}\exp\left(-\frac{F\psi_o}{RT}\right)}, \quad (1)$$

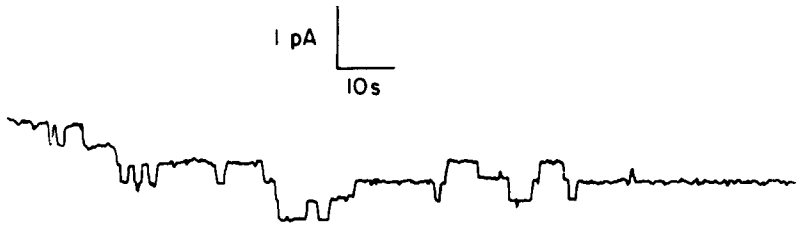

FIGURE 1. Single-channel conductance fluctuations. Channel fluctuations were recorded in bilayers composed of 70% PE/30% CL with symmetrical solutions containing 0.1 M KCl. The mean single-channel conductance from the above record is 16.0 ± 1.2 pmho (SD). The holding voltage was −20 mV.

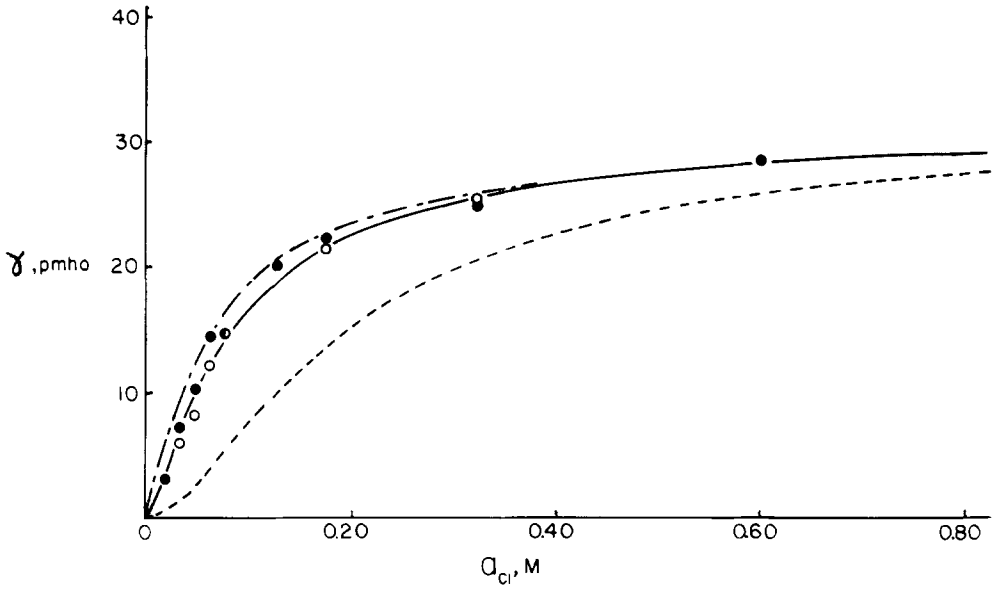

FIGURE 2. Conductance-activity relation in neutral and charged membranes. Single-channel conductances were measured at +40 mV in bilayers composed of 95% PE/5% PC (open circles) or 70% PE/30% CL (solid circles) in KCl buffers of various salt concentrations. Cl^- activity was calculated from Robinson and Stokes (1955). Each point represents the mean of 10–50 determinations; SEM in all cases was smaller than the diameter of the points. The curves were drawn according to Eqs. 1 and 2, using a value of 32 pmho for γ^m; 0.075 M for K, and $\sigma_o = 0$ (— · — · —), $\sigma_o = 2 \times 10^{-3}$ charges/Å^2 (– – –) (the measured charge density for PE/CL membranes) and $\sigma_o = 2.5 \times 10^{-4}$ charges/Å^2 (——) (the best fit).

where γ^m is the maximum conductance, a is the ion activity in the bulk medium, K is the apparent dissociation constant, ψ_o is the surface potential and F, R, and T have their usual meanings. The surface potential term must be included, since in the presence of any surface charge the bulk activity, a, is

not the same as at the membrane-solution interface and, hence, not the same as at the channel "mouth." The surface potential, ψ_o, is related to the surface charge density, σ_o, by

$$sinh\left(\frac{F\psi_o}{2RT}\right) = \frac{136\,\sigma_o}{\sqrt{a}}. \quad (2)$$

The value of σ_o for a given membrane composition can be determined by means of the nonactin method of McLaughlin et al. (1970).

In both neutral and charged bilayers, the data can be described well by the same parameters: the maximum conductance, γ^m, is 32 pmho; the half-saturation constant, K, is 0.075 M and the surface charge density, σ_o, is 2.5 $\times 10^{-4}$ electronic charges/Å^2. Included in Fig. 2 are theoretical curves calculated with the above values of γ^m and K and the measured values of the surface charge density for a neutral membrane ($\sigma_o = 0$) and the PE/CL membrane ($\sigma_o = 2 \times 10^{-3}$ charges/Å^2). These findings are significant for two reasons. First, the channel does not "see" the same surface charge density as the bulk membrane. Second, that, aside from surface potential effects, the conductance-activity relation follows a linear hyperbola indicates that the channel can be occupied by at most one ion at a time (Läuger, 1973).

