Voltage-dependent Chloride Conductance of the Squid Axon Membrane and Its Blockade by Some Disulfonic Stilbene Derivatives

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ABSTRACT When giant axons of squid, Sepioteuthis, were bathed in a 100 mM Ca-salt solution containing tetrodotoxin (TTX) and internally perfused with a solution of 100 mM tetraethylammonium-salt (TEA-salt) or tetramethylammonium-salt (TMA-salt), the membrane potential was found to become sensitive to anions, especially Cl−. Membrane currents recorded from those axons showed practically no time-dependent properties, but they had a strong voltage-dependent characteristic, i.e., outward rectification. Cl− had a strong effect upon the voltage-dependent membrane currents. The nonlinear property of the currents was almost completely suppressed by some disulfonic stilbene derivatives applied intracellularly, such as 4-acetoamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and as 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), which are blockers of chloride transport. On the basis of these experimental results, it is concluded that a voltage-dependent chloride-permeable channel exists in the squid axon membrane. The chloride permeability (Pc) is a function of voltage, and its value at the resting membrane (Em = −60 mV) is calculated, using the Goldman-Hodgkin-Katz equation, to be $3.0 \times 10^{-7}$ cm/s.

INTRODUCTION

It was demonstrated in a previous paper (Inoue, 1980) that squid giant axons can develop all-or-none action potentials in a CaCl₂ solution containing tetrodotoxin (TTX) under internal perfusion with a K⁺-free solution and that the Ca²⁺ current associated with the spike is completely blocked by internally applied tetraethylammonium ion (TEA⁺). The development of such TTX-insensitive action potentials has been interpreted in terms of an entry of Ca²⁺ through the K⁺ channel (Inoue, 1980, 1981). The resting potential of those axons is kept at a negative level, although the equilibrium potential across the K⁺ channel becomes inside-positive. Therefore, it has been suggested that an ionic channel that is neither the Na⁺ nor the K⁺ channel may exist in the axon membrane and play a leading role in generating the resting potential when the conductances of.
the two cationic channels are suppressed or reduced. The aim of the present study was to characterize the third ionic channel in the squid axon membrane.

It was found in a preliminary survey that the axon membrane behaved like a Cl-sensitive electrode when both the Na\(^+\) and the K\(^+\) channels had been blocked by TTX and by TEA\(^+\) (or tetramethylammonium ion [TMA\(^+\)]). This observation led me to imagine that the third ionic channel could be a Cl\(^-\) channel. It seemed worthwhile to test this idea because Cl\(^-\) channels have been characterized in other excitable tissues (cf. Hutter and Warner, 1972; Hagiwara and Takahashi, 1974; Fukuda, 1975; Palade and Barchi, 1977; White and Miller, 1981; Barish, 1983; Blatz and Magleby, 1983). Most squid axons used in the present work were internally perfused with solutions of 100 mM TEA-salt (or TMA-salt), and were immersed in 100 mM Ca-salt solutions containing 1 \(\mu\)M TTX. The effects of changing the anionic compositions of both internal and external fluid media on the membrane potential and on the membrane current were studied. The effects on these electrical properties of some disulfonic stilbene derivatives (SITS, DIDS), which are known as anion-transport blockers, were also examined. This paper describes the experimental results that characterize the third ionic channel as being a Cl\(^-\) channel.

Some of these results have been reported at the 1983 Seiriken conference (Japan).

**METHODS**

Experiments were performed on giant axons of squid, *Sepioteuthis lessoniana*, obtained from the Kitanada Fisherman's Cooperative Association, Naruto City, Japan. The diameter of the axons used was between 400 and 600 \(\mu\)m. Before starting internal perfusion, axoplasm was squeezed out with a roller (Baker et al., 1962). The axon was then transferred to a Lucite chamber where internal perfusion with a glass cannula was conducted. No protease was used at the initiation of internal perfusion. The flow rate of internal perfusion was kept at \(\sim 20 \mu\)l/min. The electrolyte solutions used are given in Table I. The letter \(M\) in the table represents either TEA\(^+\) or TMA\(^+\), and \(X\) indicates the concentration of Cl\(^-\). SITS or DIDS was dissolved in each testing solution just before using it. TTX was provided by the Sankyo Chemical Co., Tokyo, Japan. All other salts, acids, bases, and reagents were purchased either from the Nakarai Chemicals Ltd., Kyoto, Japan, or from the Wako Pure Chemicals Ltd., Osaka, Japan. Axons of which both the Na\(^+\) and the K\(^+\) channels were blocked by TTX and by TEA\(^+\) (or TMA\(^+\)) are referred to as TTX-TEA axons (or TTX-TMA axons).

