A Chloride Channel from Lobster Walking Leg Nerves

Characterization of Single-Channel Properties in Planar Bilayers

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ABSTRACT A novel, small conductance of Cl^- channel was characterized by incorporation into planar bilayers from a plasma membrane preparation of lobster walking leg nerves. Under conditions of symmetrical 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, single Cl^- channels exhibit rectifying current–voltage (*I-V*) behavior with a conductance of 19.2 ± 0.8 pS at positive voltages and 15.1 ± 1.6 pS in the voltage range of −40 to 0 mV. The channel exhibits a negligible permeability for Na^+ compared with Cl^- and displays the following sequence of anion permeability relative to Cl^- as measured under near bi-ionic conditions: I^- (2.7) > NO_3^- (1.8) > Br^- (1.5) > Cl^- (1.0) > $CH_3CO_2^-$ (0.18) > HCO_3^- (0.10) > gluconate (0.06) > F^- (0.05). The unitary conductance saturates with increasing Cl^- concentration in a Michaelis-Menten fashion with a K_m of 100 mM and γ_{max} = 33 pS at positive voltage. The *I-V* curve is similar in 10 mM Tris or 10 mM HEPES buffer, but substitution of 100 mM NaCl with 100 mM tetraethylammonium chloride on the *cis* side results in increased rectification with a 40% reduction in current at negative voltages. The gating of the channel is weakly voltage dependent with an open-state probability of 0.23 at −75 mV and 0.64 at +75 mV. Channel gating is sensitive to *cis* pH with an increased opening probability observed for a pH change of 7.4 to 11 and nearly complete inhibition for a pH change of 7.4 to 6.0. The lobster Cl^- channel is reversibly blocked by the anion transport inhibitors, SITS (4-acetamido, 4′-isothiocyanostilbene-2,2′-disufonic acid) and NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid). Many of these characteristics are similar to those previously described for small conductance Cl^- channels in various vertebrate cells, including epithelia. These functional comparisons suggest that this invertebrate Cl^- channel is an evolutionary prototype of a widely distributed class of small conductance anion channels.

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INTRODUCTION

Chloride channels that are not directly gated by agonist molecules are known to be widely distributed in many cells (Frizzell, 1987; Gogelein, 1988). However, the function and regulation of agonist-independent Cl^- channels in neural cells have received relatively little attention. Abnormalities in the Cl^- conductance of skeletal muscle fibers are known to produce abnormal muscle action potentials (Adrian and Bryant, 1974) and more recent work has suggested that protein kinase–mediated regulation of Cl^- currents may be important in the normal function of epithelia (Frizzell, 1987; Hwang et al., 1989; Li et al., 1989) and cardiac myocytes (Bahinski et al., 1989; Harvey and Hume, 1989). Since most Cl^- channels do not exhibit the profound voltage- and time-dependent behavior of cation-selective channels in excitable cells, their contribution has often been dismissed as leak current in macroscopic electrophysiology. However, at the microscropic level patch clamp experiments have begun to reveal the characteristics of single Cl^- channels in isolation. These functional studies suggest that Cl^- channels comprise a diverse class of channel proteins to be considered along with Na^+-, K^+-, and Ca^{2+}-selective channels as effectors and regulators of secretion and signaling.

This report describes the single-channel properties of a relatively low conductance (~20 pS) Cl^- channel that is readily incorporated into planar lipid bilayers from a plasma membrane preparation of lobster walking leg nerve. This particular membrane vesicle preparation was previously developed by Correa et al. (1987) to study the actions of various neurotoxins on $^{22}Na^+$ flux mediated by TTX-sensitive Na^+ channels. Since this preparation is enriched in TTX-binding sites, it is likely that the Cl^- channel we observe originates from the axon plasma membrane; however, it is also possible that it may derive from Schwann cell membranes. To our knowledge, Cl^- channels have not been previously described in crustacean axons, although a macroscopic Cl^- current has been documented in squid giant axons (Inoue, 1985), and single Cl^- channels have been characterized in cultured vertebrate neurons (Franciolini and Nonner, 1987; Franciolini and Petris, 1988), Schwann cells (Gray et al., 1984), and oligodendrocytes (Barres et al., 1988). Several of the functional characteristics of the lobster Cl^- channel described in this paper are similar to those of small conductance Cl^- channels from various vertebrate tissues, including epithelia. Our results on the single-channel properties of a Cl^- channel from an invertebrate species suggest that Cl^- channels are an ancient class of proteins with distinctive characteristics that have been conserved in evolution. A preliminary version of this work has been presented in abstract form (Lukács and Moczydlowski, 1989).

METHODS

Membrane Preparation

Plasma membrane vesicles from walking leg nerves of the North American lobster, *Homarus americanus*, were isolated essentially as described by Correa et al. (1987). After dissecting and rinsing 6–7 g wet weight of nerves from six lobsters in S1 solution (0.33 M sucrose, 2 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5, at 0–4°C), the nerves were homogenized at 2 ml S1/g

tissue in four separate aliquots with a Tissumizer (Tekmar Co., Cincinnati, OH) at 12,000 rpm for 30 s. The homogenate was adjusted with S1 solution to a volume of 10 ml per g of wet nerve and homogenized (six strokes) with a glass-teflon homogenizer in 15-ml portions. After centrifugation at 85,000 *g* for 30 min the pellet was resuspended in S1 and homogenized as before. This homogenate was layered on 30 ml of solution containing 1.12 M sucrose, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and centrifuged in a swinging bucket rotor at 65,000 *g* for 1 h. Plasma membrane vesicles were collected from the top of the 1.12 M sucrose layer, diluted threefold with 10 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.5, and centrifuged at 85,000 *g* for 30 min. The pellet was resuspended in 0.3 M sucrose, 10 mM Tris-HCl, 0.1 mM EGTA, pH 7.4, at a final protein concentration of 3–4 mg/ml, frozen in liquid nitrogen in small aliquots, and stored at −70°C.

Planar Bilayers and Cl^- Channel Incorporation

Planar bilayers composed of a mixture of 25 mg/ml neutral lipids in decane (8:2 bovine brain phosphatidylethanolamine/1,2-diphytanoylphosphatidylcholine) were painted with a fire-polished glass capillary over a 200-μm hole drilled in a polystyrene chamber. Bilayer formation was monitored by the increase in membrane capacitance during membrane thinning (final value = 150–200 pF). The volume of the *cis* and *trans* chambers was 1.2 and 0.6 ml, respectively. Chloride channels were normally incorporated in the presence of a symmetrical solution of 100 mM NaCl, 10 mM Tris base adjusted to pH 7.4 with 8.2 mM HCl. Lobster nerve membrane vesicles were added to the *cis* chamber (1.5–5 μg/ml) and continuously stirred until a Cl^- channel incorporated as indicated by a stepwise increase in current (~1 pA) monitored at ±50 mV. Neither an osmotic gradient nor additional Ca^{2+} were necessary for Cl^- channel incorporation. In most of the attempts this incorporation procedure resulted in bilayers containing multiple Cl^- channels. Single-channel bilayers were obtained in only 10–20% of the experiments.

Electrical Measurements and Data Analysis

Current was measured with a high-gain amplifier circuit based on patch clamp design as described previously (Guo et al., 1987). Contact with the aqueous chamber solutions was made via Ag/AgCl electrodes through small agar bridges containing 0.2 M KCl, 0.1 mM EDTA. The *trans* chamber, connected to the input of an operational amplifier used as a current-to-voltage converter, was defined as virtual ground. Applied potentials were corrected for electrode asymmetry and junction potentials. In this paper we follow the convention that positive currents, plotted upward on chart records, correspond to current moving *cis* → *trans* or Cl^- movement *trans* → *cis*. Reversal potentials were determined by linear interpolation between two and four current values on either side of the reversal. The current output was filtered with an 8-pole low pass Bessel filter at 50 or 100 Hz (−3 db corner frequency) for analysis and display. Recordings of single-channel experiments were stored on VCR tape using a VR-10 digital data recorder from Instrutech Corporation (Elmont, NY). Measurements of unitary current and open state probability, and construction and fitting of dwell time histograms was performed with the aid of an Atari computer system and Instrutech interface designed for single-channel analysis (Affolter and Sigworth, 1988). Dwell time histograms of closed- and open-state events were compiled using a 50% threshold criterion from single-channel records digitized at 2 kHz. The resulting frequency density histograms were fit to a sum of exponentials according to the method of Sigworth and Sine (1987) which uses a log-time binning procedure.