As a further test of the single-ion nature of the channel, we have investigated the concentration dependence of the Br^-:Cl^- permeability ratio. Läuger (1973) has shown that, for a single-ion channel, the permeability ratio measured under biionic conditions is independent of ion concentration. This is not true for a multiion channel, for which the permeability ratio will vary with concentration as the saturation of the channel changes (Eisenman et al., 1978; Urban and Hladky, 1979). Fig. 3 shows that (P_{Br^-}/P_{Cl^-}) is indeed independent of ion concentration and has a value of 0.68 ± 0.03. Furthermore, (P_{Br^-}/P_{Cl^-}) does not depend upon the side of the membrane to which a given ion is added, an indication that Hille's (1975 *b*) "peak energy offset" approximation is valid for this channel.

Voltage Dependence of the Open Channel

To model the free-energy profile of the open channel, one must study the voltage dependence of the open-channel conductance. We have previously shown (White and Miller, 1979) that at 0.1 M Cl^-, γ is independent of voltage in the range +50 to −50 mV. We have now extended the range from +100 to −120 mV by using instantaneous *I-V* curves, which reflect the single-channel behavior. In Fig. 4, the voltage dependence of the channel conductance is presented at a saturating Cl^- concentration (1 M). Fig. 4 shows that in this extended voltage range, γ is indeed slightly voltage dependent. Note that between +50 and −50 mV the voltage dependence is weak, but as the voltage becomes highly negative, the conductance clearly increases with voltage. This behavior puts constraints on the construction of free energy profiles for the channel.

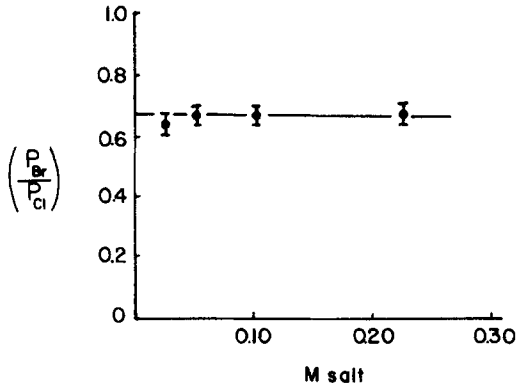

FIGURE 3. Concentration independence of the permeability ratio. Planar bilayers were formed as described in Methods in either KCl or KBr buffers and modified by incorporating *Torpedo* microsacs into the bilayer by membrane fusion. After the conductance had stabilized, biionic conditions were established by perfusing the *cis* chamber with either KBr or KCl buffer of identical ionic strength. The permability ratio, P_{Br}/P_{Cl}, was then determined by the method of tail currents (Coronado et al., 1980). Each point represents the mean ± SD of 3–10 determinations. The line corresponds to the mean of all determinations, 0.68 ± 0.03.

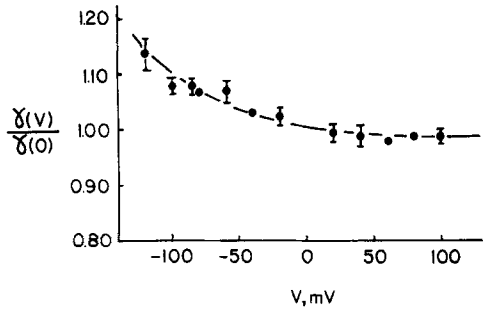

FIGURE 4. Conductance-voltage relation of the open channel in PE/CL membranes. Instantaneous *I-V* curves were obtained in 1.0 M KCl buffer, and, after the background conductance was subtracted, the conductance relative to the interpolated value at zero voltage was determined. Each point represents the mean ± SD of three determinations. The solid curve was obtained from Eq. 3, using the parameters from the model shown in Fig. 10.

SCN^- Block is Voltage Dependent

When SCN^- is added to either the *cis* or the *trans* chamber, the voltage-dependent Cl^- conductance is inhibited (Fig. 5). This inhibition occurs within the 10-s stirring time of the chamber and is fully reversed by removing the SCN^- by perfusion. SCN^- has no effect on the "leak" conductance measured at large positive voltages (Miller and White, 1980). If the inhibition is due to the blocking of the channel by SCN^-, then the effect should be voltage

dependent (see Appendix). As has been pointed out (Coronado and Miller, 1979), one cannot simply measure the macroscopic inhibition constant for SCN^- as a function of voltage, because this parameter is a combination of the single-channel dissociation constant and the voltage-dependent equilibrium constant for channel opening. Therefore, the observation of a voltage-dependent macroscopic blocking constant does not imply that the blocking reaction itself is voltage-dependent. Only when other voltage-dependent equilibria are eliminated can voltage-dependent blocking be unambiguously demonstrated.

There are two ways of eliminating the contribution of the channel-opening equilibrium constant: by direct measurement of the single-channel conductance as a function of voltage in the presence of the blocking ion, as has been done for the Cs^+ blocking of the sarcoplasmic reticulum K^+ channel (Coronado and Miller, 1979), or by analyzing the instantaneous *I-V* curves of many-channel membranes (Woodhull, 1973; Adelman and French, 1978). As shown

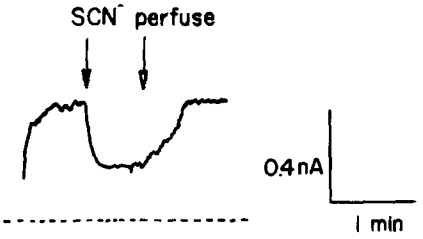

FIGURE 5. Effect of SCN^- on the voltage-dependent conductance. Planar bilayers (70% PE/30% PG) were formed in 100 mM KCl buffer, and channels were inserted as in Fig. 3. The voltage was clamped to −40 mV (note the increase in current as the channels open), and after the current had reached a steady value, KSCN was added to the *cis* chamber to a final concentration of 6 mM. After the current had reached a new steady-state level, the *cis* chamber was perfused with SCN^--free buffer. The dashed line represents the zero-current level.