It is known that internally applied Cl\(^-\) causes a deterioration of the electrical properties of the membrane, including excitability (cf. Tasaki et al., 1965). The Cl\(^-\) effects are explained in terms of breakage of salt linkages among charged groups at the inner membrane layer by strong interactions between the charged groups and counterions having a large lyotropic number (cf. Tasaki, 1968; Inoue et al., 1976). The rate of development of the adverse effects thus depends on the concentration of Cl\(^-\) and on the duration of intracellular treatment with Cl\(^-\). In order to delay the development of adverse effects, the internal solution was switched from one containing no Cl\(^-\) to another containing Cl\(^-\) only when measurements at high [Cl\(^-\)] were required. The internal solution was switched back to the Cl\(^-\)-free one immediately after the measurements were done. Even though this precaution was taken, irregular and anomalous shapes frequently appeared in the membrane currents internally perfused with a Cl\(^-\)-containing solution (see for example, record c in Fig. 3).
A glass pipette electrode (70 μm o.d.) filled with a 1-M KCl solution in contact with an Ag/AgCl wire was used as an internal voltage pick-up electrode. The tip of the electrode (~0.5 mm in length) was filled with asbestos fibers, which improved the stability of the tip potential (Conti et al., 1984). A platinized platinum wire (30 μm diam) was inserted into the electrode to lower the electric impedance. A similar type of electrode immersed in the external fluid was used as a reference point for potential measurements. For voltage clamp, a platinized platinum wire (70 μm diam) was introduced into the axon. Platinized silver blocks were used as a current-measuring electrode with a guard system (Hodgkin et al., 1952). The length of the central current-measuring electrodes was 6.0 mm, and that of the guard electrodes was 5.5 mm. Voltage pulses were delivered from a home-made programmable pulse generator, which was originally designed by Dr. Stühmer (Max-Planck-Institut, Göttingen, Federal Republic of Germany). The duration of the voltage pulses was 50–200 ms, and the pulse interval was 1 s.

Voltage-clamp current signals from an axon were amplified, and their high-frequency components were cut off at <3 kHz. The signals were then digitized at a sampling rate of 250–1,000 μs/point with a 12-bit A/D converter (model S-210; Autonics Co., Shiki, Japan) and transferred to a computer (9826; Hewlett-Packard Co., Palo Alto, CA). The signals thus stored in the computer were displayed on a computer screen. All records shown in

### TABLE I

**Composition of Solutions**

| Internal solutions | M⁺ | Cl⁻ | Phosphate⁺ (pH 7.3) | HEPES-M (pH 7.5) | Glycerol | % by volume |
|--------------------|----|-----|---------------------|------------------|----------|-------------|
| 100 M-0 Cl         | 100 | —   | 100                 | —                | 10       |             |
| 100 M-X Cl         | 100 | X   | 100 - X             | —                | 10       |             |
| 100 M-100 Cl       | 100 | 100 | —                   | 2                | 10       |             |

| External solutions | Ca²⁺ | Mg²⁺ | Cl⁻ | MeSO₄ | SO₄²⁻ | HEPES-Ca (pH 7.8) | TTX | Glycerol | % by volume |
|--------------------|------|------|-----|-------|-------|------------------|-----|----------|-------------|
| 100 Ca-XCl         | 100  | —    | X   | (200 - X)/2 | —     | 5                | 1   | 9        |             |
| 100 MgCl₂          | 100  | 200  | —   | —     | —     | 20               | 0.3 | 9        |             |
| 100 MgSO₄          | 100  | 100  | —   | —     | —     | 20               | 0.3 | 9        |             |

| Na⁺ | K⁺ | Ca²⁺ | Mg²⁺ | Cl⁻ | SO₄²⁻ | MeSO₄ | HEPES-Ca (pH 7.8) | % by volume |
|-----|----|------|------|-----|-------|-------|-------------------|-------------|
| ASW | 450 | 9    | 10   | 60  | 539   | 30    | —                 | 10          |
| ASW-0 Cl | 450 | 9    | 10   | 60  | 60    | 479   | —                 | 10          |

M: TEA⁺ or TMA⁺.
X: concentration of Cl⁻.
MeSO₄: methylsulfate ion.
this paper were obtained by making hard copies from the computer screen. A steady state current value associated with a voltage pulse was obtained by averaging the values of the last 10 points at the end of the pulse.

