ANION CONDUCTANCES OF THE GIANT AXON OF SQUID *SEPIOTEUTHIS*

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ABSTRACT Anion conductances of giant axons of squid, *Sepioteuthis*, were measured. The axons were internally perfused with a 100-mM tetraethylammonium-phosphate solution and immersed in a 100-mM Ca-salt solution (or Mg-salt solution) containing 0.3 μm tetrodotoxin. The external anion composition was changed. The membrane currents had a large amount of outward rectification due to anion influx across Cl^- channels of the membrane (Inoue, 1985). The amount of outward rectification depended on the species of anion used and was strongly influenced by temperature and internal pH. In contrast to the anion conductances themselves, the conductance relative to Cl^- (g_A/g_{Cl}) was found to be quite stable against changes in the membrane potential, temperature, and pH. It is therefore suggested that each g_A/g_{Cl} is an intrinsic quantity of the Cl^- channel of the squid axon membrane. The sequence and values of g_A/g_{Cl} obtained in this study were NO_3^- (1.80) > I^- (1.40) > Br^- (1.07) > Cl^- (1.00) > $MeSO_3^-$ (0.46) > $H_2PO_2^-$ (0.33) > CH_3COO^- (0.29) > SO_4^{2-} (0.06).

INTRODUCTION

The chloride conductance (g_{Cl}) present in the squid axon membrane shows a large amount of outward rectification, and is irreversibly blocked by disulfonic stilbene derivatives such as 4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS)[1] and diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) (Inoue, 1985), which block anion transport in the erythrocyte membrane (Knauf and Rothstein, 1971; Fairbanks et al., 1971; Cabantchik and Rothstein, 1972; Ho and Guidotti, 1975; Rothstein et al., 1976), and in the sarcoplasmic reticulum membrane (Kasai and Kometani, 1979), and also open the potassium channel of the squid axon membrane (Inoue, 1986). In the present study, conductances of the Cl^- channel for various foreign anions were measured, and effects of temperature and pH on the anion conductances were studied. The results show that, whereas the anion conductances are strongly influenced by temperature and pH, the anion conductances relative to g_{Cl} (g_A/g_{Cl}) are independent of those extrinsic factors. Values of g_A/g_{Cl} are summarized in this paper together with values of permeability ratio (P_A/P_{Cl}), determined from the amount of shift of the membrane potential associated with anion substitutions. Characteristic properties of the Cl^- channel are thereby discussed.

METHODS

Experiments were conducted at the Marine Biological Station of the Institute for Enzyme Research, Tokushima University, Naruto, Japan. Hindmost giant axons of squid *Sepioteuthis lessoniana* were dissected out in ~5-cm lengths from a portion near the stellate ganglion, of which diameter ranged between 400 and 550 μm. The axons of this species of squid were very useful for studying such small conductances as g_{Cl}, because the nonspecific leakage current remaining after blockade of g_{Cl} was very small. This advantage was probably related to the fact that the axons have a long smooth surface with a small number of axonal branches, so that current leak through the cut ends of nerve branches could be kept at a low level.

Internal perfusion was conducted in a Lucite chamber with a glass cannula after squeezing out axoplasm on a silicone rubber pad with a small rubber-covered roller (Baker et al., 1962). Internal perfusion fluid contained 100 mM tetraethylammonium (TEA)–phosphate and 10% (by volume) glycerol. External fluid contained 100 mM Ca-salt (or Mg-salt), 10 mM Tris-Hepes buffer, 9% (by volume) glycerol, and 300 nM tetrodotoxin (TTX). Thus, ionic currents passing through both K and Na channels were blocked by TEA and TTX. pHs of the internal and the external solutions were adjusted to 7.2 and 7.8 by TEA-phosphate and by Tris-Hepes, respectively, except when changes in pH were required. Eight species of Ca-salts, i.e., $CaCl_2$, $Ca(NO_3)_2$, CaI_2, $CaBr_2$, $Ca(SCN)_2$, $Ca(MeSO_3)_2$, $Ca(H_2PO_2)_2$, and $Ca(CH_3COO)_2$, and two species of Mg-salts, i.e., $MgCl_2$ and $MgSO_4$, were used for studying the anion conductances. SITS or DIDS was used for blocking the Cl^- channel.