in the Appendix, these two methods are equivalent when the blocking reaction is very fast as compared with channel gating relaxations. It is difficult to make detailed and accurate single-channel measurements for the *Torpedo* Cl^- channel, so we have studied the SCN^- block by the second method. Fig. 6 shows instantaneous *I-V* curves in the presence and absence of 12 mM KSCN on the *cis* side. Note that the control curve is linear, whereas in the presence of SCN^- the current is diminished (relative to the control) at all voltages, with the effect becoming more pronounced as the voltage is made increasingly negative. Fig. 7 shows that the data follow a simple blocking model (Eq. A-5) well; from the plot we obtain the "electrical distance," δ, of the blocking site from the *cis* side, as well as the apparent dissociation constant for SCN^-, $K(0)$, at zero voltage. The averages from all experiments in 200 mM KCl were $\delta = 0.35 \pm 0.02$ and $K(0)_{cis} = 8.5 \pm 0.4$ mM (SEM of 15 determinations).

When SCN^- is added to the *trans* side, voltage-dependent block is also seen

(Fig. 7); however, the electrical distance (also measured from the *cis* side), ϵ, for this site is 0.65 ± 0.03 and $K(0)_{trans}$ = 27 ± 3 mM in 200 mM KCl. This implies that there are two different blocking sites, one accessible from the *cis* side only and one accessible from the *trans* side only. Each of these sites experiences ~35% of the voltage drop from its respective side of the membrane. In all experiments, the values of δ and ϵ were independent of both SCN^- (2–30 mM) and Cl^- (100–300 mM) concentration.

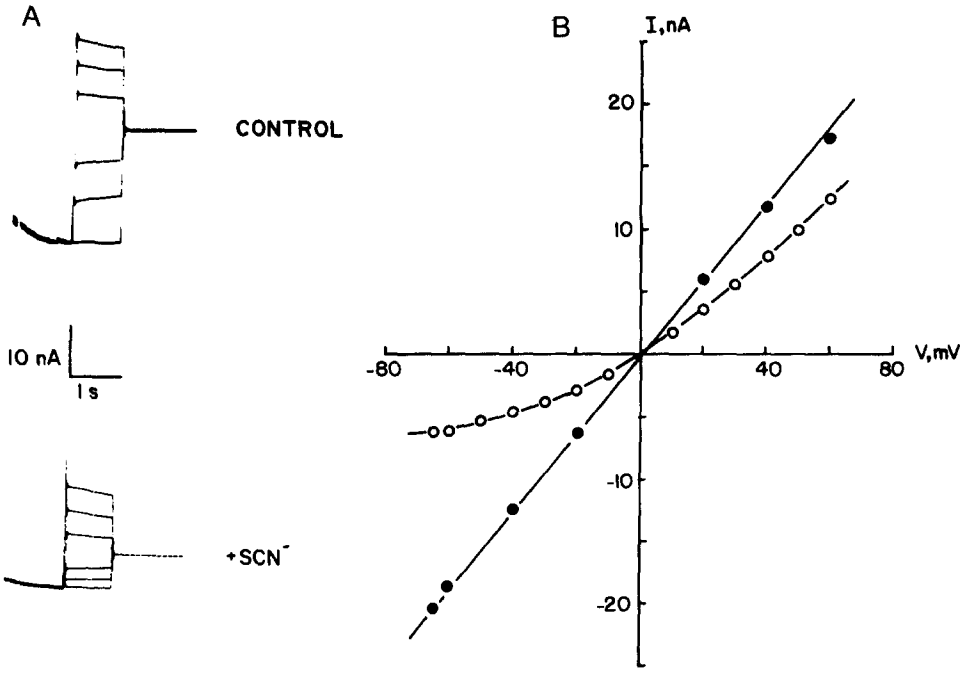

FIGURE 6. Voltage dependence of the SCN^- block in 200 mM Cl^-. A planar bilayer (70% PE/30% PG) was modified as described in the legend to Fig. 3. (*A*) The instantaneous *I-V* curve was obtained as described in Methods from +60 to −60 mV in 20-mV steps ("control"); 12 mM KSCN was added to the *cis* chamber, and the instantaneous *I-V* curve was obtained again ("+SCN"). (*B*) The data from *A* with points taken at additional voltages. Solid circles: control; open circles: $+SCN^-$.

SCN^- Block is Competitive with Cl^-

If SCN^- blocks by interacting reversibly with a conduction site within the channel, one would expect competition between Cl^- and SCN^- for that site. This competition would be manifested by an increase in $K(0)_{cis}$ and $K(0)_{trans}$ when the Cl^- concentration is increased. Fig. 8 shows that this is the case for *cis* blocking. Similar behavior is seen for *trans* blocking (data not shown). From the zero intercept one can obtain $K^*(0)_{cis}$ (or $K^*(0)_{trans}$), the "true" dissociation constant for the SCN^--channel site interaction, and, from the slope, the ratio

of the SCN^- and Cl^- affinities. However, measurements below 75 mM Cl^- are difficult to make, and so the extrapolation must be made with data from experiments with high Cl^- concentrations. This introduces a large error in the determination of both $K^*(0)$ and K_{Cl}. $K^*(0)_{cis}$ is on the order of 1–5 mM, whereas $K^*(0)_{trans}$ is on the order of 5–10 mM. The dissociation constant for Cl^- measured by this technique is on the order of 15–50 mM for both the *cis* and *trans* sites.