Experiments were carried out at a temperature of 11–12°C.

RESULTS

Effect of Cl⁻ on \( E_m \)

The membrane potential (\( E_m \)) was recorded from TTX-TMA axons. In each experiment, an axon that was immersed in a 100 mM Ca-salt solution containing a certain concentration of Cl⁻ was initially internally perfused with 100 TMA-0 Cl. After reaching a steady level of \( E_m \), the internal Cl⁻ concentration ([Cl⁻]₀) was raised by switching the internal solution to one containing Cl⁻. As shown in Fig. 1, the membrane underwent depolarization and reached a new steady level after raising [Cl⁻]. \( E_m \) at the steady state depended upon [Cl⁻]₀. When [Cl⁻]₀ was lowered to 0 mM, \( E_m \) returned to the original negative level within 1 min.

In Fig. 2 (left), steady values of \( E_m \) at various external Cl⁻ concentrations ([Cl⁻]₀) are plotted against a logarithmic scale of [Cl⁻]. The same symbols represent the data taken from the same axon. On the right, the data are replotted against a logarithmic scale of [Cl⁻]. In each diagram, the slope of the \( E_m \) changes becomes steeper and approaches the Nernst slope as the Cl⁻ concentration rises. On the other hand, \( E_m \) tends to deviate from the chloride potential (\( E_{Cl} \)) when the Cl⁻ concentration decreases. These features of the \( E_m \) changes are systematic. That is, \( E_m \) seems to be affected not only by Cl⁻ but also by MeSO₄ and by phosphate ion. In fact, the data fit curves that are drawn according to the following equation:

\[
E_m = \frac{RT}{F} \ln \left[ \frac{[\text{Cl}^-]_i + 0.3[\text{phosphate}]}{[\text{Cl}^-]_o + 0.2[\text{MeSO}_4]_o} \right].
\]
Here, $R$, $T$, and $F$ have their common meaning.

It should be noted that the coefficients of 0.2 and 0.3 in the equation represent only approximate values for the anion selectivity of the membrane for two reasons. (a) Phosphate ion is treated as if it were univalent for convenience, while it involves both univalent and divalent forms at pH 7.3. Therefore, the coefficient 0.3 does not represent a defined meaning such as a relative permeability of phosphate ion to Cl$^-$ from a thermodynamic point of view. (b) The contribution of cations, i.e., Ca$^{2+}$ and TMA$^+$, to $E_m$ is neglected. Besides MeSO$_4^-$ and phosphate ion, these cations can contribute to the deviation of $E_m$ from $E_{Cl}$, especially at low [Cl$^-$], where the Cl$^-$ conductance becomes very small. In fact, replacement of external Ca$^{2+}$ by Mg$^{2+}$ brought about a small depolarization when the internal solution was 100 TEA-0 Cl, as seen in Fig. 4.

In spite of such an approximate expression for the anion selectivity, the data in Fig. 2 clearly demonstrate that the membrane behaves like a Cl$^-$-sensitive electrode under these particular conditions and suggest that both MeSO$_4^-$ and phosphate ion influence $E_m$ by acting as foreign anions. In other words, the data suggest that Cl$^-$, MeSO$_4^-$, and phosphate ion permeate the same ion-conductive channels in the membrane according to their own permeation ability, which should reflect the membrane conductance.