An internal voltage electrode was a glass capillary (~70 μm in outer diameter and 5 cm in length) filled with a 1 M KCl solution in contact with a Ag-AgCl wire. A small portion at the tip (~0.5 mm in length) was filled with asbestos fibers tightly held by the glass wall of the electrode by means of gentle heating. An electrically floating platinized platinum wire (20 μm in diameter) was inserted to the electrode up to the asbestos tip to lower the electrode resistance. A similar type of asbestos tip glass capillary electrode was used as an external reference of potential measurements. This asbestos tip electrode system could effectively improve the stability of the liquid junction potential at the tip of the electrodes (Conti et al., 1984). An internal current electrode was platinized platinum wire, 70 μm in diameter. External current electrodes consisted of three pairs of platinized silver blocks. The lateral length of a pair of central current measuring electrodes was 6.5 mm, and that of the other two pairs of guard electrodes (Hodgkin et al., 1952) was 5.5 mm.

A standard voltage clamp system (Moore and Cole, 1963) was used. Series resistance was not compensated because the anionic currents were

[1] *Abbreviations used in this paper:* DIDS, diisothiocyanostilbene-2,2′-disulfonic acid; $MeSO_3$, methanesulfonate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; TEA, tetraethylammonium; TTX, tetrodotoxin.

very small and showed practically no time dependence. Voltage pulses were delivered from a homemade programmable pulse generator build with a microcomputer (AIM 65, Rockwell International Co., Anaheim, CA). Current signals from a nerve were digitized at a rate of 100–1,000 μs per point by means of a 12-bit A/D converter (S-210, Autonics Co., Shiki, Saitama, Japan), and transferred to a computer (model 9826, Hewlett-Packard Co., Palo Alto, CA) and analyzed. Current records could be printed out with a graphic printer (model 2671G, Hewlett-Packard Co.).

All experiments except those studying the temperature effects on the anion conductances were carred out at 11–12°C.

RESULTS

Chloride Current of Squid Axon Membrane

The chloride current (I_{Cl}) of the squid axon membrane became evident when the cationic currents flowing across both Na and K channels had been blocked, and those passing through nonspecific parts of the membrane had been minimized. To meet these requirements, the internal solution contained a TEA-salt, and the external solution contained a Ca-salt (or Mg-salt) containing TTX. Fig 1 *a* shows one set of current records obtained under these conditions at given voltages between −90 and +100 mV at every 10-mV step. Nonspecific leakage currents associated with the same voltage pulses, which remained after blocking currents passing through Cl^- channels by intracellular application of SITS are shown in Fig. 1 *b*. It is seen from these records that the greater part of the outwardly rectifying currents observed under these environmental conditions was produced by Cl^- influx through the Cl^- channels.

Anion Conductances of the Cl^- Channel

The outwardly rectifying current was influenced both by alterations in the external Cl^- concentration and by replacement of the external Cl^- with other anions. The outward current increased when the external Cl^- was replaced by Br^-, I^-, or NO_3^- (Fig. 2 *a*), and decreased when replaced by $MeSO_3^-$, $H_2PO_2^-$, or CH_3COO^- (Fig. 2 *b*). The outwardly rectifying current almost disappeared

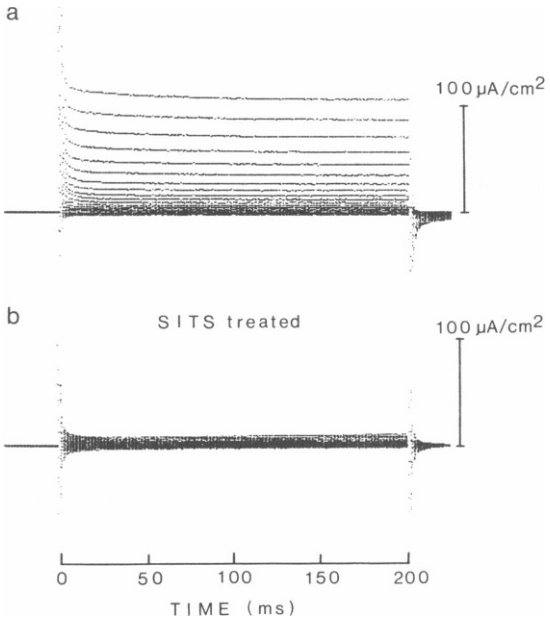

FIGURE 1 (*a*) Membrane currents associated with stepwise changes in the membrane potential demonstrating I_{Cl} obtained from an axon internally perfused with a 100-mM TEA-phosphate solution and bathed in a 100-mM $CaCl_2$ solution containing 0.3 μM TTX. Voltages of the step pulses were −90 ~ +100 mV at 10-mV intervals. E_m was −56 mV and the holding potential was −60 mV. (*b*) Membrane currents obtained from the same axon at the same voltages after application of 100 μM SITS to the axon interior, showing nonspecific leakage currents after blocking I_{Cl}. E_m was −21 mV, and the holding potential was −20 mV.

when the external Cl^- was replaced by SO_4^{2-} (not in the figure, see Inoue, 1985).