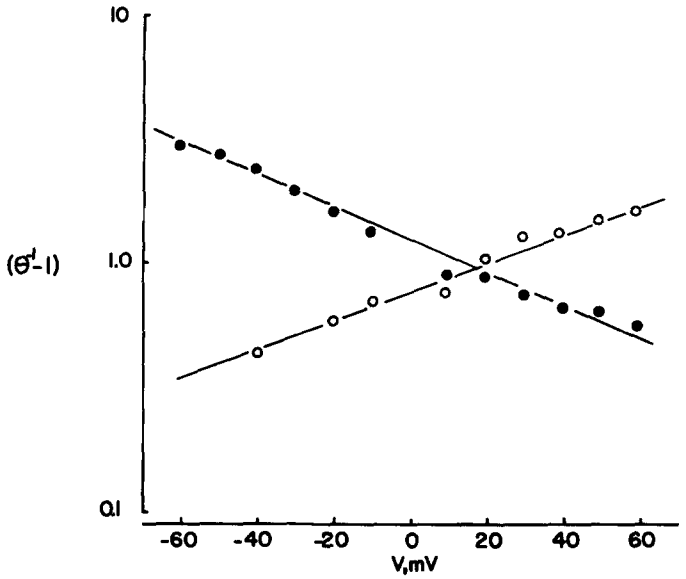

FIGURE 7. Analysis of blocking data. Instantaneous *I-V* curves such as the one in Fig. 6 were analyzed as described in the Appendix. θ is defined as the channel-mediated conductance in the presence of SCN^- relative to the conductance in the absence of SCN^-. (Solid circles) Voltage-dependent block in 200 mM Cl^- with 12 mM KSCN on the *cis* side. The solid curve corresponds to Eq. A-5 with $\delta = 0.39$ and $K(0)_{cis} = 9.9$ mM. (Open circles) Voltage-dependent block in 200 mM Cl^- with 25 mM KSCN on the *trans* side. The solid curve corresponds to Eq. *A*-5 with $\epsilon = 0.63$ and $K(0)_{trans} = 33.5$ mM.

SCN^- Block Decreases the Single-Channel Conductance

To confirm that the effect of SCN^- represents true channel blocking, we have investigated the effect of SCN^- on the single-channel conductance. Although detailed measurements are not possible due to the difficulty of obtaining membranes with only a few channels, the reduction of channel conductance by SCN^- is confirmed in Fig. 9. At +30 mV the control single-channel conductance, γ_o, was 16.3 pmho, whereas in the presence of 6 mM SCN^- on both sides of the membrane, the value of γ was 7.9 pmho. The value of γ/γ_o obtained, 0.48, is in good agreement with the value predicted from Eq. A-2, 0.47. We do not expect to see the discrete nature of the block, as was observed

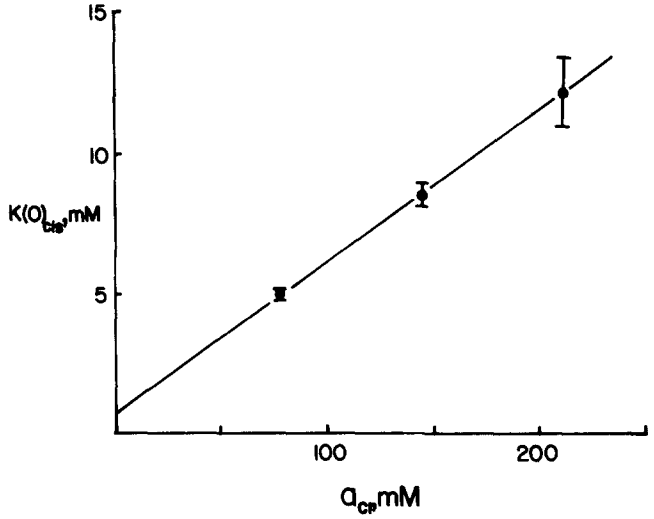

FIGURE 8. Competition between SCN^- and Cl^- for the blocking site. The apparent dissociation constant for *cis* SCN^- blocking, $K(0)_{cis}$, was determined at several Cl^- concentrations. Each point represents the mean ± SEM of five to seven determinations. Cl^- activity was calculated from Robinson and Stokes (1955), and the solid line is of the form $K(0)_{cis} = K^*(0)_{cis}\,[1 + (Cl^-/K_{Cl})]$, with $K^*(0)_{cis} = 1$ mM and $K_{Cl} = 30$ mM.

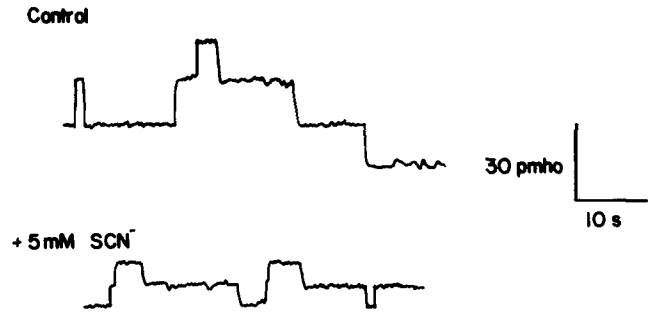

FIGURE 9. Effect of SCN^- on the single-channel conductance. Single channels were measured in 70% PE/30% PG membranes in 100 mM KCl buffer at +30 mV in the presence and absence of 6 mM KSCN on both sides of the membrane. The control single channel conductance, γ_o, is 16.3 ± 0.6 pmho, and that in the presence of SCN^-, γ, is 7.9 ± 0.7 pmho.

by Neher and Steinbach (1978), because of the limit of the low time resolution of the recordings and the rapid nature of the blocking reaction.