Similar anion effects on $E_m$ were also observed in TTX-TEA axons.
Effect of Cl\textsuperscript{-} on \(I_m\)

Small ionic currents that remain after blocking both Na\textsuperscript{+} and K\textsuperscript{+} channels have been regarded as leakages in the squid axon membrane. Three sets of records in Fig. 3 display such leakages taken at three different Cl\textsuperscript{-} compositions as given to the left of the records. The external cation was 100 mM Ca\textsuperscript{2+}, and the internal cation was 100 mM TEA\textsuperscript{+}. The pulse protocols employed in the three measurements were the same except that the holding potential was adjusted to a level near \(E_m\) in each set of measurements. Curves a, b, and c represent the current-voltage relations at the steady state for records a, b, and c, respectively. The currents show practically no time-dependent characteristics, but they have a strong voltage dependence, i.e., outward rectification. As can be seen in the figure, Cl\textsuperscript{-} had a strong effect upon the voltage-dependent currents. The total replacement of the external anion (MeSO\textsubscript{4}) with Cl\textsuperscript{-} doubled the outward currents, whereas it did not increase the inward currents (see a and b). Note that the chord conductances for the outward currents in b are roughly proportional to those in a. However, replacement of the internal phosphate ion with Cl\textsuperscript{-} greatly increased the inward currents without changing the outward currents significantly (b and c). A proportional relation in the chord conductances is recognized for the inward current components in this case. (The small decay in the currents seen in record c at large depolarizations [greater than +70 mV] is due to the adverse effect of internal Cl\textsuperscript{-}, as mentioned in the Methods.) The membrane conductances at the reversal potentials calculated from the current-voltage relations for curves a, b, and c are 120, 140, and 220 \(\mu\text{S/cm}^2\), respectively. These values are much smaller than the resting conductance of normal axons, which is \(\sim 1 \text{ mS/cm}^2\). Therefore, it is understood that the anion sensitivity of the membrane becomes evident when all the cation permeabilities have been suppressed.

The outwardly rectifying leakages were more effectively suppressed when SO\textsubscript{4}\textsuperscript{2-} was used as the external anion. This series of experiments was done with 100-mM Mg-salt solutions externally instead of 100-mM Ca-salt solutions. The Mg-salt solutions contained 20 mM HEPES-Ca buffer (pH 7.8). The internal solution was 100 TEA-0 Cl. As shown in Fig. 4, a large amount of outward rectification was also observed when the axon was bathed in a 100 mM MgCl\textsubscript{2} solution (see curve a). \(E_m\) was \(-37.8\) mV, which was \(\sim 10\) mV less negative than that measured in the 100 mM CaCl\textsubscript{2} solution. The outward rectification decreased to approximately half when half of the MgCl\textsubscript{2} (50 mM) was replaced by MgSO\textsubscript{4} (curve b), and it almost completely disappeared when MgCl\textsubscript{2} was totally replaced by MgSO\textsubscript{4} (curve c). The membrane depolarized associated with the anion substitutions. \(E_m\) was \(-26.2\) mV at 50 mM SO\textsubscript{4}\textsuperscript{2-} and \(+9.2\) mV at 100 mM SO\textsubscript{4}\textsuperscript{2-}. The insensitivity of the inward currents to the external anion substitutions is also evident in these three curves.

It is clear from these results that the major part of the outward rectifying currents in TTX-TEA axons (or TTX-TMA axons) is attributed to anion influx across the membrane. Thus, a rectifying anion conductance actually exists in the axon membrane. The conductance depends upon the species of anion; the sequences are \(g_{\text{Cl}} > g_{\text{MeSO}_4} > g_{\text{SO}_4}^2\) and \(g_{\text{Cl}} > g_{\text{phosphate}}\). We may refer to the ani-
FIGURE 3. (Top) Time courses of membrane currents obtained from a TTX-TEA axon at three different anion compositions. The Cl⁻ concentrations of both the external and the internal solutions are given to the left of each set of records. The external cation was 100 mM Ca²⁺ and the internal cation was 100 mM TEA⁺. The external anion was MeSO₄⁻ or Cl⁻, and internal anion was phosphate ion or Cl⁻. The holding potentials for records a, b, and c were -20, -46, and -14 mV, respectively. (Bottom) The current-voltage relations at steady state (at the end of pulses) for records a (open squares), b (open circles), and c (open triangles). For further details, see text.
onic conductance channel as a Cl⁻ channel, since it is highly permeable to Cl⁻, which is the major anion in physiological circumstances.

