Modification of K Conductance of the Squid Axon Membrane by SITS

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ABSTRACT The effects of 4-acetamido-4' -isothiocyanostilbene-2,2'-disulfonic acid (SITS) on the K conductance, gK, were studied in internally perfused giant axons from squid, Doryteuthis. SITS at 3–200 μM was applied intracellularly by adding the reagent to the internal perfusion fluid. Three remarkable changes in gK were noted: (a) there was a slowing of the opening and closing rates of the K channel in the whole voltage region; (b) K channels modified with SITS started to open at voltages below -100 mV, and thus 30% of total K channels were open at the level of normal resting potential (approximately -60 mV) after the maximal drug effect was attained (<30 μM); (c) there was a disappearance of gK inactivation that became distinct at relatively high temperature (>8°C). These drug effects depended solely on the drug concentration, not on factors such as repetitive alterations of the membrane potential, and the changes in gK were almost irreversible. Another disulfonic stilbene derivative, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), had similar effects on gK, but the effects were ~1.5 times stronger. These changes in gK were somewhat similar to alterations in gNa produced by an application of veratridine, batrachotoxin, and grayanotoxin, which are known as Na channel openers.

INTRODUCTION

Some disulfonic stilbene derivatives, such as SITS and DIDS, are known as blockers of anion transport across plasma membranes of erythrocytes (cf. Knauf and Rothstein, 1971; Fairbanks et al., 1971; Cabaniss and Rothstein, 1972; Clarke, 1975; Ho and Guidotti, 1975; Rothstein et al., 1976) and of sarcoplasmic reticulum (cf. Kasai and Kometani, 1979; Kasai and Taguchi, 1981). It was shown in a previous article (Inoue, 1985) that these reagents have an irreversibly suppressive effect on the voltage-dependent gCl found in the squid axon membrane. It was also recognized in the previous study that application of the drug caused irreversible alterations in gK in addition to the blockade of gCl (Inoue, 1985). The present study focused on the drug effects on gK of the squid axon membrane. Three remarkable alterations appeared in gK upon intracellular application of SITS (or DIDS): (a) a slowing of the time-dependent gK change, (b) the appearance of a steady gK at a voltage more negative than -100 mV, and...
(c) the disappearance of $g_{K}$ inactivation. The results suggest that these reagents can be used as tools for opening $K$ channels without depolarizing the membrane.

**METHODS**

Giant axons from squid, *Doryteuthis bleekeri*, were obtained from the Tomoura Fishermen's Cooperative Association, Tokushima, Japan. The diameter of the axons was between 450 and 650 μm. Internal perfusion was performed with a glass cannula in a Lucite chamber after squeezing the axoplasm out with a small roller (Baker et al., 1962). Details of the internal perfusion are given in an earlier article (Inoue, 1985). The internal perfusion fluid contained 300 mM KF, 60 mM K-phosphate (pH 7.2), and 4.8% (by volume) glycerol. This solution will be referred to as 360 K in this article. When blockade of $K^{+}$ current, $I_{K}$, across the membrane was required, tetraethylammonium (TEA)-phosphate (pH 7.2) was added to 360 K. The artificial seawater (ASW) used as external fluid contained 423 mM NaCl, 9 mM KCl, 10 mM CaCl$_2$, 23 mM MgCl$_2$, 25 mM MgSO$_4$, and 6 mM Tris-HCl buffer (pH 7.8). In one series of experiments, the $K^{+}$ concentration of ASW was varied by iso-osmotical replacement of KCl with NaCl or of NaCl with KCl. Two solutions having a simpler ionic composition than ASW were also used as external fluid; they are referred to as 60 K/450 Na and 240 K/270 Na. 60 K/450 Na contained 60 mM KCl, 450 mM NaCl, 30 mM CaCl$_2$, and 10 mM Ca-HEPES buffer (pH 7.8). 240 K/270 Na contained 240 mM KCl, 270 mM NaCl, 30 mM CaCl$_2$, and 10 mM Ca-HEPES buffer (pH 7.8). Although the two solutions contained no Mg salts, the removal of Mg$^{2+}$ had no significant effect on $I_{K}$. An increase in the Ca$^{2+}$ concentration from 10 to 30 mM also had no effect on $g_{K}$. The external solutions contained 300 nM tetrodotoxin (TTX) to eliminate currents passing through Na channels unless otherwise indicated.