DISCUSSION

The results of this study demonstrate that the *Torpedo* Cl^- channel is an excellent system for studying the mechanism of anion conduction. The channel

has well-defined properties (voltage-dependent gating, ionic selectivity, blockade by impermeant ions) that can be studied in detail on both the macroscopic and microscopic levels. This has allowed an investigation into the nature of ion conduction through an anion channel by the approach that has proved so fruitful in the study of cation channels.

Effect of Surface Potential on the Conduction Process

That an ion channel's conduction process may be insensitive to surface potential was first reported by Begenisich (1975). His work on *Myxicola* giant axons demonstrated that, although the gating processes of both the Na^+ and K^+ channels are affected by changes in bulk surface potential, the instantaneous *I-V* curves, which reflect the conduction process, are not. In addition, Horn and Patlak's (1980) elegant work on the single-channel conductance properties of the acetylcholine-gated channel from cultured rat muscle demonstrated that the single-channel current as a function of Na^+ ion activity follows a simple linear hyperbola. Animal cell membranes carry a substantial negative surface charge, and so one would not expect this behavior unless the channel did not sense the bulk membrane surface potential.

The data of Fig. 2 demonstrate that the open-channel properties of the *Torpedo* Cl^- channel are not affected by the bulk membrane surface potential. The channel senses the same surface potential in neutral (PE/PC) and charged (PE/CL) membranes, even though the bulk surface potentials differ by ~45 mV at 75 mM, corresponding to a fivefold lower surface Cl^- concentration in charged than in neutral membranes; clearly, the conductance-activity curves do not reflect this difference. There are several possible explanations for this behavior, all of which depend on a shielding of the bulk surface charge at the channel mouth. One could imagine that, when the channels are inserted into the planar bilayer by fusion, the vesicle lipids and the planar bilayer lipids do not mix, resulting in patches of membrane with a different charge density than the bulk membrane. If this were the case, then one would expect that not only the conduction properties of the channel would be insensitive to the lipid composition of the bilayer but that the gating properties should also not be affected by the make-up of the planar bilayer. We have found that the conductance-voltage curves are quite sensitive to the lipid composition of the bilayer, with a shift of 10–20 mV on the voltage axis between PE/PC membranes and PE/CL membranes (unpublished observation).

As an alternative to the patching model, one can assume that the channel protein is surrounded by the bulk lipids but that the architecture of the channel is such that the channel mouth is isolated from the membrane surface. As a crude estimate for the distance required to bring about this isolation, we note that the Debye length is 9.6 Å in 0.1 M KCl. Therefore, if the channel mouth is 15–20 Å from the membrane surface, it would fail to sense most of the surface potential. One could imagine several configurations of the channel protein that could give rise to such an isolation. For example, a flat disk of protein could surround the mouth, making it look like a plate with a hole in the middle when viewed along an axis perpendicular to the bilayer plane.

Another possibility is that the entire channel protein could extend a substantial distance into the aqueous phases on both sides of the membrane. Although these possibilities are mere speculation, they are useful for setting up more rigorous models involving deviations from the Gouy-Chapman smeared charge model. (See Nelson and McQuarrie [1975] for such an approach.)

A Model for the Conduction Process

Table I summarizes the data obtained from the SCN^--blocking experiments described in Results. The most surprising result is that there are two separate blocking sites in the channel, one accessible from the *cis* side only and one accessible from the *trans* side only. SCN^- cannot go from one site to the other (Miller and White, 1980), indicating that it is a truly impermeant ion. It can enter the channel; it can leave the channel; but it cannot traverse the channel. The free energy barriers between the two sites are too great for SCN^- to cross.

TABLE I
SUMMARY OF BLOCKING DATA

	cis Block	*trans* Block
δ or ϵ	0.35±0.02	0.65±0.03
$K^*(0)$, *mM*	1–2	2–4
K_{Cl}, *mM*	15–50	15–50

The voltage dependence of the SCN^- block was determined for both *cis* and *trans* additions of SCN^- at several Cl^- concentrations. The locations of the *cis* (δ) and the *trans* (ϵ) blocking sites were found to be independent of SCN^- and Cl^- concentration. $K^*(0)$ is the extrapolated dissociation constant at zero Cl^- as described in the legend of Fig. 8, and K_{Cl} was obtained from the slope of the same plots. The values of δ and ϵ represent the mean ± SEM of 15–20 determinations, while the values of $K^*(0)$ and K_{Cl} were obtained by least-squares analysis, and the ranges represent the uncertainties in the determination of the slopes and intercepts by this method. Note that both δ and ϵ are measured from the *cis* side of the membrane.

The results presented here are consistent with the notion that the *Torpedo* Cl^- channel can be occupied by at most one ion at a time. Läuger (1973) has shown that a single-ion channel must show linear hyperbolic saturation of conductance with increasing salt concentration and concentration-independent biionic permeability ratios. The Cl^- channel fulfills both of these criteria (Figs. 2 and 3). In addition, the block by SCN^- is purely voltage dependent, another necessary condition for a single-ion channel. We see no discontinuity in the blocking effectiveness as the direction of current flow changes (zero voltage in the case shown in Fig. 7). Finally, the concentration independence of δ and ϵ for the SCN^- block is a further demonstration of the single-ion nature of the channel. This is in contrast to the squid axon K^+ channel, where Adelman and French (1978) found that the value of δ for the Cs^+ block varies with Cs^+ concentration. They interpreted this to mean that more than one Cs^+ ion at a time could be accommodated in the channel. Similar results have

been obtained for the inward rectifying K^+ channel of starfish eggs (Ciani et al., 1980). Both of these channels are known to act as multiion pores.