RESULTS

Incorporation and Identification of Single Cl^- Channels

In preliminary bilayer experiments with the lobster nerve membrane preparation of Correa et al. (1987) we observed two types of conductance activity in the presence of 0.2 M symmetrical KCl. One class of unitary channel fluctuations exhibited a relatively large conductance with brief openings similar to the lobster K^+ channel previously described by Coronado et al. (1984). The second class of fluctuations

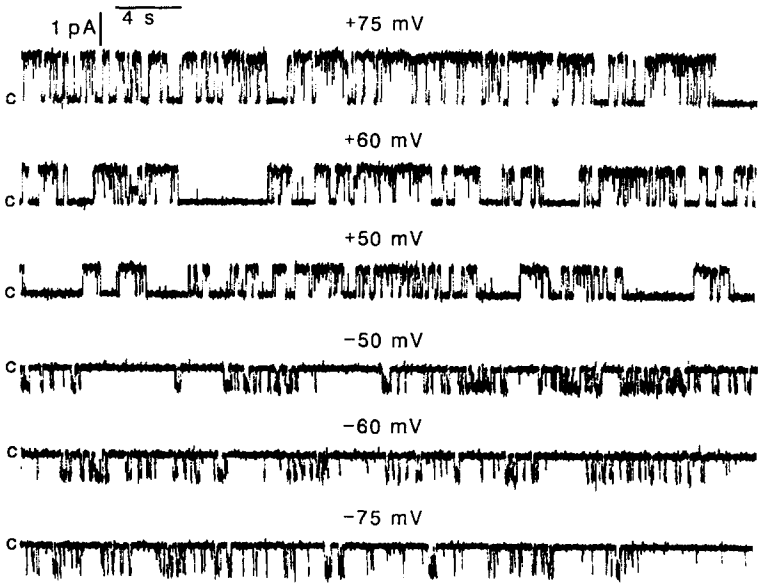

FIGURE 1. Typical current records of a single Cl^- channel. A Cl^- channel from lobster axon vesicles was incorporated into a planar lipid bilayer as described in Methods. The solution on both sides of a bilayer was 10 mM Tris-HCl, pH 7.4, 100 mM NaCl. Current records are filtered at 50 Hz (8-pole Bessel filter, −3 dB corner frequency). The closed state is indicated by *c*. Positive currents flowing *cis* to *trans* (Cl^- flux *trans* to *cis*) are plotted upward. The steady-state probability of the open state, P_o, was calculated from 70–150-s records at the indicated holding voltages P_o = 0.64, +75 mV; 0.60, +60 mV; 0.51, +50 mV; 0.21, −50 mV; 0.19, −60 mV; 0.17, −75 mV.

corresponded to a small conductance channel (~20 pS) with longer lasting opening bursts that were well resolved at 100-Hz filtering. Experiments in the presence of a KCl gradient across the bilayer indicated that the fast-gating, large conductance channel was K^+-selective and the slow, small conductance channel was Cl^- selective. Substitution of KCl solutions with NaCl permitted study of the Cl^- channel in isolation, since the fast K^+ channel, having selectivity properties of a delayed rectifier K^+ channel, is practically impermeable to Na^+ (Coronado et al., 1984).

Fig. 1 shows steady-state current fluctuations from a bilayer containing a single Cl^- channel recorded in the presence of symmetrical 100 mM NaCl, 10 mM Tris-Cl,

pH 7.4. This channel primarily fluctuates between a closed, zero-current level and a major open state; however, several distinct substate levels were occasionally observed. The behavior shown in Fig. 1 is typical of >95% of single Cl^- channels recorded from the lobster preparation. The single-channel gating activity is characterized by a bursting pattern of open-state activity with many brief unresolved closures separated by longer closed-state events lasting 0.1 second to several seconds in duration. In <5% of the records the channel spontaneously shifted into a faster gating mode characterized by the more rapidly flickering behavior shown in Fig. 2. In some cases, the flickering behavior of Fig. 2 was present from the moment of incorporation. Various manipulations of the ionic conditions described in this paper did not appear to influence the probability of fast (Fig. 2) vs. slow gating (Fig. 1).

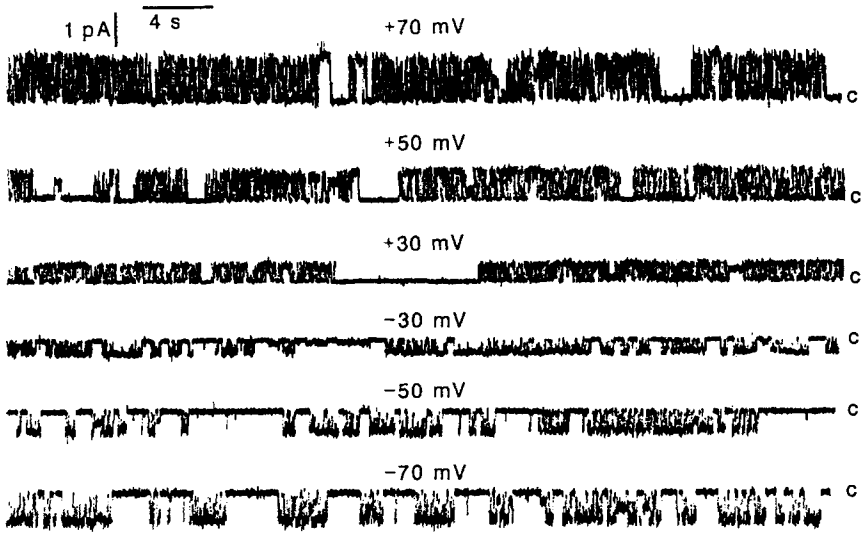

FIGURE 2. Current records of a single Cl^- channel gating in a rapidly flickering mode. Records from a different single-channel bilayer are shown under the same conditions as described in Fig. 1. Open-state probability was calculated from 55–90-s records at various holding voltages: P_o = 0.39, +70 mV; 0.47, +50 mV; 0.48, +30 mV; 0.39, −50 mV; 0.38, −70 mV.

Phenomenologically, this behavior may be characterized as another example of "gear shifting" or "mode" activity that has been described for other types of ion channels (Moczydlowski and Latorre, 1983; Patlak and Ortiz, 1989).

Incorporation of Cl^- channels under the conditions of Fig. 1 was reproducible from day to day using freshly thawed membrane preparations stored at −70°C for up to 3 mo. The experiments described here were carried out with four different membrane preparations using commercially available lobsters obtained at various times throughout the year. The major difficulty encountered in this study was that of limiting the number of incorporated channels. Most experiments resulted in bilayers containing two or more Cl^- channels which are less desirable for detailed analysis. The usual procedures of dilution and sonication of the preparation or increasing the

percentage of phosphatidylcholine in the lipid mixture did not appear to markedly improve the frequency of obtaining single-channel bilayers. Nevertheless, the success rate was sufficient to permit an initial study of anion permeation, gating, and pharmacology.

Since this particular Cl^- channel has not been previously described either in artificial membranes or in the native axon, it was important to establish criteria to define the orientation of the channel with respect to the *cis* and *trans* chambers. Addition of vesicles to only one of the chambers, defined as *cis* in bilayer experiments, is not completely reliable since the incorporation procedure does not always result in the same orientation.

Fortunately, the Cl^- channel exhibits a readily determined asymmetry with respect to voltage that permits assignment of orientation on the basis of a functional property of the channel. As shown in the records of Figs. 1 and 2, lobster Cl^- channels display an obvious current rectification. Under conditions of symmetrical 0.1 M NaCl, this channel usually incorporates such that unitary currents were visibly larger at a positive voltage of +60 mV (*trans* chamber = ground) than unitary currents at −60 mV. This behavior is illustrated by the control *I-V* curve in Fig. 3 *A* that reverses at 0 mV and similar results in Fig. 6 with either 10 mM Tris-HCl or 10 mM HEPES-NaOH (pH 7.4) as the buffer. The results in Fig. 3 *A* show that the *I-V* curve of the open state is approximated by a linear, ohmic relationship at positive voltages with a slope conductance of 19.2 ± 0.8 pS (±SD, $n = 12$) in symmetrical 0.1 M NaCl. However, at negative voltages the *I-V* curve is concave, resulting in a lower conductance. If the *I-V* behavior at 0 to −40 mV is fit by a linear relation, the corresponding slope conductance in this voltage range is 15.1 ± 1.6 pS, giving a rectification ratio of 1.3 for positive to negative currents at low voltage. In our experience, the channel usually incorporated with the positive-rectifying orientation; however, in some cases a negative-rectifying *I-V* curve indicated a reverse orientation that was exploited in additional tests of functional asymmetry (e.g., Fig. 7 *C*). In the subsequent discussion, the positive-rectifying orientation is defined as the normal or reference orientation of the lobster Cl^- channel.

Since this Cl^- channel has not been physiologically characterized in an intact preparation, we cannot unambiguously assign the *cis* or *trans* sides of the channel with respect to the intracellular or extracellular aspects of the cell membrane. However, by analogy to a growing number of similar outward-rectifying Cl^- channels from other species and tissues, we propose in the Discussion that the *trans* chamber corresponds to the extracellular side and the *cis* chamber corresponds to the intracellular side. If this suggestion is correct, our chosen voltage convention of *trans* = ground would correspond to the normal cellular convention. Negative-going currents in our experiments would thus correspond to an inward cellular current or an outward net flux of Cl^-.