SITS was obtained from ICN Nutritional Biochemicals, Cleveland, OH. DIDS was purchased from the Wako Pure Chemical Co., Osaka, Japan. These chemicals were dissolved in testing solutions before they were used. TTX was provided by the Sankyo Chemical Co., Tokyo, Japan.

Both the internal and external voltage pick-up electrodes consisted of glass pipettes with a small portion of the tip (0.5 mm in length) filled with asbestos fibers, which were tightly held by the glass wall of the pipettes by means of gentle heating. The electrodes were filled with a 1 M KCl solution making contact with an Ag/AgCl wire. This electrode system effectively prevented any convectional movement of solutions in and out of the electrodes, thereby yielding a stable liquid junction potential (Conti et al., 1984). The internal electrode was a long glass capillary (~70 μm in outer diameter and 5 cm in length) containing an electrically floating platinized platinum wire (20 μm in diameter) along the whole length up to the asbestos tip. The electric resistance of the internal electrode was 260 kΩ, and that of the external electrode was 25 kΩ. The internal electrode was introduced into an axon together with an internal current electrode, a platinized platinum wire 70 μm in diameter. The external current electrodes consisted of three pairs of silver blocks having a half-cylindrical convex surface, which was platinized. Three electrodes in each side were fixed in a Lucite block that was movable in a direction perpendicular to the axon. The axon could be placed in the center of the cylindrical electrodes to the right of the electrodes. The distance between the center axis of the axon and the electrode on either side was 2.5 mm. The lateral length of the central current-measuring electrodes was 6.5 mm, and that of the guard electrodes (Hodgkin et al., 1952) was 5.5 mm. One central electrode had a small hole in its top center through which the external voltage electrode was introduced to the external fluid. The tip of the external voltage electrode could be placed very close to the axon, so that the electric resistance in series with the membrane between the internal and external voltage electrodes, which was estimated to
be $\sim 2 \Omega \cdot \text{cm}^2$, became much smaller than the minimum resistance of K channels of the membrane ($20-40 \Omega \cdot \text{cm}^2$).

A standard voltage-clamp system (Moore, 1971) was used. A series resistance of 0.5 $\Omega \cdot \text{cm}^2$ was compensated. The linear leakage current and capacitive transient current could be compensated with a four-component analog transient generator. Voltage pulses were generated by means of a microcomputer (AIM 65, Rockwell International Co., Anaheim, CA) programmed according to various pulse protocols. Signals from a nerve were sampled at a rate of 20–1,000 $\mu$s per point with a 12-bit A/D converter (S-210, Autonics Co., Shiki, Saitama, Japan) and were transferred to a computer (model 9826, Hewlett-Packard Co., Palo Alto, CA). The signals thus stored were displayed onto a computer screen; hard copies of the signals could be made with a graphic printer (2671G, Hewlett-Packard Co.).

**RESULTS**

**General Description of the Effects of SITS on $g_K$**

SITS effectively modified the membrane properties only when it was applied internally. In all the experiments described in this article, SITS (or DIDS) was dissolved in the internal perfusion fluid. Fig. 1 shows an example of the effects of SITS on the membrane potential and on the membrane current, $I_m$, associated with step depolarizations from a holding level ($-70 \text{ mV}$) under voltage clamp. The external solution was ASW containing no TTX, and the internal solution was $360 \text{ K}$. Control records were taken before the application of SITS. After the addition of $100 \mu\text{M}$ SITS, the resting potential, $E_m$, which was originally $-60.9 \text{ mV}$, immediately started to shift in the negative direction and reached a steady level of $-72.1 \text{ mV}$ within 2 min, while SITS was applied for a period of 3 min. This change in $E_m$ was irreversible. The shape of the action potential gradually changed during the SITS application, which involved (a) gradual prolongation, (b) the disappearance of the afterhyperpolarizing potential, and (c) the appearance of a slow falling phase toward the resting level. The peak potential decreased by 2 mV. The SITS application did not, however, produce significant changes in the rising phase or in the threshold voltage of the action potential. (The threshold current to evoke the action potential increased.) In the current records, a marked SITS effect was seen in $I_K$ but not in $I_Na$. Therefore, it is suggested that the prolongation of the action potential was attributed to the slowing down of the $g_K$ change, which is described below.