Anion conductances at each test voltage were calculated from the I-V relations. The relative anion conductance, i.e., the ratio of the anion conductance to the Cl^- conductance, are plotted against the membrane potential in Fig. 3. The figure shows both the chord conductances (*open symbols*) and the slope conductances (*solid symbols*) calculated from the curves drawn according to curve fittings with cubic functions. We may assume that both the chord conductance ratios and the slope conductance ratios are

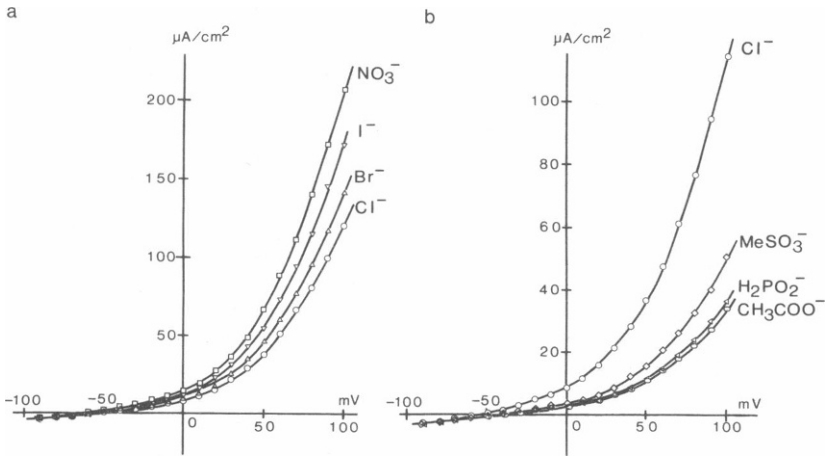

FIGURE 2 Current-voltage relations under various external anion compositions. Curves in *a* and those in *b* were obtained from different axons, respectively. The axons were internally perfused with a 100-mM TEA-phosphate solution, and bathed in 100-mM Ca-salt solutions.

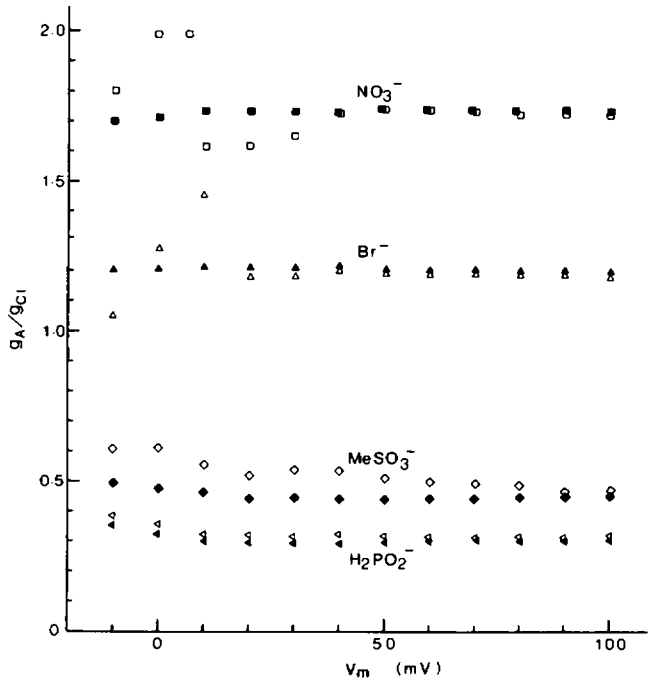

FIGURE 3 Voltage dependence of anion conductances relative to g_{Cl} obtained from some data in Fig. 2. (*Open symbols*) Chord conductance ratios; (*solid symbols*) slope conductance ratios.

independent of the membrane potential. The chord conductances at +80 mV were used in calculating g_A/g_{Cl} to avoid a large contribution of nonspecific leakage currents, and these values of g_A/g_{Cl} were used as the relative anion conductances in this paper. The chord conductances corrected by subtracting the nonspecific leakage (SITS-insensitive) conductance were also obtained, and used in calculating g_A/g_{Cl} (see Table I). However, the correction became significant only for SO_4^{2-}, for which conductance was similar size to the nonspecific leakage conductance.