**Blockade of the Anion Conductance by SITS**

It is well established that some disulfonic stilbene derivatives, such as SITS and as DIDS, which are amino-reactive reagents, produce a specific and irreversible inhibition of the anion transport of the erythrocyte membrane (cf. Knauf and Rothstein, 1971; Rothstein et al., 1976) and of the sarcoplasmic reticulum membrane (cf. Kasai and Kometani, 1979; Kasai and Taguchi, 1981). The

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**FIGURE 4.** Effect of replacements of external MgCl₂ with MgSO₄ on the current-voltage relation of the leakage. The Mg-salt compositions of the external fluid are given in the figure. The external solutions contained 20 mM HEPES-Ca buffer and 300 nM TTX. The internal solution was 100 TEA-0 Cl. The current data represented by the solid triangles were taken after an intracellular treatment with SITS. Details for the data are given in the next section.
following observations show that these reagents have a strong suppressive effect on the leakage of the squid axon membrane.

In the experiment in Fig. 5, the “control” records were taken with 100 Ca-200 Cl externally and with 100 TEA-0 Cl internally. $E_m$ was $-44.0$ mV. The internal solution was then switched to one containing 100 μM SITS. The currents started to decrease immediately after the onset of SITS and reached a level shown by the “SITS” records within 5 min. The membrane depolarized by $\sim25$ mV during this period; that is, the anion sensitivity of $E_m$ greatly deteriorated. No further suppression of the currents was produced either by a prolonged treatment with SITS or by an increase in the SITS concentration. There was also no recovery after washing out SITS from the axon interior. Therefore, it is suggested that the “SITS” records represent the currents after the maximal and irreversible inhibition attained by SITS. The current-voltage relations for the two records show that the outward rectification almost disappeared after the SITS treatment. The chord conductances after SITS are not more than 90 μS/
cm² in the whole voltage region in this case. This value is ~70% of the conductance at $E_m$ of the "control" data.

On the other hand, the SITS treatment produced only a small decrease in the leakage currents when the major portion of the outwardly rectifying currents had been suppressed by the replacement of the external 2 Cl⁻ with SO₄²⁻, as can be seen from curves c and d in Fig. 4. These facts can be interpreted as suggesting that the Cl⁻ channel is almost impermeable to SO₄²⁻ and that SITS inhibits all the anion permeabilities of the Cl⁻ channel.

![Graph showing K⁺ currents before (control) and after (SITS) an internal application of 200 μM SITS. The open and solid circles represent the current-voltage relations at the steady state of the K⁺ currents (200 ms after the depolarizations) before and after SITS, respectively. The internal solution was 100 K⁺-0 Cl⁻ and the external solution was 100 Ca⁺-200 Cl⁻.](image)

**Figure 6.** Records showing K⁺ currents before (control) and after (SITS) an internal application of 200 μM SITS. The open and solid circles represent the current-voltage relations at the steady state of the K⁺ currents (200 ms after the depolarizations) before and after SITS, respectively. The internal solution was 100 K⁺-0 Cl⁻ and the external solution was 100 Ca⁺-200 Cl⁻.

The SITS concentration tested was between 10 and 500 μM. While the higher SITS concentration brought about a faster inhibition of the leakages, the final level of the currents was independent of the SITS concentration. Externally applied SITS had a suppressive effect on the leakages, but the effect was much weaker (<1/100) than that of internally applied SITS.

Fig. 6 shows the effect of SITS on K⁺ currents ($I_K$). This experiment was done to determine if the K⁺ channel might contribute to the nonlinear property of the leakage, since the K⁺ conductance also has a nonlinear property, the so-called
delayed rectification. 100 K-0 Cl was used as the internal solution in this experiment. The bathing solution was 100 Ca-200 Cl. The "control" records display the currents associated with step depolarizations from a holding level of -70 mV. They exhibit well-known characteristics of \( I_k \), i.e., an initial rise followed by a decay toward a steady level, which differ greatly from the time courses of the leakage. When SITS was applied internally, there were rapid and irreversible changes in \( I_k \). These involved a slowing of the rising phase and a disappearance of the decay of \( I_k \). However, the steady state \( I_k \) (at 200 ms after the depolarizations) still remained after the SITS treatment; it decreased slightly at voltages below 40 mV and increased at 60 mV, as can be seen from the current-voltage relations. In other words, the SITS-sensitive component of the leakage does not involve a current contributed by the K\(^+\) channel. DIDS had similar effects on the leakages and on \( I_k \).