Fig. 2 shows the effect of SITS on $I_m$ in an axon internally perfused with $360 \text{ K}$ bathed in $60 \text{ K}/450 \text{ Na}$ containing TTX. The records in A–C were taken with the same pulse procedure; i.e., a prepulse of $-70 \text{ mV}$ and $500 \text{ ms}$ was applied before the depolarizing pulses, which ranged between $-40$ and $+70 \text{ mV}$ at $10$-mV intervals. The records in D were obtained with a prepulse to $-150 \text{ mV}$. SITS at $30 \mu\text{M}$ was applied for a period of 7 min in this experiment. The records in A, which were obtained before the SITS application, represent normal characteristics of $I_K$. After the SITS application, the rising phase of $I_K$ became slow, and, as can be seen from the records in B, each time-dependent $I_K$ was superposed upon a certain steady (DC) current. This steady component appeared immediately after the addition of SITS, and reached a constant level within a short period ($<3 \text{ min}$), whereas the slowing of the time-dependent $I_K$ proceeded.
FIGURE 1. Effects of internally applied SITS on the time course of $E_m$ associated with the action potential, and on $I_m$ under voltage clamp. Test pulses of $-40$ to $+70$ mV were given in 10-mV increments from a holding level of $-70$ mV. The internal solution was 360 K; the external solution was ASW containing no TTX. 100 $\mu$M SITS was applied for a period of 3 min. The control and SITS records were taken before and after the SITS treatment, respectively. Temperature, 7.6°C.

FIGURE 2. Effect of SITS on $I_K$ associated with depolarizations to $-40$ to $+70$ mV, given in 10-mV increments. The holding potential was $-40$ mV. The external solution was 60 K/450 Na, and the internal solution was 360 K for records A, B, and D, and 360 K containing 30 mM TEA-phosphate for record C. 30 $\mu$M SITS was applied internally for 7 min. The control records were taken before the SITS treatment; and the other records were taken after SITS. Records A–C were obtained with the same pulse procedure shown beneath record A; and the records in D were obtained with the pulse procedure shown beneath the records. The duration of the prepulse was 500 ms. Temperature, 6.9°C.
Therefore, the appearance of the steady component is considered to be a different process from the slowing of the $g_K$ activation. Nevertheless, the fact that the steady component was completely suppressed, together with the time-dependent one by internally applied 30 mM TEA (C), strongly suggests that the steady component is a part of $g_K$. The records in Fig. 2D demonstrate that the steady component could be selectively suppressed by an application of a large negative prepulse. Another remarkable effect of SITS was seen in the decaying $I_K$ after the peak (Fig. 3). The decay of $I_K$ disappeared almost completely within 5 min after the addition of 100 µM SITS.

Three major SITS effects on $g_K$ have been shown. These drug effects depended solely on the drug concentration. No other factors, such as voltage changes or repetitive stimulation, etc., influenced the rate. Further details of these SITS effects are described in the following sections.