With these properties in mind, and with the data of Fig. 4, we can construct a plausible free-energy profile of the channel using Eyring rate theory (Hille, 1975 *b*). We have chosen to model the channel as a three-barrier, two-site system that can be occupied by at most one ion at a time. This is the simplest configuration that fits the blocking data, which require at least two sites. The position of these free-energy wells are given by δ and ϵ, the electrical distances of the blocking sites measured from the *cis* side. To obtain information about the individual peak heights and well depths, we must examine the voltage dependence of the channel conductance. It can be shown that for a three-barrier, two-site channel with wells at δ and ϵ, under saturating ion concentration, the ratio of the single-channel conductance at a given voltage to that at zero voltage is given by

$$\frac{\gamma^m(V)}{\gamma^m(0)} = \frac{\sinh(z\Psi)}{(z\Psi)} \frac{(1 + A + B)(1 + C)}{WX + BY} \tag{3}$$

$$W = A \exp[-(1 - \epsilon)\, z\Psi] + \exp(\delta z\Psi)$$

$$X = \exp[-(\epsilon - \delta)z\Psi] + C \exp[(\epsilon - \delta)z\Psi]$$

$$Y = C \exp[(\epsilon - \delta - 1)z\Psi] + \exp[(\delta - \epsilon + 1)z\Psi],$$

where $\Psi = FV/2RT$ and z is the valence of the ion. A, B, and C are ratios of the rate constants defined in Fig. 10. A is the ratio of entry rates into the channel (k_{01}/k_{32}), B is the ratio of rates for leaving the well at ϵ (k_{10}/k_{12}), and C is the ratio of rates over the central barrier (k_{21}/k_{12}). By fixing δ and ϵ, as well as C (Table I), we can construct free-energy profiles that give the behavior seen in Fig. 8. Many combinations of values for A and B that give the asymmetry shown in Fig. 8 can be found, but they all have the same characteristics, namely, the entry rate ratio, A, is in the range 1.2–1.5 and the parameter B is in the range 1.7–2.3. These small differences, corresponding to only 0.1–0.5 kcal/mol energy differences in the barrier heights, are enough to give the behavior seen.

To assign numerical values to the peak heights and well depths, one can use absolute rate theory to obtain the single-channel conductance at saturation and the channel's dissociation constant for Cl^- in terms of the individual rate constants. Since the energy level differences between the individual peaks are small ($\leq$0.5 kcal/mol), we will use as a first approximation Läuger's (1973) approach for symmetrical channels. It has been shown (Coronado et al., 1980) that for a channel with n equal barriers and $(n - 1)$ equal wells, the maximum single-channel conductance, γ^m, and the dissociation constant, K, can be expressed in terms of the peak heights and the well depths:

$$\gamma^m = \frac{e^2}{n(n-1)h} \exp\frac{(-(p - w))}{RT} \tag{4}$$

and

$$K = \frac{1}{n-1}\exp(w/RT), \tag{5}$$

where p and w are the Gibbs free energies of the peaks and wells (with respect to the aqueous solutions, using a 1 M standard state), h is Planck's constant, and e is the electronic charge. Using a value of 32 pmho for γ^m and 0.075 M for the dissociation constant, we obtain a value of $p = 7.1$ kcal/mol and $w = -1.1$ kcal/mol. After making the minor corrections on the individual peaks

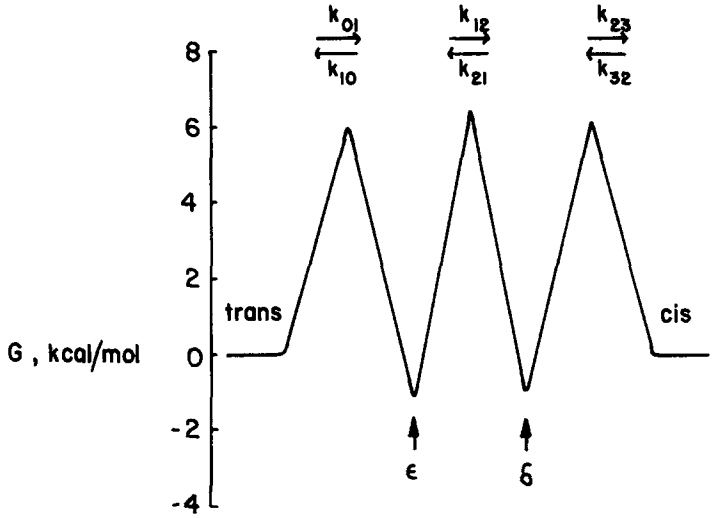

FIGURE 10. Three-barrier model for the *Torpedo* Cl^- channel. The diagram represents an Eyring free-energy profile that must be negotiated by a Cl^- ion during its passage through the channel. The profile has been constructed to be consistent with the data presented in this paper: the slight asymmetry in the conductance-voltage relation, the two SCN^--blocking sites 35% of the way in from the *cis* and the *trans* sides, an apparent dissociation constant of 75 mM, and a maximum conductance of 32 pmho.

and wells, we obtain the profile for Cl^- conduction shown in Fig. 10. For SCN^- the wells would be ~2.5 kcal/mol deeper ($K_{SCN}/K_{Cl}/ \sim 0.01$) and the peak heights at least 2 kcal/mol higher ($P_{SCN}/P_{Cl} < 0.05$; Miller and White 1980), meaning that the barrier height for SCN^- is at least 4.5 kcal/mol higher than for Cl^-.

We emphasize that the model in Fig. 10 is an idealized conception of the free-energy profile across the channel. It is the simplest model consistent with all of the data presented here. More complicated models obviously are possible, but we see no need to put in additional barriers or wells at this time.