### I\(_{cl}\) under More Normal Conditions

An anion conductance having a large amount of outward rectification has been identified under somewhat abnormal conditions. This characteristic is so remarkable that it is expected that such an anion conductance can also be detected under more normal conditions. In attempting to detect the outwardly rectifying anion currents, Cl\(^-\) in artificial seawater (ASW) that contained 300 nM TTX was replaced by MeSO\(_4\) and partly by SO\(_4^{2-}\). The Cl\(^-\)-free ASW is referred to as ASW-0 Cl. A 400 mM K-glutamate solution was used as the internal solution. This K\(^+\) solution contained 60 mM TEA-phosphate in order to eliminate outward K\(^+\) currents. This solution is referred to as 400 K-60 TEA-0 Cl. Fig. 7 shows the effect of the anion replacement on the leakage currents. The curve represented by the solid circles displays the steady state current-voltage relation obtained with ASW externally and with 400 K-60 TEA-0 Cl internally. The replacement of ASW with ASW-0 Cl decreased the currents to a level represented by the solid squares. The effect of the anion replacement became more evident as the voltage rose. For example, while the conductance decreased <10% at -60 mV, it decreased by 23% at 0 mV and by 36% at +100 mV. Therefore, the rectifying Cl\(^-\) influx was actually demonstrated under these more normal conditions. A similar effect of anion replacement on the outward currents was encountered after switching the internal 400 K-60 TEA-0 Cl to 100 TEA-0 Cl, as can be seen from the curves represented by the open symbols. Furthermore, it is evident that the amount of decrease in the current at each voltage produced by anion replacement observed with 400 K-60 TEA-0 Cl internally is close to that seen after the switching the internal solution to 100 TEA-0 Cl, whereas the switching of the internal solution greatly reduced the total current. This suggests that the rectifying anion conductance, which is mainly attributed to anion influx, is fairly stable against alterations of the salt composition of the axon interior.

### The Chloride Permeability

Attempts were made to estimate absolute values of the chloride permeability (\( P_{cl} \)) of the axon membrane. If Cl\(^-\) currents (\( I_{cl} \)) are estimated, \( P_{cl} \) can be calculated using the Goldman-Hodgkin-Katz equation for an univalent anion:
The component of leakage in TTX-TEA axons that was blocked by SITS was regarded as a current passing through Cl-permeable channels. It should be noted that there is some ambiguity in this way of estimating $I_{Cl}$ because it is not certain whether or not the component of the leakage that is blocked by SITS is attributed solely to the chloride conductance channel, even when the two major cation conductances have been suppressed by TTX and by TEA. If the latter is the case, $I_{Cl}$ thus estimated must involve a small current component through some unknown ion-conductive sites that are also blocked by SITS. However, no
appropriate way of judging between the two possibilities has been found at present.

100 TEA-100 Cl was used as the internal solution in order to estimate inward \( I_{C_l} \), which is produced by efflux of Cl\(^-\). Axons were intracellularly treated with 100 \( \mu \)M SITS for a period of 10 min. During this period, the axons were internally perfused with 100 TEA-0 Cl. Considerable difficulties arose in this series of experiments because the axons had to be exposed to internal Cl\(^-\) twice, i.e., before and after SITS. Anomalous currents were frequently encountered during the second exposure to internal Cl\(^-\). Accordingly, only 5 experiments were done successfully out of 17 trials.

Fig. 8 (left) shows an example of current data obtained before (open triangles) and after (solid triangles) an internal application of 100 \( \mu \)M SITS. The holding potential was -4 mV in the two measurements. The internal solution was 100 TEA-100 Cl, and the external solution was 100 Ca-100 Cl. (Right) Current-voltage relation for the Cl currents obtained by subtracting \( I_2 \) from \( I_1 \) at corresponding voltages. For further details, see text.
linear portion of the curve to the voltage axis is almost 0 mV. This means that $P_{\text{Cl}}$ has a constant value below $-30$ mV. (Note that the Cl$^{-}$ concentrations both inside and outside the axon are equal.) $P_{\text{Cl}}$ at the linear region calculated from the data is $2.8 \times 10^{-7}$ cm/s.