As mentioned previously, definitive assignment of anion selectivity was made in the presence of NaCl or KCl concentration gradients. This is illustrated in Fig. 3 *A*, where single-channel *I-V* data pooled from 7–12 bilayers are plotted under conditions of symmetrical 0.1 M NaCl and also in the presence of a gradient of 0.2 M NaCl *cis*/0.1 M NaCl *trans*. This experiment shows that the observed reversal

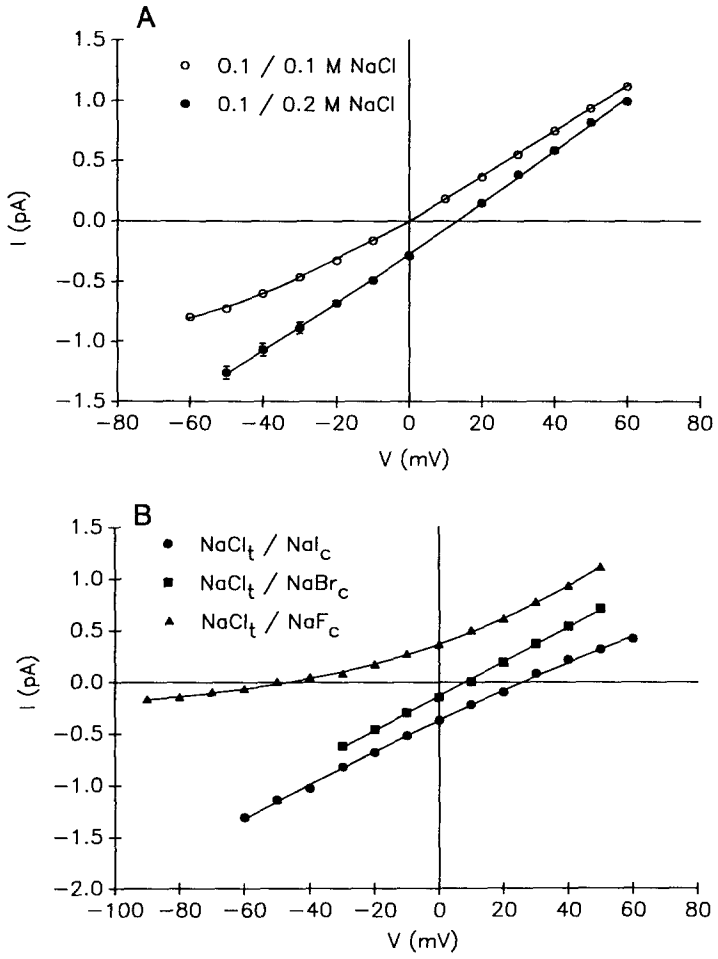

FIGURE 3. *I-V* relations of single Cl^- channels demonstrating rectification in the presence of symmetrical NaCl and selectivity for halide anions. All solutions contained 10 mM Tris-HCl, pH 7.4. *A*, Unitary currents were measured at various voltages in the presence of symmetrical 100 mM NaCl (○) or 200 mM NaCl *cis*, 100 mM NaCl *trans* (●). Data points are means of 7–12 channels and each current value is averaged from 4 to 10 determinations on the same channel. Error bars not shown are smaller than the size of the data point. *B*, Halide selectivity under nearly bi-ionic conditions. Cl^- channels were incorporated in symmetrical 100 mM NaCl and the *cis* chamber was subsequently perfused with either 100 mM NaF (▲), 100 mM NaBr (■), or 100 mM NaI (●). Solid lines are drawn by eye.

potential of $V_0 = +15.5 \pm 1.0$ mV is very close to the predicted value of +15.2 mV for a perfect Nernst Cl^- electrode, using tabulated values of NaCl activity coefficients to calculate Cl^- activity (Robinson and Stokes, 1959). If we assume that our reversal potential measurement, V_0, is accurate to within 1 mV, a lower limit of the permeability ratio, $P_{Na}/P_{Cl} \geq 40$, can be estimated from a form of the Goldman-

Hodgkin-Katz equation (Hodgkin and Katz, 1949):

$$V_0 = (-RT/F) \ln [(P_{Cl}[Cl]_t + P_A[A]_t + P_{Na}[Na]_c)/(P_{Cl}[Cl]_c + P_A[A]_c + P_{Na}[Na]_t)], \quad (1)$$

where $RT/F = 25.4$ mV at 22°C, the subscripts on the concentration terms refer to *cis* (c) and *trans* (t), and P_{Cl}, P_A, and P_{Na} are the respective permeabilities for Cl^-, a test anion (if present) and Na^+. This result implies that the lobster axon Cl^- channel discriminates almost perfectly in favor of Cl^- over Na^+. In the following analysis of anion selectivity, the permeability for Na^+ was therefore assumed to be zero in calculations based on the Goldman-Hodgkin-Katz equation.

Permeability Ratios of Halides and Other Anions

Fig. 3 *B* shows the results of experiments in which normally oriented Cl^- channels recorded in the presence of symmetrical 0.1 M NaCl were tested for permeation of other halide anions by perfusing the *cis* chamber with 0.1 M NaI, NaBr, or NaF. These experiments are performed under conditions that are nearly bi-ionic except for 8 mM Cl^- due to HCl used in preparation of 10 mM Tris-HCl, pH 7.4, as the buffer. Under these conditions, if the test anion is equally permeant as Cl^-, a reversal potential near 0 mV is expected; if the test ion is totally impermeant, the current should theoretically reverse at −59.8 mV. Fig. 3 *B* shows that the reversal potential for F^- is about −50 mV, indicating a low P_F/P_{Cl} ratio. In contrast, the reversal potentials for Br^- and I^- are positive, indicating that these halides are more permeant than Cl^-. The halide selectivity sequence for this channel measured under near bi-ionic conditions is: I^- (2.7) > Br^- (1.5) > Cl^- (1.0) > F^- (0.049), with the values in parentheses being the relative permeability as calculated from Eq. 1 using activities based on available activity coefficients (Robinson and Stokes, 1959) and reversal potential data listed in Table I. According to the terminology of Eisenman's selectivity theory (Eisenman and Horn, 1983), this Cl^- channel exhibits a low field-strength permeability sequence for halides, in which lower dehydration energy of the larger anions predominates over stronger coulombic interaction energy of the smaller anions in determining selectivity. This selectivity behavior is different from the strong Cl^- preference previously described for the dimeric Cl^- channel from *Torpedo californica* (Miller and White, 1980), but is almost identical to that measured for the Gaba- and glycine-activated Cl^- channels of mouse neurons (Bormann et al., 1987) and is also similar to that of various other mammalian Cl^- channels (Franciolini and Nonner, 1987; Reinhardt et al., 1987; Halm et al., 1988).

Several other small inorganic and organic anions were also tested for their relative permeability in an attempt to estimate the cutoff diameter of the largest permeant molecule. The experiments of Fig. 4, carried out using the same protocol as the near bi-ionic conditions of Fig. 3, indicate that the small anion, NO_3^-, is more permeant than Cl^-, but that the larger monovalent anions, HCO_3^-, $CH_3CO_2^-$, isethionate ($HOCH_2CH_2SO_3^-$), and gluconate [$HOCH_2(CH_2OH)_4COO^-$] are less permeant. The measured reversal potential for isethionate (−56.7 ± 2.3 mV) is equivalent to the predicted value for $P_A/P_{Cl} = 0$, suggesting a permeation cutoff diameter of ~5 A. However, the *I-V* data of Fig. 4 *B* indicate that a slightly smaller divalent anion,

SO_4^{2-}, is also impermeant and appears to block the residual Cl^- current at negative voltages, since we did not observe any reversal current at −80 mV in three experiments. Fig. 4 *B* also shows that a slightly larger anion, gluconate, is apparently more permeant than isethionate. These results imply that molecular size alone cannot be used to estimate the size of the sieving region of the channel. Binding interactions with sites in the channel or ion–ion interactions between permeant ions also appear to be important in determining the permeability ratio of various anions. Further support for the latter possibility is given in Table I, where permeability ratios determined under mixed ionic conditions with [200 mM Cl^- *cis*]/[100 mM Cl^- *trans* + 100 mM test anion *trans*] are compared with those determined under the near bi-ionic conditions of Figs. 3 *B* and 4, *A* and *B*. These data show that all of the

TABLE I

Reversal Potentials and Permeability Ratios for Near Bi-ionic and Mixed Conditions

		Lobster bi-ionic		Lobster mixed		Mouse	
Anion	Diameter	V_0 ± SE (*n*)	P_A/P_{Cl}	V_0 ± SE (*n*)	P_A/P_{Cl}	Gly-R P_A/P_{Cl}	Gaba-R P_A/P_{Cl}
	Å	*mV*		*mV*			
I^-	4.3	+24.7 ± 1.1(4)	2.7	−18.8 ± 2.6(5)	3.2	1.8	2.8
Br^-	3.9	+9.3 ± 1.9(4)	1.5	−11.3 ± 3.6(4)	2.1	1.4	1.5
Cl^-	3.6	0.0	1.0	0.0	1.0	1.0	1.0
F^-	2.7	−50.0 ± 1.0(3)	0.049	+7.0 ± 1.1(4)	0.52	0.025	0.02
NO_3^-	2.5	+13.4 ± 4.6(4)	1.8	−10.3 ± 2.9(5)	2.1	1.9	2.1
HCO_3^-	4.5	−42.3 ± 1.0(4)	0.10	n.d.	n.d.	0.11	0.18
$CH_3CO_2^-$	4.5	−33.7 ± 4.7(3)	0.18	n.d.	n.d.	0.035	0.08
Gluconate	6.0	−48.3 ± 1.5(3)	0.06	+8.8 ± 3.0(4)	0.38	n.d.	n.d.
Isethionate	5.0	−56.7 ± 2.8(3)	<0.01	n.d.	n.d.	<0.01	<0.01

Reversal potentials, V_0, for lobster Cl^- channels were determined under near bi-ionic conditions by perfusing the *cis* chamber with 10 mM Tris-HCl, pH 7.4, 100 mM Na^+ salt of the test anion after incorporation in symmetrical 10 mM Tris-HCl, pH 7.4, 100 mM NaCl. For mixed conditions: *cis*, 10 mM Tris-HCl, pH 7.4, 200 mM NaCl; *trans*, 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 100 mM Na^+ salt test anion. Permeability ratios were calculated from Eq. 1 as described in the text. Activity coefficients were taken from Robinson and Stokes (1959) except in the case of bicarbonate and gluconate, where activities equivalent to acetate were assumed. The anion diameters for halides are Pauling radii (Hille, 1984). For the other species, the minimum diameter that passes the anion was measured from Corey-Pauling-Koltun models and rounded to the nearest 0.5 Å. The listed permeability ratios for mouse Gly-R and Gaba-R Cl^- channels are taken from Bormann et al. (1987) as measured under similar near bi-ionic conditions. n.d., not determined.

tested anions exhibit a significantly larger permeability ratio under the mixed-ionic condition as compared with the near bi-ionic condition. In particular, F^- and gluconate appear to exhibit 10- and 7-fold increases in relative permeability, respectively, under the mixed ionic conditions. Concentration-dependent permeability ratios are one of the characteristics of multi-ion channels that can simultaneously hold more than one permeant ion (Hille, 1984, p. 265). It is also possible that the results of Table I could be explained by an asymmetric energy profile of a single-ion channel (Eisenman and Horn, 1983), since the test ion was present on opposite sides of the channel in the bi-ionic vs. the mixed experiments. However, in the case of I^-, F^-, HCO_3^-, and gluconate, we found that the bi-ionic reversal potential measured on

reverse-oriented channels did not differ by more than 2 mV from that determined for normally oriented channels. This suggests that the increase in relative permeability observed under mixed-ionic conditions is primarily due to effects of multi-ion occupancy rather than an asymmetric energy barrier for permeation.