**Effect on $\tau_n$**

In studying the effect of SITS on the time-dependent $g_K$ in a wide voltage region, the values of the time constant at the rising phase of $I_K$, $\tau_n$, and those at the falling phase of $I_K$, $\tau_{tail}$, at various levels of test voltage were estimated by means of curve-fittings. Axons studied were internally perfused with 360 K and immersed in 60 K/450 Na, and the internal SITS concentration was successively raised. The rising phase of $I_K$ associated with each depolarizing pulse after a hyperpolarizing prepulse of $-150$ mV and 500 ms was approximated by the following equation:

$$I_K = \bar{I}_K[1 - \exp(-t/\tau_{n})]^p.$$  

Here, $\bar{I}_K$ represents the maximal level of each $I_K$. The parameter $p$, which deviated from a value of 4 when a negative prepulse was given (Cole and Moore, 1960) and when the SITS effect proceeded, was determined by a curve-fitting to the experimental points at a test voltage of +20 mV in each set of records. This value of $p$ was used in estimating $\tau_n$ for other records in the same set. On
the other hand, the rapidly decaying phase of $I_K$ (tail $I_K$) associated with step hyperpolarizations from a holding level of $-40$ mV was approximated by a single-exponential function.

Two examples of curve-fitting data for the rising phase of $I_K$ before an application of SITS (control) and at the application of 100 $\mu$M SITS are presented in Fig. 4A. In Fig. 4B, values of $\tau_a$ thus estimated for the records taken at various

![Figure 4](image)

**Figure 4.** (A) Two examples of curve-fitting data showing the effect of SITS on the rising phase of $I_K$ at various test voltages given from a prepulse level of $-150$ mV. The data were obtained from an axon internally perfused with 360 K and bathed in 60 K/450 Na at 9.3°C. The circles indicate the experimental points at SITS concentrations of 0 (control) and 100 $\mu$M. Note the difference in time scales. The smooth curves were drawn according to the equation $I_K = I_K[1 - \exp(-t/\tau)]^p$. Values of $\tau_a$ and $p$ are given in the figure. (B) $\tau_a$ as a function of test voltage at various SITS concentrations.

SITS concentrations are plotted against test voltages. It is seen that $\tau_a$ increased at all test voltages, roughly maintaining a proportionality, whereas the slowing of the $g_K$ activation increased as the SITS concentration was raised.

A similar slowing effect of SITS was also recognized in the tail currents, which represent the closing process of K channels (Fig. 5). It is understood from these results that SITS slowed the opening and closing rates of the K channel in the whole voltage region.
Effect on the Steady $g_K$

The effect of SITS on $E_m$ was studied at various external K+ concentrations. In Fig. 6, values of $E_m$ before and after an application of 50 $\mu$M SITS for 3 min are plotted against K+ concentrations of ASW. The SITS effect became more distinct as the K+ concentration was lowered or as $E_m$ became more negative. The K+ sensitivity of the membrane at a normal resting state, which was gained by means of the SITS treatment, is also demonstrated by a trace of $E_m$ in the figure. At higher K+ concentrations (above 50 mM), where the membrane behaved like a K+-sensitive electrode, the SITS treatment did not produce any change in $E_m$.

The effect of SITS on steady $I_K$ was studied in another series of experiments with axons bathed in 240 K/270 Na. The holding potential was set to near $E_m$, which was -11 mV. Currents at a steady state at various test voltages were measured. A linear leakage component at -150 mV was compensated with the analog transient generator (see Methods). SITS was applied internally until the maximal effect on steady $I_K$ was attained; the internal solution was then changed back to the original 360 K.

Fig. 7A illustrates an example of the effect of SITS on the relation of steady $I_K$ to test voltages. Two remarkable changes can be seen: one is an increase in the steady $I_K$, and the other is a large shift in the voltage at which steady $I_K$ went to zero in the negative direction. The magnitude of the shift was 46 mV in this case, i.e., -64 to -110 mV. Because the maximal drug effect was attained by this SITS application, the chord conductances at the linear portion of the I-V curve marked by SITS can be regarded as the maximum steady $g_K$. Furthermore, the inactivation of $g_K$ is completely suppressed at this concentration of SITS, as
will be demonstrated in the following section. This means that the maximum steady $g_k$ is nearly equal to the maximum $g_K$, $g_k$. Therefore, the values of the chord conductance relative to the maximum steady $g_k$ obtained from the curve (SITS) are considered to represent, roughly, the fraction of open K channels. These values are plotted against voltages in Fig. 7B (solid circles). (The open circles are the chord conductances of the control data divided by the same $g_k$.