When the external Cl^- was partly replaced with foreign anions, the conductance changed almost linearly with the extent of the anion replacement. This conductance change was reversible for the replacements of the foreign anion with Cl^-.

A replacement of the external Cl^- by SCN^- gave a transient increase in the outward current followed by a rapid decline. Further exposure to SCN^- caused a gradual increase in the nonspecific (SITS-insensitive) current, and a subsequent loss of the outward rectification. When the SCN^- effect proceeded to this stage, which generally occurred within 3 min, no recovery of the outward rectification was brought about by reintroduction of the original $CaCl_2$ solution to the axon exterior. The degradation of the membrane properties by SCN^- was found to be enhanced when depolarizing pulses were applied to the membrane.

Effect of Temperature on g_{Cl}

The outwardly rectifying I_{Cl} was strongly influenced by temperature (Fig. 4). The average Q_{10} for g_{Cl} obtained from three individual experiments was 2.7 ± 0.1 (mean ± SD), which is more than twice of that of the free Cl^- mobility which is 1.3 (Robinson and Stokes, 1959). On the other hand, the Q_{10} of the nonspecific conductance which remained after the SITS application was 1.3.

Effect of pH on g_{Cl}

Alterations of the internal pH changes produced irreversible changes in g_{Cl}. Therefore, the pH effects were studied either in alkaline side or in acid side in each experiment. On the alkaline side, g_{Cl} gradually decreased as the pH was elevated; there was, at the same time, irreversible degrada-

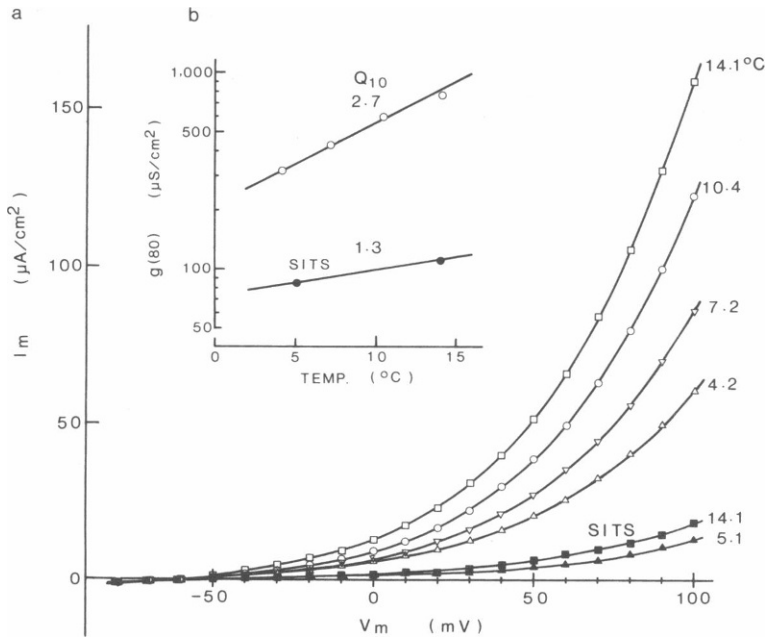

FIGURE 4 (*a*) Current-voltage relations obtained at various temperatures. The axon was internally perfused with a 100-mM TEA-phosphate solution and bathed in a 100-mM $CaCl_2$ solution. (*Solid symbols*) Data obtained after intracellular application of 100 μM SITS for a period of 10 min. (*b*) Chord conductances at +80 mV as a function of temperature, showing that the Q_{10} of g_{Cl} is 2.7 and that nonspecific leakage conductance that remained after the SITS application is 1.3. Note that the Q_{10} of free diffusion of ions is 1.3.