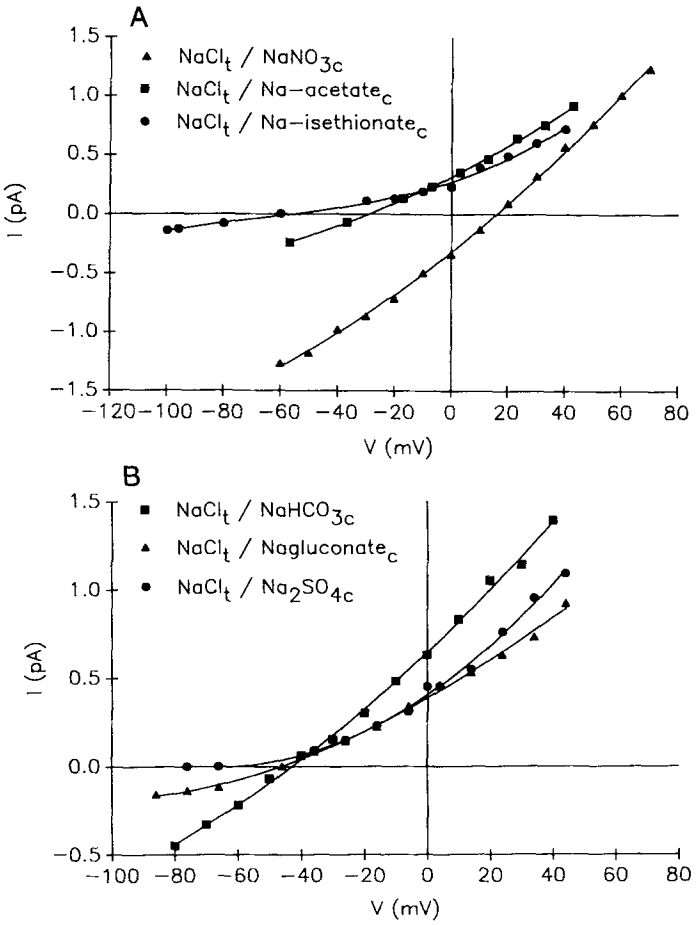

FIGURE 4. Selectivity of various anions under nearly bi-ionic conditions. Single Cl^- channels were first incorporated in symmetrical 10 mM Tris-HCl, pH 7.4, 100 mM NaCl and the *cis* chamber was subsequently perfused with a similar solution in which NaCl was replaced by 100 mM of various Na^+ salts: *A*, $NaNO_3$ (▲), Na-acetate (■), Na-isethionate (●). *B*, $NaHCO_3$ (●), Na-gluconate (▲), Na_2SO_4 (●). The final pH of the *cis* solution was 7.4 except for the solutions containing Na-acetate and $NaHCO_3$, which were pH 8.0.

Saturation Behavior at High Cl^- Concentration

Many ion-selective channels exhibit current saturation with increasing permeant ion concentration, reflecting occupation of one or more distinct ion binding sites within the channel. An investigation of this behavior for the lobster Cl^- channel studied in

the presence of increasing symmetrical concentrations of Cl^- is summarized in Fig. 5. At low positive or negative voltages the *I-V* behavior can be approximated by ohmic relationships indicated by linear regression fits to lines intersecting at 0 mV. At Cl^- concentrations less than ~200 mM, positive rectifying *I-V* behavior is evident in the larger slope conductance at positive vs. negative voltage. However, at higher Cl^- concentration, single-channel *I-V* curves showed much less rectification and could be adequately fit by an ohmic relationship over the voltage range of −50 to +50 mV (e.g., Fig. 5 *A*, 475 mM Cl^-). To provide a phenomenological description of saturation behavior, the measured slope conductance at low positive or negative voltage is plotted as a function of Cl^- concentration in Fig. 5, *B* and *C*. This representation of the *I-V* data shows current saturation at both positive and negative voltages. These data were well described by the Michaelis-Menten relationship as indicated by the fitted curves and double-reciprocal plots in Fig. 5, *B* and *C*. At positive voltage (Fig. 5 *B*) the Michaelis-Menten fit gave parameters of K_m = 101 mM, γ_{max} = 32.7 pS for the Cl^- concentration at half-saturation and the maximal conductance, respectively. The corresponding parameters at negative voltage were K_m = 264 mM and γ_{max} = 39.6 pS. This apparent Michaelis-Menten behavior implies that the channel contains at least one Cl^- binding site. Similar saturation behavior has been observed for the *Torpedo* Cl^- channel (K_m = 75 mM, γ_{max} = 32 pS; White and Miller, 1981) and the Gaba-activated (K_m = 155 mM, γ_{max} = 72 pS) and glycine-activated (K_m = 108 mM, γ_{max} = 92 pS) Cl^- channels (Bormann et al., 1987).

Effect of HEPES and Tris Buffers

Common zwitterionic buffers such as HEPES and MOPS have previously been shown to block Cl^- channels in *Drosophila* neurons (Yamamoto and Suzuki, 1987) and a human epithelial cell line (Tabcharanchi and Hanrahan, 1989). Since such buffer effects could interfere with analysis of the functional properties of Cl^- channels, and since knowledge of such effects is important for establishing proper conditions for physiological studies in intact axons, we examined the effect of HEPES and Tris. Fig. 6 presents a comparison of single-channel *I-V* curves taken in the presence of symmetrical 0.1 M NaCl and either 10 mM Tris-HCl or 10 mM HEPES-NaOH at pH 7.4. Recordings in the presence of HEPES or MOPS did not exhibit increased noise or flickering relative to the Tris recordings, but there was a slight reduction in the unitary currents that might be attributed to an inhibitory effect of HEPES. In the presence of HEPES, the calculated slope conductance is 17.9 ± 1.5 pS (±SD, n = 6) in the positive voltage range and 12.5 ± 2.0 pS in the voltage range of −40 to 0 mV. After correcting for increased conductance expected for 8 mM additional Cl^- content of the Tris buffer, the only significant effect of HEPES is a 10% current reduction at negative voltages. To examine whether Tris buffer might also affect the Cl^- currents, we compared *I-V* curves taken in the presence of 1 mM Tris buffer with those taken at 10 mM Tris. We did not observe a significant difference in the *I-V* behavior at these two Tris concentrations (not shown), suggesting that the positive-rectifying *I-V* behavior at subsaturating Cl^- concentration is due to an asymmetry in Cl^- permeation through the channel, which is independent of Tris.