Comparisons with Other Anion Conductances

Although it has not been possible to study other anion-specific channels in the detail presented here, there is evidence that SCN^- blocks anion conductances of biological membranes. The phenomenon of "anomalous mole-fraction dependence" of conductance when SCN^- is used to replace Cl^- has been observed in several systems, among them frog skeletal muscle (Hutter and Padsha, 1959), crayfish inhibitory synapse (Takeuchi and Takeuchi, 1971), and stingray muscle (Hagiwara and Takahashi, 1974). When small quantities of SCN^- are added, the conductance decreases, but as more SCN^- is added, the conductance increases. At low concentrations, SCN^- acts as a blocker, whereas at high concentrations it seems to be permeant. We do not observe this behavior in the *Torpedo* Cl^- channel. When all of the Cl^- is replaced by SCN^-, the voltage-dependent conductance is eliminated (Miller and White, 1980). In addition, the *Torpedo* channel is much more selective than these other anion pathways, which allow rather nonselective permeation by a variety of anions (Miller and White, 1980; Wright and Diamond, 1977).

These differences notwithstanding, the *Torpedo* Cl^- channel is an excellent system for the study of mechanisms of channel-mediated anion conduction. The processes underlying the kinetics and thermodynamics of voltage-dependent channel gating can be studied as well. Finally, the planar bilayer system itself enables one to control not only the ionic composition of the bathing medium but also the protein concentration and lipid composition of the bilayer, making modifications of the channel environment possible.

APPENDIX

Analysis of Single-Channel Blocking from Macroscopic I-V Curves

A simple model for voltage-dependent blocking of ion channels has been presented by Woodhull (1973). In brief, one assumes that the blocking ion moves part of the way across the membrane to form the blocking complex. The electric field acting on the charged ligand would contribute to the standard free energy of the binding reaction. Thus, $K(V)$, the dissociation constant for the SCN^--binding reaction, should vary exponentially with the voltage according to

$$K(V)_{cis} = K(0)_{cis} \exp\left(-\frac{z\delta FV}{RT}\right) \text{ for } cis \text{ blocking} \qquad (A\text{-}1\ a)$$

$$K(V)_{trans} = K(0)_{trans} \exp\left[\frac{z(1-\epsilon)FV)}{RT}\right] \text{ for } trans \text{ blocking,} \qquad (A\text{-}1\ b)$$

where $K(0)$ is the zero-voltage dissociation constant, δ and ϵ are the fractions of the total electrical potential drop (measured from the *cis* side) experienced at the blocking site for *cis* or *trans* additions of SCN^-, respectively; z, F, V, R, and T have their usual meanings. Note that if there is a single site accessible from both the *cis* and the *trans* sides, $\delta = \epsilon$. If the blocking of the single channel follows a simple binding scheme,

then we obtain the following

$$\frac{\gamma}{\gamma_o}(V) = \left[1 + \frac{[B]_{cis}}{K(0)_{cis}} \exp\frac{(z\delta FV)}{RT} + \frac{[B]_{trans}}{K_{trans}} \exp\frac{(z(\epsilon - 1)FV)}{RT}\right]^{-1}, \qquad (A\text{-}2)$$

where γ and γ_o are the single-channel conductances in the presence and absence of the blocker, respectively, and [B] is the concentration of the blocking ion.

To relate the macroscopic to the microscopic behavior, one must assume that the blocking-unblocking reaction is much faster than the channel gating. If this is true, then the instantaneous *I-V* curve will reflect the single-channel blocking-unblocking. This is a reasonable assumption for the *Torpedo* Cl^- channel, where the relaxation time for the opening-closing reaction is 200 ms and greater (data not shown), much slower than any binding-unbinding reactions. At the end of the −85-mV prepulse, essentially all of the channels are open and the macroscopic conductance is given by

$$g(-85) = N\gamma + g_b, \qquad (A\text{-}3)$$

where N is the total number of channels and g_b is the ohmic "leak" conductance. Immediately after the application of the test voltage, V, all of the channels remain open, so we can define $\theta(V)$ as

$$\theta(V) = \frac{g(V, [B]) - g_b}{g(V, 0) - g_b} = \frac{\gamma}{\gamma_o}(V). \qquad (A\text{-}4)$$

We thus obtain

$$[\theta^{-1}(V) - 1] = \left\{\frac{[B]_{cis}}{K(0)_{cis}} \exp\frac{(z\delta FV)}{RT} + \frac{[B]_{trans}}{K(0)_{trans}} \exp\frac{(z(\epsilon - 1)FV)}{RT}\right\}. \qquad (A\text{-}5)$$

This formula allows one to obtain both the position of the blocking site(s) and the voltage-independent dissociation constant. In most cases, SCN^- is added to only the *cis* or only the *trans* side, which further simplifies the analysis. In this case, when $\ln(\theta(V)^{-1} - 1)$ is plotted vs. V, the slope yields δ (or ϵ), and the intercept the zero-voltage dissociation constant.

We are grateful to Dr. R. Coronado for comments and suggestions on the manuscript and to Dr. Ramón Latorre for a preprint of his paper on conduction through hemocyanin channels.[1] This research was sponsored by National Science Foundation grant BNS76-23212, National Institutes of Health (NIH) grant R01-AM-19826-03, NIH training grant GM-00212-20 (to M. M. White) and NIH Research Career Development Award K04-AM-00354-02 (to C. Miller). This is publication 1,348 from the Graduate Department of Biochemistry, Brandeis University.

Received for publication 4 February 1981.

REFERENCES

ADELMAN, W. J., and R. J. FRENCH. 1978. Blocking of the squid axon potassium channel by external Cs^+ ions. *J. Physiol. (Lond.).* **276:**13–25.