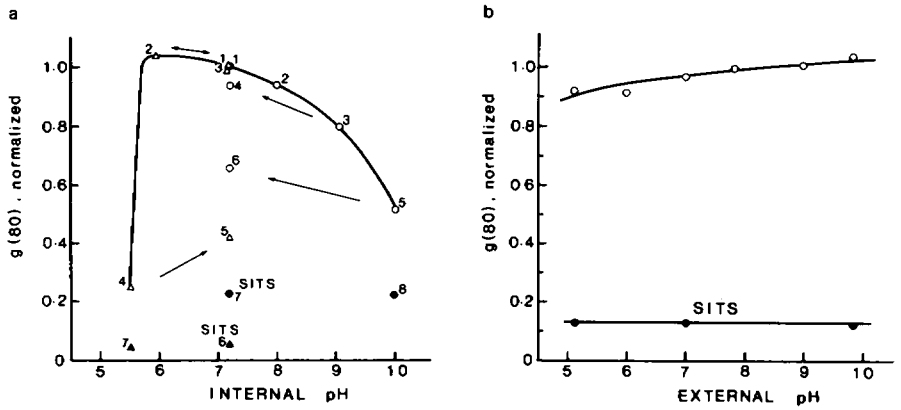

FIGURE 5 (*a*) Effect of changes in the internal pH g_{Cl} at +80 mV. The external pH was maintained at 7.8. The results obtained from two axons are represented. The values of g_{Cl} are normalized to g_{Cl} at pH 7.2. The numbers put by the symbols indicate the sequence of conductance measurements. (*Solid symbols*) Data obtained after intracellular treatment with 100 μM SITS. The external pH was maintained at 7.8. (*b*) Effect of changes in the external pH on g_{Cl} (*open circles*) and on nonspecific leakage conductance (*solid circles*). The internal pH was kept at 7.2.

tion of g_{Cl} and increase of the nonspecific leakage conductance (Fig. 5 *a*). On the acid side, g_{Cl} unchanged or increased slightly as the pH was lowered to 5.8. When the pH was lowered below 5.5, however, there was a sudden decrease in g_{Cl} ; this change was largely irreversible (4 to 5). The nonspecific leakage conductance kept at a low level even when the pH was lowered below 5.5.

In contrast, alterations of the external pH between 5.0 and 10.0 produced only a small change in g_{Cl} (Fig. 5 *b*). An irreversible decline of g_{Cl} gradually took place either when the pH was reduced below 5.0 or elevated above 10.0.

Relative Anion Conductances

Though g_{Cl} was dependent on both temperature and internal pH, the relative anion conductances, g_A/g_{Cl}, were found to be quite stable against changes in these parameters. Data demonstrating the stability of the relative anion conductance (g_{MeSO_3}/g_{Cl} in these cases) against temperature changes and pH changes are presented in Fig. 6.

From the experimental results just described, it was assumed that the relative anion conductances represent intrinsic properties of the Cl^- channel of the squid axon membrane. The values, g_A/g_{Cl}, and the corrected values, g_A^*/g_{Cl}^*, obtained after subtracting the SITS-insensitive components, are summarized in Table I. A conspicuous difference between the uncorrected and corrected values is apparent only for g_{SO_4}/g_{Cl}. The column, ΔE_m, shows the magnitudes of the shift of the membrane potential associated with the total replacement of Cl^- of the external solution by foreign anions. The permeability ratios, P_A/P_{Cl}, were calculated according to the equation $P_A/P_{Cl} = \exp(-\Delta E_m F/RT)$. Here, F, R, and T have their common meaning. It should be noted that there is a consistency between the values of g_A/g_{Cl} and those of corresponding P_A/P_{Cl}.

DISCUSSION

In the squid axon membrane, g_{Cl} showed a strong outwardly rectifying characteristic. The amount of the rectification was much larger than that calculated with a constant Cl^- permeability from the Goldman-Hodgkin-Katz equation according to the unequal distribution of Cl^- across the membrane. It has been shown in a previous report (Inoue, 1985) that the rectification is produced by the voltage dependence of the Cl^- permeability.

The Q_{10} of g_{Cl} of the squid axon was 2.7. This value is larger than the Q_{10}s of the temperature sensitive g_{Cl} found in frog skeletal muscle (Harris, 1965) and stingray muscle (Hagiwara and Takahashi, 1974), which are ~2. This increased temperature sensitivity of g_{Cl} of the squid axon suggests that open-close rates of the Cl^- channel are strongly related to temperature.

It is known that chloride conductances of muscle membranes are sensitive to extracellular pH (Hutter and War-