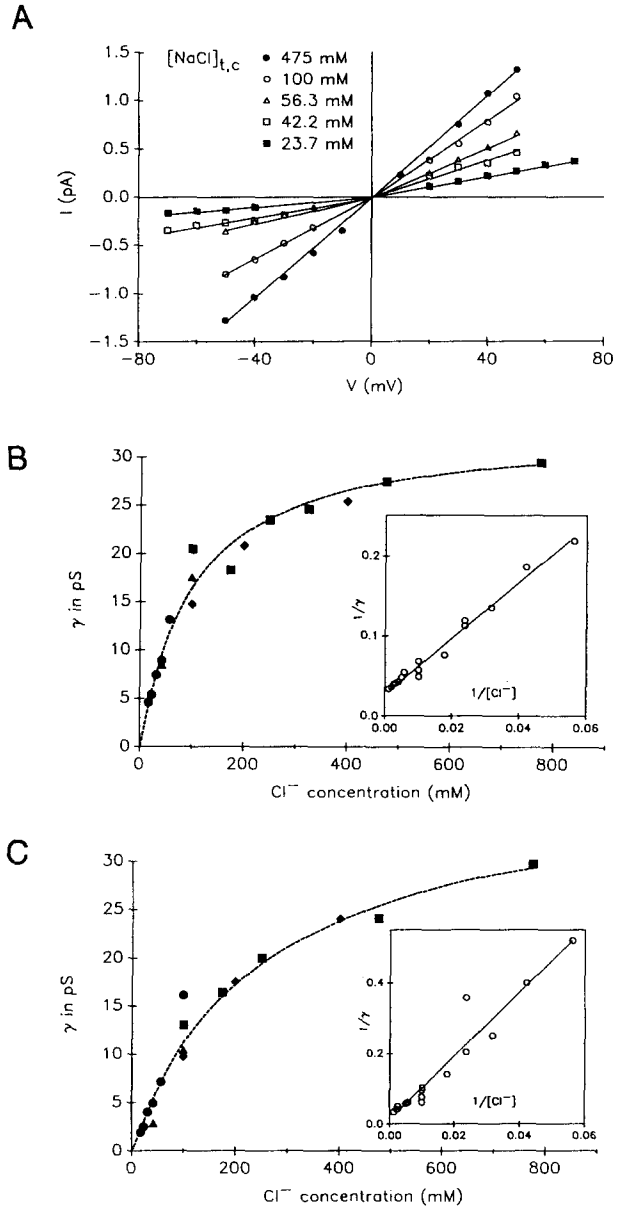

FIGURE 5. Single-channel conductance as a function of Cl^- concentration. Single Cl^- channels were incorporated in the presence of symmetrical 10 mM Tris-Cl, pH 7.4 and 100 mM NaCl. NaCl concentration was subsequently increased or decreased by addition of 3 M NaCl or dilution with H_2O. Slope conductance was determined separately for positive and negative voltages by linear regression. *A*, Single-channel *I-V* data at various symmetrical NaCl concentrations: ■, 23.7 mM; □, 47.2 mM; △, 56.3 mM; ○, 100 mM; ●, 475 mM. *B*, Slope conductance vs. $[Cl^-]$ at positive voltage. *C*, Slope conductance vs. $[Cl^-]$ at negative voltage. Data in *B* and *C* are fit to a rectangular hyperbola. The insets show the same data plotted in double reciprocal form. *B*, K_m = 101 mM, γ_{max} = 32.7 pS; *C*, K_m = 264 mM, γ_{max} = 39.6 pS.

Asymmetric Current Inhibition by TEA

The K^+ channel blocker, tetraethylammonium (TEA^+), is a common constituent of physiological buffer solutions used to isolate and record Cl^- currents. Since some Cl^- channels exhibit a significant permeability for monovalent cations (Franciolini and Nonner, 1987) including TEA^+ (Manning and Williams, 1989), we examined the effect of TEA^+ on the lobster Cl^- channel. Fig. 7 *A* shows current records from a bilayer containing a single, normally oriented Cl^- channel before and after replacement of 0.1 M NaCl in the *cis* chamber with 0.1 M TEA-Cl. This exchange of TEA^+ for Na^+ resulted in a marked current reduction that was greater at −80 than at +60 mV. The gating kinetics of channel opening were not obviously affected by TEA^+ (not shown). Single-channel *I-V* curves taken in the presence of 0.1 M TEA-Cl *cis*/0.1

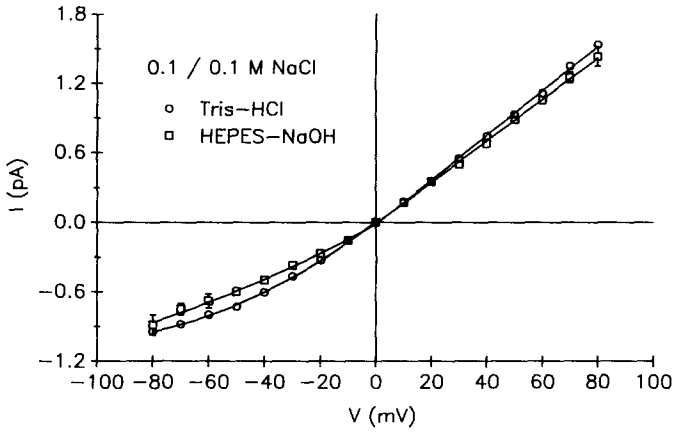

FIGURE 6. Comparison of single-channel *I-V* behavior in Tris and HEPES buffers. Sincle Cl^- channels were incorporated in the presence of symmetrical 100 mM NaCl and 10 mM Tris-Cl, pH 7.4 (O) or 10 mM HEPES-NaOH, pH 7.4 (□). Data points are means of 7–12 channels and each current value is averaged from 4 to 10 determinations on the same channel. Error bars not shown are smaller than the size of the data point. Solid lines are drawn by eye at negative voltage or according to a linear fit at positive voltage.

M NaCl *trans* are compared with those for symmetrical 0.1 M NaCl for normally oriented (Fig. 7 *B*) and reverse-oriented (Fig. 7 *C*) Cl^- channels. For the normally oriented channels, *cis* TEA^+ results in an average current reduction of 40 ± 4% ($n = 3$) at negative voltages and 11 ± 2% at positive voltages. However, no effect of TEA^+ was observed on the reverse-oriented channel, indicating that the effect of TEA^+ is asymmetric. The effect of *cis* TEA^+ in causing a more pronounced positive rectification of the single-channel current suggests that cations normally used as counterions, such as Na^+, might be partially responsible for the positive rectification of the channel observed in the presence of symmetrical NaCl. Thus, two alternative hypotheses may explain the rectifying *I-V* behavior in symmetrical NaCl: (*a*) rectification is due to an intrinsically asymmetric energy barrier profile for Cl^- permeation; (*b*) rectification is due to an effect of counterions such as Na^+ acting from the *cis* side

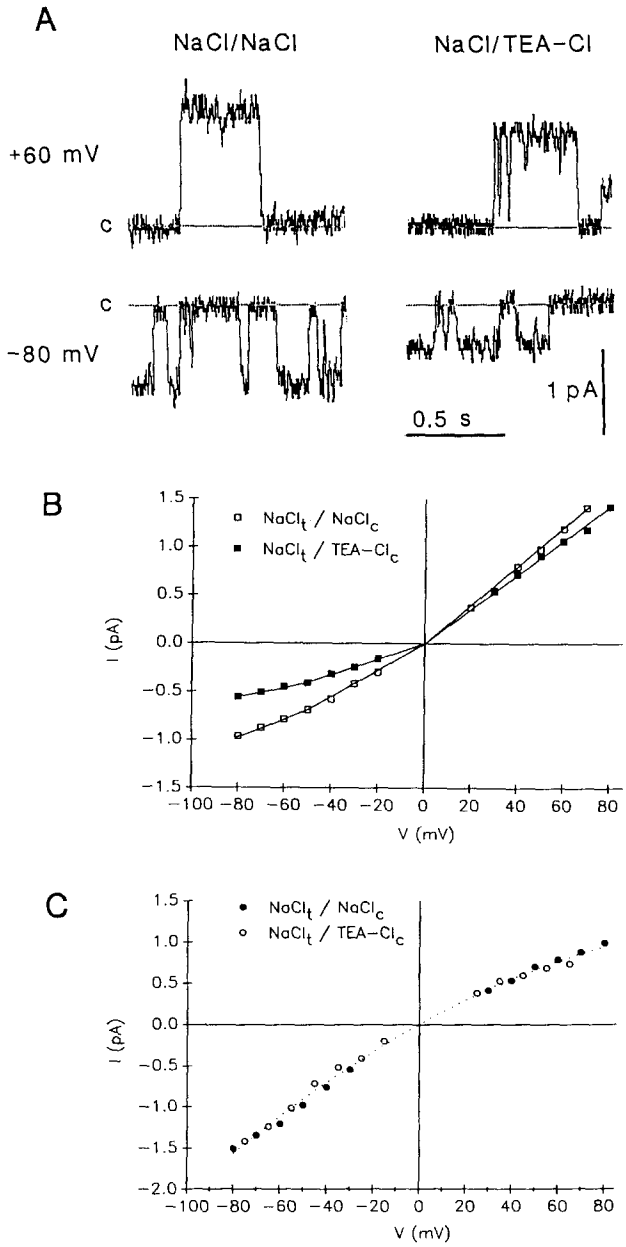

FIGURE 7. Asymmetric effect of TEA on single-channel *I-V* behavior. *A*, Cl^- channel was incorporated in symmetrical 10 mM Tris-Cl, pH 7.4, 100 mM NaCl and current fluctuations were recorded at the indicated holding potentials. The *cis* chamber was then perfused with 10 mM Tris-Cl, pH 7.4, 100 mM TEA-Cl and the *I-V* relation was measured again. *B*, Current records of a normally oriented Cl^- channel are shown before (□) and after (■) perfusion with *cis* TEA-Cl. *C*, Single-channel *I-V* relation of a backward-oriented channel before (○) or after (●) perfusion with *cis* TEA-Cl.

superimposed on a symmetrical energy barrier profile for Cl^-. Further experiments will be required to distinguish these and other possibilities such as asymmetric surface charge.