ARMSTRONG, C. M. 1975. K^+ pores of nerve and muscle membranes. *In* Membranes, a Series of Advances, Vol. III. G. Eisenman, editor. Marcel Dekker, Inc., New York. 325–358.

BEGENISICH, T. 1975. Magnitude and location of surface changes in *Myxicola* giant axons. *J. Gen. Physiol.* **66:**47–65.

CAHALAN, M., and T. BEGENISICH. 1976. Sodium channel selectivity. Dependence on internal permeant ion concentration. *J. Gen. Physiol.* **68:**111–125.

CIANI, S., S. KRASNE, and S. HAGIWARA. 1980. A model for the effects of potential and external K^+ concentration on the Cs^+ blocking of inward rectification. *Biophys. J.* **30:**199–204.

COMFURIUS, P., and R. F. A. ZWAAL. 1977. The enzymatic synthesis of phosphatidylserine and purification by CM-cellulose column chromatography. *Biochim. Biophys. Acta.* **488:**36–42.

CORONADO, R., and C. MILLER. 1979. Voltage-dependent Cs^+ block of a K^+ channel from sarcoplasmic reticulum. *Nature (Lond.).* **280:**807–810.

CORONADO, R., R. L. ROSENBERG, and C. MILLER. 1980. Ionic selectivity, saturation, and block in a K^+-selective channel from sarcoplasmic reticulum. *J. Gen. Physiol.* **76:**425–446.

EISENMAN, G., J. SANDBLOM, and E. NEHER. 1978. Ionic selectivity, saturation, binding, and block in the gramicidin A channel: a summary of recent findings. *In* Membrane Transport Processes, Vol. 2. D. C. Tosteson, Yu. A. Ovchinnikov, and R.Latorre, editors. Raven Press, New York. 285–312.

HAGIWARA, S., and K. TAKAHASHI. 1974. Mechanism of anion permeation through the muscle fibre membrane of an elasmobranch fish, *Taeniuva lymma* (stingray). *J. Physiol. (Lond.)* **238:** 109–127.

HILLE, B. 1975 *a*. Ionic selectivity, saturation, and block in Na^+ channels. A four-barrier model. *J. Gen. Physiol* **66:**535–560.

HILLE, B. 1975 *b*. Ionic selectivity of Na^+ and K^+ channels of nerve membranes. *In* Membranes, a Series of Advances, Vol. III. G. Eisenman, editor. Marcel Dekker, Inc., New York. 255–323.

HORN, R., and M. S. BRODWICK. 1980. Acetylcholine-induced current in perfused rat myoballs. *J. Gen. Physiol.* **75:**297–321.

HORN, R., and J. PATLAK. 1980. Single channel currents from excised patches of muscle membranes. *Proc. Natl. Acad. Sci. U. S. A.* **77:**6930–6935.

HUTTER, O. F., and S. M. PADSHA. 1959. Effect of nitrate and other anions on the membrane resistance of frog skeletal muscle. *J. Physiol. (Lond.).* **146:**117–132.

LÄUGER, P. 1973. Ion transport through pores: a rate theory analysis. *Biochim. Biophys. Acta.* **311:** 423–441.

LEWIS, C. A., and C. F. STEVENS. 1979. Mechanism of ion permeation through channels in a postsynaptic membrane. *In* Membrane Transport Processes, Vol. 3. C. F. Stevens and R. W. Tsien, editors. Raven Press, New York. 133–151.

MCLAUGHLIN, S. G. A., G. SZABO, G. EISENMAN, and S. M. CIANI. 1970. Surface charge and conductance of phospholipid membranes. *Proc. Natl. Acad. Sci. U. S. A.* **67:**1268–1275.

MILLER, C. 1978. Voltage-gated cation conductance channel from fragmented sarcoplasmic reticulum: steady-state electrical properties. *J. Membr. Biol.* **40:**1–23.

MILLER, C., and M. M. WHITE. 1980. A voltage-gated Cl^- conductance channel from *Torpedo* electroplax membrane. *Ann. N. Y. Acad. Sci.* **341:**534–551.

MUELLER, P., and D. O. RUDIN. 1969. Biomolecular lipid membranes. Techniques of formation, study of electrical properties, and induction of ionic gating phenomena. *In* Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stampfli, editors. Springer-Verlag, Berlin. 141–156.

NEHER, E., and J. H. STEINBACH. 1978. Local anaesthetics transiently block currents through single acetylcholine receptor channels. *J. Physiol. (Lond.).* **277:**153–176.

NELSON, A. P., and D. A. MCQUARRIE. 1975. The effect of discrete charges on the electrical properties of a membrane. I. *J. Theor. Biol.* **55:**13–27.

ROBINSON, A., and R. H. STOKES. 1955. Electrolyte Solutions. Academic Press, Inc., New York.

TAKEUCHI, A., and N. TAKEUCHI. 1971. Anion interaction at the inhibitory postsynaptic membrane of the crayfish neuromuscular junction. *J. Physiol. (Lond.)*. **212:**337–351.

URBAN, B. W., and S. B. HLADKY. 1979. Ion transport in the simplest single file pore. *Biochim. Biophys. Acta.* **554:**410–429.

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

WHITE, M., M., and C. MILLER. 1981. Chloride permeability of membrane vesicles isolated from *Torpedo* electroplax. *Biophys. J.*, In press.

WOODHULL, A. M. 1973. Ionic blockage of Na^+ channels in nerve. *J. Gen. Physiol.* **61:**687–708.

WRIGHT, E. M., and J. M. DIAMOND. 1977. Anion selectivity in biological systems. *Physiol. Rev.* **57:**109–156.