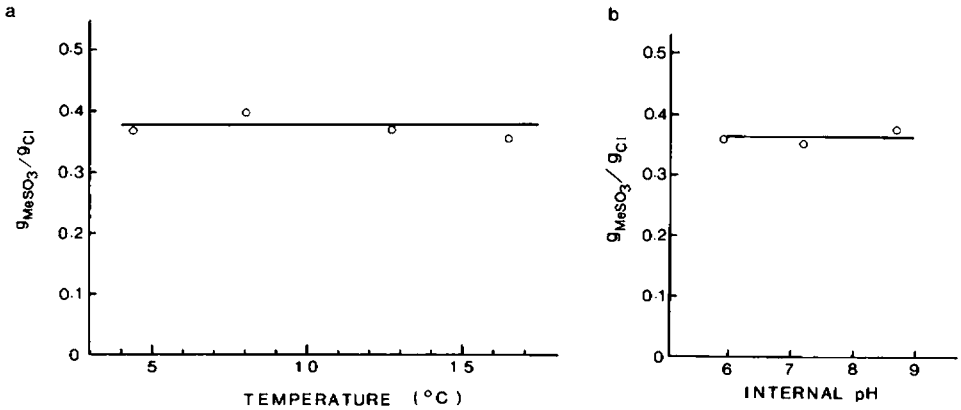

FIGURE 6 (*a*) A relative anion conductance (g_{MeSO_3}/g_{Cl}) as a function of temperature. (*b*) Same as a function of internal pH, demonstrating relative anion conductance as insensitive to temperature and internal pH, respectively.

TABLE I
RELATIVE ANION CONDUCTANCES AND RELATIVE ANION PERMEABILITIES OF THE CHLORIDE CHANNEL

Anion	No.	g_A/g_{Cl}		g_X^*/g_{Cl}^*		ΔE_m (mV)		P_A/P_{Cl}
		Mean	*(SD)*	*Mean*	*(SD)*	*Mean*	*(SD)*	
NO_3^-	7	1.65	(0.13)	1.80	(0.16)	−12.2	(2.4)	1.62
I^-	8	1.36	(0.19)	1.40	(0.23)	−8.5	(2.7)	1.40
Br^-	8	1.06	(0.07)	1.07	(0.13)	−1.0	(2.0)	1.07
$MeSO_3^-$	11	0.48	(0.06)	0.46	(0.07)	21.9	(5.1)	0.42
$H_2PO_2^-$	12	0.37	(0.03)	0.33	(0.04)	26.5	(3.9)	0.35
CH_3COO^-	9	0.35	(0.03)	0.29	(0.04)	27.0	(3.7)	0.34
SO_4^{2-}	3	0.20	(0.02)	0.06	(0.03)	39.5	(7.4)	

ner, 1967*a*; Moore, 1969; Dipolo, 1972; Hagiwara and Takahashi, 1974). The apparent pK of those tissues is 5.3. The apparent pK of squid axon g_{Cl} was found to be slightly lower than 5.5, but g_{Cl} was sensitive only to internal pH.

In contrast to the individual anion conductances, the values of the relative anion conductances were independent of environmental conditions such as membrane potential, temperature, and pH. This suggests that the ion filter property of the Cl^- channel is not influenced by these factors, whereas the gating kinetics are strongly affected by them.

However, the consistency between the conductance ratio and the permeability ratio is a remarkable property of the Cl^- channel of the squid axon membrane. In Cl^- channels of other living tissues, both the values and the sequence of the conductance ratios generally differ from those of permeability ratios (Harris, 1958; Hodgkin and Horowicz, 1959; Hutter and Padsha, 1959; Adrian, 1961; Spurway, 1965; Hutter and Warner, 1967*b*; Takeuchi and Takeuchi, 1971; Hagiwara and Takahashi, 1974; Palade and Barchi, 1977). The inconsistency between the conductance and permeability ratios is interpreted by assuming that those Cl^- channels, have multiple binding sites in the ionic pathway (Hille and Schwarz, 1978; White and Miller, 1981; Bormann et al., 1987), so that the permeability related to both the partitioning of counter ions and ion mobilities can differ from the conductance determined by ion mobilities.

In squid axons, the stability of inner membrane protein layer is related to the lyotropic number of internal anions (Tasaki et al., 1965; Inoue et al., 1976). F^- stabilizes the protein structure, and is most favorable in maintaining the channel activities. On the contrary, SCN^- causes destruction of the salt linkages of the protein layer and brings about a rapid deterioration of the channel activity. The Cl^- channel of the squid axon membrane was shown to be irreversibly destroyed by SCN^-, although the Cl^- channel was highly permeable to SCN^-. Considering that the degradation of the Cl^- channel was enhanced by depolarization, the protein structure of the Cl^- channel at the inner membrane side might be destroyed by means of strong salting-in effect of SCN^-.

Though the physiological role of the Cl^- channel of the squid axon membrane is unknown, it contributes to the resting potential to a certain extent. 14–28% of the resting membrane conductance is produced by g_{Cl} (Inoue, 1985).

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