Weak Voltage Dependence of Gating

The current records of Figs. 1 and 2 suggest that the open-state probability of the lobster Cl^- channel is higher at large positive voltages vs. a negative voltage of equal magnitude. Fig. 8 summarizes measurements of the time-averaged open-state probability at various voltages compiled from 3–18 bilayers. The majority of Cl^- channels exhibited an open-state probability of ~0.64 from +40 to +75 mV, and showed a gradual decrease in open-state probability at negative voltages to a value of 0.23

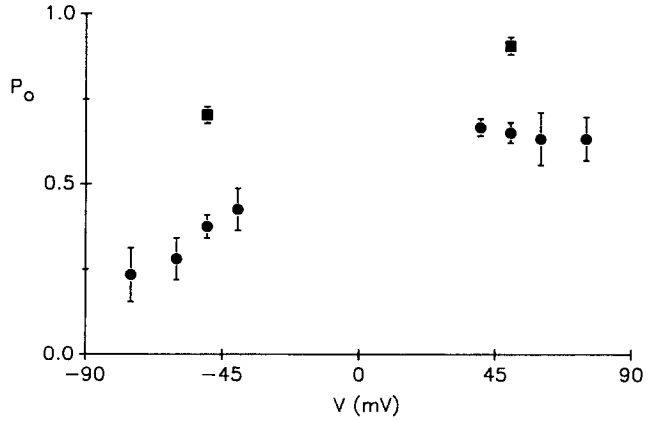

FIGURE 8. Voltage dependence of Cl^- channel gating. The open-state probability, P_o, of single channels was measured using 1–3 min segments of current records taken at various voltages under conditions of symmetrical 100 mM NaCl. (●) Data points correspond to the mean ± SEM averaged for the following number of experiments at various voltages (n, mV): 3, −75,; 3, −60; 15, −50; 6, −40; 5, +40; 18, +50; 3, +60; 3, +75. (■) Data points correspond to similar measurements ($n = 4$) at +50 and −50 mV for Cl^- channels that exhibited an unusually high opening probability.

measured at −75 mV, the most negative voltage studied. In 4 of 22 bilayers we observed Cl^- channels that appeared to be open almost all of the time at +50 mV, suggesting that there might be an activated form of the channel that can be retained in the process of membrane isolation, storage, and incorporation. This observation is indicated by pooling data for such channels separately as denoted by the solid squares in Fig. 8.

Previously studied examples of Cl^- channels are characterized by complex gating kinetics that include several open and closed states in addition to multiple substates (Coronado and Latorre, 1982; Blatz and Magleby, 1986). Detailed dwell time analysis of the lobster Cl^- channel in bilayers is hampered by this channel's small conductance and filtering ($f_c = 50$–100 Hz) that must be used to resolve pA currents across large capacitance (100–200 pF) bilayers. However, to provide

baseline parameters of dwell times that may be useful in fingerprinting this channel in comparison with other Cl^- channels, we constructed dwell time histograms of closed- and open-state events at +50 mV using sampling and analysis procedures described in Methods. Frequency density histograms (not shown) of open-state dwell times resolved two lifetime components of 29 ± 4 and 220 ± 28 ms (±SE, $n = 16$ channels). The closed-state histograms displayed three components with time constants of 9.3 ± 1.3, 140 ± 18, and 810 ± 120 ms. Thus, the minimum gating scheme for this channel must include transitions between at least three closed and two open states.

Dependence of gating on pH

The gating of Cl^- channels from various sources has been found to be modulated by Ca^{2+} (Li et al., 1989) or pH (Hanke and Miller, 1983; Gray et al., 1984). The activity of lobster nerve Cl^- channels does not appear to be dependent on Ca^{2+} or Mg^{2+} since concentrations of these ions tested up to 2 mM did not affect the single-channel behavior. The addition of 0.5 mM EGTA to both sides of the bilayer, which reduced the free Ca^{2+} to submicromolar levels, also had no effect (not shown).

In contrast to the lack of effect of Ca^{2+}, the probability of channel opening was found to be a sensitive function of the pH on the *cis* side of the channel. Fig. 9 *A* shows current records at +40 and −40 mV from a single-channel bilayer where the pH was consecutively raised from pH 7.4 to 8.0 to 11.2 by the addition of known amounts of 0.5 N NaOH. This resulted in an apparent increase in the mean burst time at both positive and negative voltage as indicated by the decrease in the number of long closed-state events at higher pH. Fig. 9 *B* shows the results of an experiment on a different bilayer where a decrease in the *cis* pH from 7.4 to 6.0 resulted in a virtually complete inactivation of the channel after stirring was complete. In additional experiments we found that the apparent activation of the channel in the range of pH 7.4–11 was a reversible phenomenon; however, once the inactivation phenomenon of Fig. 9 *B* occurred by changing the pH to ≤6, normal channel activity could not be revived by a subsequent pH increase. The irreversibility of this phenomenon suggests that the structure of the channel is altered at low pH by a process such as denaturation or loss of a subunit or cofactor. The dependence of the lobster Cl^- channel on pH is summarized by the data in Fig. 9 *C*, where the time-averaged open-state probability at +40 and −40 mV is plotted as a function of *cis* pH. The steepness of the pH titration curve in Fig. 9 *C* is suggestive of a cooperative process. The effect of pH shown in Fig. 9 only occurred for pH changes on the *cis* side of the channel. Similar pH titrations of the *trans* chamber did not reveal a similar pH dependence (not shown). This suggests that H^+ modulation of this channel occurs at a site on the *cis* side that is outside the pore and is in equilibrium with the *cis* solution. A proton-titratable site that is within the pore would be expected to be accessible to H^+ or OH^- from either the *cis* or *trans* solutions.

Asymmetric Inhibition by SITS

Since many anion transport systems and channels are inhibited by disulfonic acid stilbene derivatives, we studied the effect of SITS (4-acetamido,4′-isothiocyanostil-

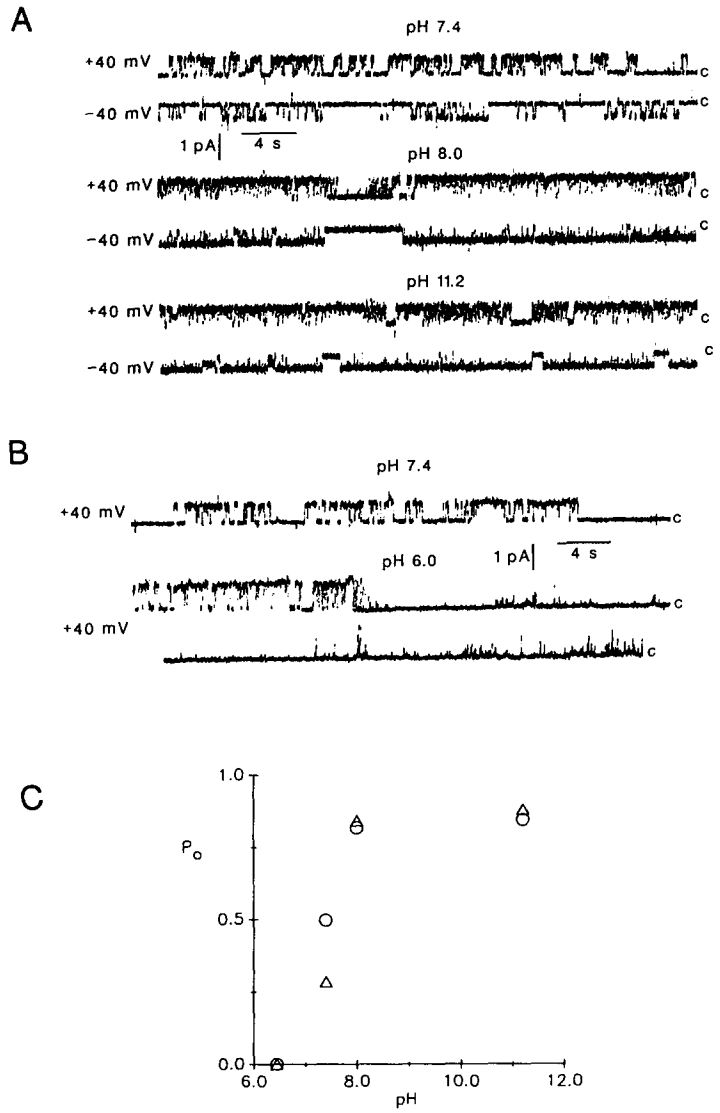

FIGURE 9. Dependence of Cl^- channel activity on *cis* pH. Cl^- channels were first incorporated under conditions of symmetrical 10 mM Tris-Cl, pH 7.4, 100 mM NaCl. The pH of the *cis* side was changed by addition of calibrated amounts of 0.5 N NaOH (*A*) or 0.2 N HCl (*B*) in separate experiments. In *B*, channel activity was recorded immediately after addition of HCl (trace labeled pH 6.0). The lag time for channel inactivation corresponds to stirring time. *C*, Titration of open-state probability vs. *cis* pH. Data points represent the mean open-state probability of two separate channels at +40 mV (○) and −40 mV (△) at the indicated pH.

bene-2,2'-disulfonic acid) on the lobster Cl^- channel. Fig. 10 shows current records from a single-channel bilayer in the presence of increasing concentrations of SITS added to the *trans* chamber. This experiment shows that SITS induces fast-flickering closures of the channel in a concentration-dependent fashion. The channel activity is

almost completely inhibited in the presence of 317 μM SITS and can be reversed by perfusion of the *trans* chamber with SITS-free solution. Similar concentrations of SITS added to the *cis* chamber resulted in no observable effect, indicating that the inhibitory site can only be reached from the *trans* side by this membrane-impermeant bis-anion. This effect of SITS is analogous to the discrete blocking effect of another disulfonic stilbene, DNDS (4,4'-dinitrostilbene-2,2'-disulfonic acid), as described for a similar Cl^- channel incorporated into planar bilayers from a plasma membrane preparation of rat colon enterocytes (Bridges et al., 1989). Bridges et al. (1989) previously analyzed the dwell time behavior of the DNDS-induced flickering events