Table II summarizes the data for inward $I_{\text{Cl}}$ obtained from five axons. The data at $-60$ mV are presented. The average value of $P_{\text{Cl}}$ of the resting membrane is $3.0 \times 10^{-7}$ cm/s. This value is $\sim 10$ times smaller than that of the frog muscle fiber estimated by Hodgkin and Horowicz (1959).

Outward $I_{\text{Cl}}$ values were estimated in axons with 100 Ca--200 Cl externally and with 100 TEA--0 Cl internally. The open circles in Fig. 9 represent values of $P_{\text{Cl}}$ for outward $I_{\text{Cl}}$ at positive voltages calculated from the data in Fig. 5. (The

Table II

| Axon   | $[\text{Cl}^-]_o$ | $[\text{Cl}^-]_i$ | $V_m$ | $I_i(-60)$ | $I_r(-60)$ | $I_o(-60)$ | $P_{\text{Cl}}(-60)$ |
|--------|-------------------|-------------------|-------|------------|------------|------------|----------------------|
| JL01831| 200               | 100               | -14   | -11.1      | -4.2       | -6.9       | 5.2                  |
| JL02831| 200               | 100               | -16   | -9.0       | -3.6       | -5.4       | 2.5                  |
| JL07833| 100               | 100               | -4    | -13.5      | -5.8       | -7.7       | 5.3                  |
| JL09831| 100               | 100               | -4    | -13.2      | -6.5       | -6.7       | 2.8                  |
| JL09832| 100               | 100               | -6    | -12.9      | -5.9       | -7.0       | 5.0                  |

| Mean   |                   |                   |       |            |            |            | 5.0                  |

FIGURE 9. Cl permeability as a function of voltage. The open circles are calculated from the data in Fig. 4, and open triangles from the data in Fig. 6.
As expected from the nonlinear current-voltage characteristic of \( I_{Cl} \), \( P_{Cl} \) is a function of voltage in the positive voltage region.

**DISCUSSION**

A voltage-dependent anion conductance has been found in the squid axon membrane that is irreversibly blocked by SITS and by DIDS. These reagents are known to selectively inhibit the anion transport through the erythrocyte membrane by binding covalently with amino groups of the anion exchange protein (cf. Rothstein et al., 1976). It is therefore suggested that the chloride conductance channel of the squid axon may have a molecular configuration similar to the anion channel of the erythrocyte. According to recent investigations on protein analysis of the erythrocyte membrane using \([^{3}H]\)SITS or \([^{3}H]\)DIDS, most \([^{3}H]\)-SITS-bound proteins or \([^{3}H]\)DIDS-bound proteins are located in the band 3 region on SDS acrylamide gels following electrophoresis; the molecular weight of the band 3 proteins is 75,000 (cf. Fairbanks et al., 1971; Clarke 1975; Ho and Guidotti, 1975); and the band 3 region actually contains a protein that has the function of anion transport (Cabantchik and Rothstein, 1972; Rothstein et al., 1976).

Values of \( P_{Cl} \) have been estimated over a wide range of voltages, while some ambiguity is left being unsolved in the \( P_{Cl} \) estimation. We can now calculate \( I_{Cl} \) at these voltages under various Cl- concentrations according to the Goldman-Hodgkin-Katz equation, given the Cl- concentrations of both external and internal fluid media. For example, let us use the data given by Hodgkin (1964) for normal axons; [Cl-] of the external fluid is 540 mM and [Cl-] of the axoplasm is between 40 and 150 mM. Values of \( I_{Cl} \) calculated from Hodgkin’s data are presented in Fig. 10. The open symbols indicate \( I_{Cl} \) calculated by taking [Cl-] as 40 and 150 mM, respectively. We have seen in Fig. 7 that the replacement of Cl- in ASW with MeSO4 decreased the outwardly rectifying currents in a voltage-dependent manner. For example, the current decreased by 45 \( \mu A/cm^2 \) at +50 mV and by 70 \( \mu A/cm^2 \) at +80 mV when the internal solution was 400 K-60 TEA-0 Cl. Assuming that the conductance of the anion channel for MeSO4 is roughly half of that for Cl- according to the data in Fig. 3, \( I_{Cl} \) in ASW for the data in Fig. 7 at +50 and at +80 mV was estimated to be 90 and 140 \( \mu A/cm^2 \), respectively. These values are in agreement with the calculated \( I_{Cl} \) at the corresponding voltages in Fig. 10. It is suggested from these considerations that, in the positive voltage region, approximately half of the outward leakage currents are due to influx of Cl- in normal axons. On the other hand, as seen in Figs. 3, 4, and 7, the Cl- influx does not contribute significantly to Cl- conductance in the negative voltage region. At the resting potential of normal axons (approximately -60 mV), the contribution of Cl- influx to depolarizing current is <10%.