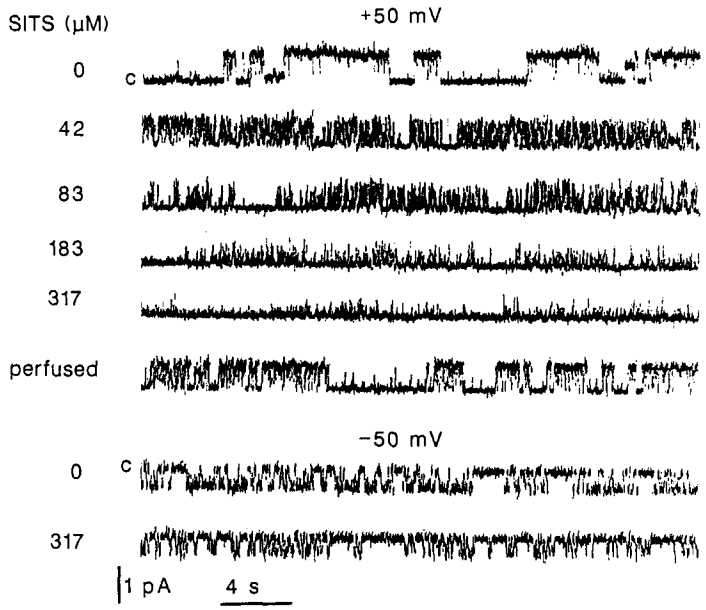

FIGURE 10. Concentration dependence and reversibility of SITS inhibition. Top trace, single channel record in the absence of SITS at +50 mV in symmetrical 10 mM HEPES-NaOH, pH 7.4, 100 mM NaCl. Successive traces show records from the same channel in the presence of indicated concentrations of SITS added to the *trans* side. Reversibility of SITS inhibition is shown after perfusion of the *trans* chamber with SITS-free solution. The lower two traces show result from the same experiment at −50 mV in the absence and presence of 317 μM SITS.

in this channel to show that the kinetics of inhibition corresponded to a one-site binding reaction. In our case, limited time response and the brief durations of the SITS-induced blocking events discouraged us from attempting a similar analysis. In contrast to the voltage-independent action of DNDS in the enterocyte Cl^- channel, the blocking effect of SITS was more potent at +50 mV that at −50 mV for the lobster Cl^- channel (Fig. 10). This behavior is summarized in Fig. 11 where the relative open-state probability is plotted as a function of SITS concentration. The data at +50 mV were fit to one-site blocking behavior with a K_I of 53 μM for SITS. The higher estimated K_I of 815 μM for SITS inhibition at −50 mV indicates that the

affinity for SITS decreases by a factor of 12 over this voltage range. If these K_I values are fit to a Woodhull (1973) model of voltage-dependent binding of a negatively charged blocker to a site in the transmembrane electric field, an electrical distance of 0.35 from the *trans* side is estimated for the SITS block, assuming a valence of -2 for SITS.

In screening various other compounds for inhibition of the lobster Cl^- channel, we found that NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid, also inhibits this channel. NPPB (50 μM) was previously shown to induce a flickering block of single Cl^- channels of cultured colon carcinoma cells studied by the patch clamp technique (Dreinhofer et al., 1988). We observed that 88 μM NPPB added to either side of the bilayer produced a complex pattern of inhibition in the lobster Cl^- channel which appeared to be due to both a decreased probability of opening and a decreased

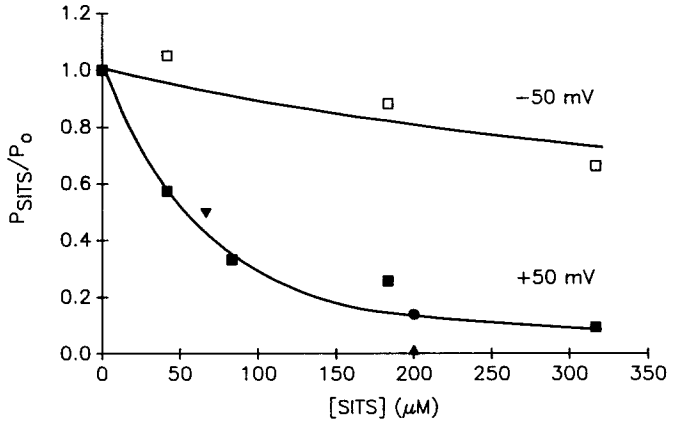

FIGURE 11. Open-state probability as a function of *trans* SITS concentration. Single-channel current fluctuations were recorded in symmetrical 10 mM HEPES-NaOH, pH 7.4, 100 mM NaCl. The ordinate value, P_{SITS}/P_O, is the ratio of the measured open-state probability in the presence of SITS to that in the absence of SITS at +50 or −50 mV. Different symbols correspond to data from four different channels.

unitary conductance (Fig. 12). The fact that this compound inhibits from either side may be expected since it is a hydrophobic weak acid that can partition in the bilayer in the neutral form and result in an unstirred layer of the drug on the opposite side of the membrane. Similar behavior of local anesthetics which are either hydrophobic neutral compounds or weak bases has previously been described for batrachotoxin-activated Na^+ channels in planar bilayers (Uehara and Moczydlowski, 1986; Wang, 1988). The inhibitory effect of NPPB on the lobster Cl^- channel was also partially reversed by perfusion (Fig. 12).

DISCUSSION

These experiments have elucidated distinctive unitary properties of lobster Cl^- channels that may be useful in identifying this channel in intact lobster neurons or

Schwann cells. These properties include rectifying *I-V* behavior in the presence of symmetrical NaCl, low permeability of monovalent cations, a low field-strength selectivity sequence for halides, saturation behavior with respect to Cl^- concentration, weak voltage dependence of gating, inhibition of channel gating by low internal pH, and block by the anion transport inhibitors SITS and NPPB. A survey of the recent literature on Cl^- channels suggest that many of the properties of this invertebrate Cl^- channel are similar to those previously described for Cl^- channels from various vertebrate tissues. In the following discussion we compare characteristics of the lobster Cl^- channel to properties that appear to be common to a class of small conductance anion channels that have been described in a variety of species and cell types.

Permeation and Gating Characteristics Distinguish Two Major Classes of I^--permeable Cl^- Channels

A large majority of anion-selective channels that have been characterized at the single-channel level display a halide permeability sequence that varies inversely with

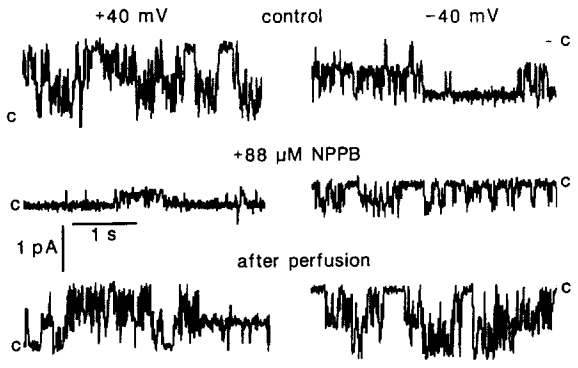

FIGURE 12. Effect of NPPB on Cl^- channel activity. Top control records at −40 and +40 mV show current fluctuations from a bilayer containing two backward-oriented Cl^- channels in the presence of symmetrical 10 mM Tris-HCl, pH 7.4, 100 mM NaCl. The middle traces were taken after the addition of 80 μM *cis* NPPB and the lower traces show partial reversibility of NPPB inhibition after perfusion of the *cis* chamber.

ionic radius: $I^- > Br^- > Cl^- > F^-$. A notable exception is the 20-pS Cl^- channel of *Torpedo californica* electroplax that also exhibits an unusual dimeric gating pattern (Miller, 1982). This *Torpedo* channel has been shown to mediate only Cl^- and Br^- current, while I^- and F^- are impermeant (Miller and White, 1980). Aside from this exception, I^--permeable Cl^- channels can be divided into two classes according to their unitary conductance and voltage dependence of gating.

Cl^- channels belonging to the first category of large conductance (BCl) anion channels exhibit an ohmic *I-V* relation in the presence of symmetrical (0.1–0.2 M NaCl with a unitary conductance in the range of 200–500 pS. Such BCl channels generally exhibit weaker ionic selectivity than small conductance (SCl) Cl^- channels in comparing Na^+ and K^+ permeability to Cl^- ($P_{Na,K}/P_{Cl} < 0.1$–0.2) and in comparing the permeability of I^- to F^- across the halide series ($P_I/P_F \simeq 1.7$–2.0). An additional striking feature of BCl channels is a high open-state probability in a narrow voltage range near 0 mV with sharply decreasing opening probability at large

positive and negative voltages. Examples of BCl channels characterized in the plasma membrane of various cells include: rat Schwann cells, 450 pS (Gray et al., 1984); cultured rat skeletal muscle, 430 pS (Blatz and Magleby, 1983); rat aorta smooth muscle, 340 pS (Soejima and Kokubun, 1988); rabbit urinary bladder, 360 pS (Hanrahan et al., 1985); amphibian skeletal muscle (Woll et al., 1987); and amphibian kidney epithelia, 360 pS (Nelson et al., 1984). In addition to these examples, a similar type of large conductance anion channel with a markedly lower P_K/P_{Cl} ratio was described in frog sarcoplasmic reticulum (Hals et al., 1989). From these studies, no consensus on the functional role of such BCl channels has been reached, although several authors have suggested that they may be related to VDAC anion channels of mitochondria or hemi-gap junction channels (Blatz and Magleby, 1983; Woll et al., 1987).

In contrast to the weak ionic selectivity and biphasic voltage dependence of BCl channels, the behavior of the 20-pS lobster Cl^- channel is similar to that of a rapidly growing number of SCl channels. Many of the SCl channels that have been studied in detail in cell-attached or excised membrane patches display an outward rectifying, single-channel *I-V* behavior. Most of these channels also display a weak voltage dependence of gating characterized by increased opening probability at positive voltage. The recognition of these two functional characteristics in the lobster Cl^- channel provides a comparative basis for proposing that the *trans* (ground) side of the bilayer corresponds to the extracellular (external) side of the channel in vivo and the *cis* bilayer chamber corresponds to the intracellular (internal) side. Selected examples of SCl channels where outward rectification and activation by depolarization have been well documented at the single-channel level include Cl^- channels of the apical membrane of rat colon epithelia (40 pS; Halm et al., 1988), cultured human colon tumor cells (50 pS; Frizzell et al., 1986), human airway epithelia (30 pS; Jetten et al., 1989), and human lymphocytes (39 pS; Chen et al., 1989). To cite similar examples in neural cell types, Franciolini and Nonner (1987) and Franciolini and Petris (1988) have described 30 pS and 62 pS Cl^- channels, respectively, in rat hippocampal neurons, which exhibit slightly outward-rectifying or ohmic *I-V* behavior and higher opening probability at positive voltage. In a glial cell type, Barres et al. (1988) identified a strongly outward-rectifying Cl^- channel in oligodendrocytes derived from rat optic nerve with a unitary conductance of 25 pS at -70 mV and 60 pS at 100 mV. Despite a majority of examples where outward-rectifying *I-V* behavior has been observed under symmetrical salt conditions, we should note that there may also be examples of SCl channels that exhibit inward rectification (e.g., Hanrahan et al., 1985).

Although the single-channel properties of invertebrate Cl^- channels have not been well studied, an outward-rectifying, macroscopic Cl^- current has been described in perfused squid axons (Inoue, 1985). The examples of SCl channels in mammalian neurons and glia cited above suggest that evolutionarily-related Cl^- channels in vertebrates and invertebrates may serve analogous functions. Since SCl channels appear to exist in both neurons and glial cells, it is possible that the lobster SCl channel that we observe in bilayers may have originated from either the axon membrane or Schwann cell membrane. Because of this uncertainty, it is difficult to propose a physiological function for the channel we have studied. Previous authors

have discussed possible functions for Cl^- channels which may also apply to the lobster. Although the Cl^- channel of rat hippocampal neurons studied by Franciolini and Nonner (1987) exhibits significant Na^+ and K^+ permeability, modeling work showed that in the absence of a cation gradient the reversal potential for current through this channel is solely determined by anions. Thus Franciolini and Nonner (1987) proposed that this Cl^--selective channel could stabilize the resting membrane potential and may also help to repolarize the nerve cell. In the squid axon, calculations by Inoue (1985) suggested that Cl^- conductance contributes ~14–28% of the total resting membrane conductance. In the case of glial cells such as astrocytes and oligodendrocytes, Ritchie (1987) and Barres et al. (1988) have suggested that Cl^- channels may assist the K^+ buffering function of glia by allowing local KCl uptake during periods of neuron firing. Future electrophysiological studies in intact lobster axons are needed to establish which of these possible cell locations and functions applies to the Cl^- channel we have described.

In most previous planar bilayer studies of small conductance Cl^- channels, the observed *I-V* behavior exhibited a characteristic negative rectification (i.e., *trans* → *cis* Cl^- flux is less than *cis* → *trans* Cl^- flux) (Coronado and Latorre, 1982; Reinhardt et al., 1987; Bridges et al., 1989; Manning and Williams, 1989). In the case of colon epithelia, a similar outward-rectifying Cl^- channel is known to exist in the intact tissue (Halm et al., 1988). This would imply that Cl^- channel incorporation occurred via fusion of rightside-out vesicles with the *cis* side of the bilayer (Reinhardt et al., 1987; Bridges et al., 1989). Because we observe positive rectification in our experiments, this suggests that vesicle fusion primarily occurs from inside-out vesicles in our case (assuming that the native channel is an outward rectifier). This situation is not without precedent because both rightside-out and inside-out incorporation have been previously noted. For example, Ca-activated K^+ channels are incorporated from rat muscle T-tubule vesicles in an inside-out orientation (Moczydlowski and Latorre, 1983) and tetrodotoxin-sensitive Na^+ channels from the same preparation are preferentially incorporated in a rightside-out orientation (Moczydlowski et al., 1984). The basis for such preferential orientation is presently unknown.

Anion Selectivity

The lobster Cl^- channel ranks among the most anion-selective of the SCl channels previously described. Some SCl channels exhibit imperfect selectivity for anions with reported $P_{Na,K}/P_{Cl}$ ratios in the range of 0.1–0.25 (Blatz and Magleby, 1985; Welsh and Liedtke, 1986; Franciolini and Nonner, 1987; Manning and Williams, 1989). Franciolini and Nonner (1987) have previously proposed that such imperfect selectivity is due to an actual requirement for a monovalent cation such as Na^+ or K^+ as a cofactor in anion permeation by a mechanism involving transient ion-pair formation in the channel. However, there are several examples of SCl channels that do appear to exhibit nearly perfect anion selectivity, as demonstrated by measured $P_{Na,K}/P_{Cl}$ ratios of ≤ 0.05. Such examples include the rat colon epithelial SCl channel (Halm et al., 1988; Diener et al., 1989) and a 40–50-pS channel in the apical membrane of dogfish rectal gland (Greger et al., 1987). The lobster Cl^- channel described here is also unique in exhibiting a high I^-/F^- selectivity as measured by

the bi-ionic $(P_I/P_{Cl})/(P_F/P_{Cl})$ ratio, which is ~55 in our case (Table I). Other SCl channels from neural and secretory cells generally exhibit values of 2.8–4.8 for this parameter (Franciolini and Nonner, 1987; Reinhardt et al., 1987; Halm et al., 1988; Diener et al., 1989).

The closest examples of Cl^- channels with a bi-ionic anion selectivity similar to that of the lobster Cl^- channel are the Gaba-R and Gly-R receptor-activated channels of mouse spinal cord neurons. The reported bi-ionic permeability ratios compared in Table I nearly match those published for the Gaba-R channel (Bormann et al., 1987). The P_I/P_F ratio of 55 measured here compares favorably with I/F permeability ratios of 72 for Gly-R and 140 Gaba-R reported by Bormann et al. (1987). This similar permeability behavior suggests that the pore structure of the lobster Cl^- channel is most related to that of the agonist-gated Cl^- channels. Other functional similarities that may be noted for these two types of anion channels include the similar K_m's for saturation with respect to Cl^- concentration, substate phenomena, and weak activation of gating by positive voltage (Bormann et al., 1987). Despite these similarities, we did not find evidence of activation of the lobster Cl^- channel in bilayers by γ-aminoisobutyric acid. In their detailed investigation of anion permeation in the Gaba-R and Gly-R channels, Bormann et al. (1987) investigation of anion permeation in the Gaba-R and Gly-R channels, Bormann et al. (1987) observed anomalous mole-fraction behavior indicative of a multi-ion conduction mechanism. Although we have not systematically investigated this possibility, we did observe that permeability ratios in the lobster Cl^- channel depend on ionic concentration and/or the orientation of the test anions. One interpretation of this observation is that the lobster Cl^- channel also functions as a multi-ion channel.

pH-dependent Gating

The effect of pH has not been thoroughly studied in most of the previously cited examples of BCl and SCl channels, but several comparisons can be made. The atypical *Torpedo* Cl^- channel was the first Cl^- channel shown to be sensitive to pH at the single-channel level (Hanke and Miller, 1983). In this case, low pH enhances opening probability by shifting the voltage activation curve to more negative voltages. In the case of a BCl-type Cl^- channel from cultured rat Schwann cells, lowering internal pH from 7.3 to 6.0 was found to reversibly close the channel (Gray et al., 1984). In cultured rat myocytes, Blatz and Magleby (1985) described a 45-pS SCl-type channel with fast gating kinetics that was also inhibited by low internal pH. By analogy to the internal effect of pH in these latter two cases, our observation of inhibition of the lobster Cl^- channel by lowering *cis* pH from 7.4 to 6.0 is consistent with our suggestion that the *cis* side corresponds to the internal side of the channel in vivo. Although these few examples are insufficient for generalization, they suggest that Cl^- channels in neural cells may be susceptible to regulation by internal pH.

Pharmacology

Cl^- channel pharmacology is presently a developing area of research with only a few examples of inhibitors that have been well characterized. However, this field has benefited from a large body of information on inhibitors of anion-exchange

transport systems. In the case of squid axon, Inoue (1985) found that the macroscopic Cl^- current was irreversibly inhibited by 100 μM internal SITS, with much less sensitivity to external application of this agent. In the case of secretory cells, external DNDS has been shown to produce a flickering block of the Cl^- channel of rat colon enterocytes with an effective K_d of 2.1 μM (Bridges et al., 1989). In this Cl^- channel the effect of amino-reactive DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) was reported to be irreversible. The reversible SITS blockade of the lobster Cl^- channel that we observed appears to occur with the same external (*trans*) side preference; however, SITS binding is of lower affinity and more voltage dependent than that described for the action of DNDS in the epithelial Cl^- channel (Bridges et al., 1989).

Another anion-exchange inhibitor, NPPB, has also been found to be an effective blocker of some epithelial SCl channels (dogfish rectal gland, Greger et al., 1987; human colon carcinoma, Dreinhofer et al., 1987) in addition to the lobster nerve Cl^- channel of this study. These similarities in the action of disulfonic stilbene derivatives and NPPB on mammalian and invertebrate Cl^- channels provide further support for the possible structural and evolutionary relationship of the lobster Cl^- channel to vertebrate Cl^- channels of epithelia and secretory cells.

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