These observations are consistent with the results of Adelman and Taylor (1961) and Brinley and Mullins (1965). It is evident from the data in Figs. 3 and 10 that the Cl- conductance at the resting potential is generated mainly by intracellular Cl-. The conductances at -60 mV calculated from the curves in Fig. 10 are 140 \( \mu S/cm^2 \) for 40 mM [Cl-], and 280 \( \mu S/cm^2 \) for 150 mM [Cl-]. According to this
calculation, the contribution of the Cl\(^-\) conductance to the resting membrane conductance is 14–28\% if the resting membrane conductance is taken as 1,000 \(\mu\)S/cm\(^2\).

Although the physiological significance of the Cl channel in the squid axon membrane is not yet clear, one possible consequence of its voltage-dependent nature is that there must be an increase in Cl\(^-\) influx during an action potential.

#### FIGURE 10.
Cl currents as a function of voltage calculated for normal axons using the values of \(P_{Cl}\) obtained in the present study and the Cl concentrations of the external fluid and the axoplasm given by Hodgkin (1964). For further details, see text.

We can roughly calculate the amount of Cl\(^-\) influx per impulse of a normal axon; the value is 0.5 pmol/cm\(^2\)-impulse. This value is in agreement with that estimated by Hill (1950) from the measurements of the volume change of an axon after repetitively elicited action potentials, but it is one order of magnitude larger than that given by Caldwell and Keynes (1960) from the \(^{36}\)Cl\(^-\) influx measurements.
At negative as well as positive voltages, the values of $P_{Cl}$ estimated in this paper are one order of magnitude larger than those estimated by the $^{36}\text{Cl}^{-}$ flux measurements (Caldwell and Keynes, 1960; Russell, 1979). However, at least from the electrophysiological point of view, the values of $P_{Cl}$ obtained in this paper are sufficient to explain the fact that the membrane can behave like a $\text{Cl}^{-}$-sensitive electrode even though nonspecific leakage pathways exist in the membrane.

Russell (1983) measured the cation-coupled $\text{Cl}^{-}$ flux across the squid axon membrane. This cation-coupled $\text{Cl}^{-}$ flux has the following characteristics: (a) it is tightly coupled not only to $\text{Na}^{+}$ uptake but also to $\text{K}^{+}$ uptake; (b) it requires cellular ATP; (c) DIDS has no effect. Obviously, these characteristics differ greatly from the properties of the $\text{Cl}^{-}$ conductance described here. The cation-coupled $\text{Cl}^{-}$ transport thus takes place in pathways other than the passive $\text{Cl}$ channel.

As seen in the record b in Fig. 5, the decay of $I_{K}$ almost disappears after the SITS treatment. If the decay of $I_{K}$ were attributed solely to the $\text{K}^{+}$ accumulation in a so-called periaxonal space, $I_{K}$ after SITS should have a similar type of decay in its time course. Thus, these results support the view that a greater part of the decay of $I_{K}$ is due to the inactivation of the $\text{K}^{+}$ conductance itself, not to the successive change in $E_{K}$ by an accumulation of $\text{K}^{+}$ in the periaxonal space (Inoue, 1981).

I wish to thank Prof. M. Kasai for suggesting the use of SITS in the present study. Thanks are also due to Mr. H. Satake for his assistance. Dr. R. Fioravanci contributed to the improvement of asbestos-tip electrodes.

This work was supported in part by a Grant-in-Aid for Special Project Research on Molecular Mechanism of Bioelectrical Response (59123005) from the Japanese Ministry of Education, Science, and Culture.

Original version received 26 December 1983 and accepted version received 4 December 1984.

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