Figure 6. Trace: time course of $E_m$ associated with switchings of the external solution between K+-free ASW (0-K ASW) and ASW containing 9 mM K⁺ before and after an internal application of 50 μM SITS. The internal solution was 360 K. In the main figure, $E_m$ is plotted as a function of K⁺ concentration of ASW before (control) and after (SITS) an internal application of 50 μM SITS for 5 min. Each symbol represents data taken from a single axon.

presented for comparison. The maximum level is ~0.4. This is mainly due to $g_k$ inactivation and partially to K⁺ accumulation in a periaxonal space. For details, see the next section.) The data well explain why SITS enhanced the K⁺ sensitivity of the membrane at its normal resting potential. For example, the value of $g_k/g_k$ at -60 mV after the SITS modification was 0.32, whereas that before SITS was 0.01. This suggests that ~30% of K channels were open at the normal resting potential of approximately -60 mV when the maximal SITS effect was attained.
It is suggested from the current traces in Fig. 3 that SITS may suppress \( g_K \) inactivation, since an accumulation of K\(^+\) in the space between the outer membrane surface and Schwann cells (periaxonal space), which is thought to bring about a decay of \( I_K \) after the peak, must occur even after the SITS application. In other words, the results in Fig. 3 suggest the existence of \( g_K \) inactivation under such a near-physiological condition, which is sensitive to SITS. This suggestion can be tested by measuring the steady state relation between \( g_K \) and the voltage of the prepulses. Fig. 8 illustrates an example of our results. The pulse procedure employed in this experiment and the current records are given in the figure. As clearly demonstrated by the control data, the steady state inactivation, \( k_{in} \), is a function of voltage. Also, inactivation is incomplete at positive voltages, as expected from the fact that \( I_K \) at any level of depolarization does not decay to zero. A slow component of \( g_K \) inactivation, having a time constant of 10 s (Ehrenstein and Gilbert, 1966; Inoue, 1981), is disregarded here, since the duration of the prepulses was 500 ms. It is also clearly demon-
It is important to note that the inactivation of $g_K$ is strongly dependent on temperature. The inactivation was hardly detectable at low temperature ($<5^\circ$C); $I_K$ decayed only slightly, and the major portion of the slowly decaying $I_K$ was characterized as being due to $K^+$ accumulation in the periaxonal space by studying instantaneous $I$-$V$ relations at the decaying phase. The inactivation became distinct when the temperature was increased above $8^\circ$C, where $I_K$ was seen to decay (Fig. 3). Therefore, the voltage-dependent characteristics of $k_n$, such as those seen in Fig. 8, could be observed only at relatively high temperature and were strongly influenced by temperature.

The records in Fig. 9 display $I_K$ traces that were normalized to their peak value, which was associated with a step depolarization from $-150$ to $40$ mV, obtained after an application of $50$ $\mu$M SITS. The values of the time constants at the decaying phase after the peak, estimated from the records, are plotted against time in minutes after the onset of SITS. The time constant stayed at an almost constant level of $\sim 40$ ms for up to $8$ min after SITS, although the magnitude of decay of $I_K$ decreased by $80\%$; it started to increase at $9$ min and was calculated to be $103$ ms at $11$ min. Since the decay of $I_K$ at $11$ min is
considered to be attributable solely to the K⁺ accumulation in the periaxonal space, the increase in the time constant observed at 9 and 10 min can be interpreted in terms of a greater contribution of the K⁺ accumulation effect to the decay of \( I_K \). In other words, the results suggest that SITS suppressed the inactivation of \( g_K \) without affecting its time constant.

DIDS had effects on \( g_K \) similar to those of SITS. The effects of DIDS were roughly 1.5 times stronger than SITS. Externally applied SITS or DIDS had only a small effect on \( g_K \). For example, externally applied SITS (500 \( \mu \)M) produced a very small amount of slowing of the \( g_K \) activation, which appeared 0.5 h after SITS was added. The resting potential and inactivation of \( g_K \) were not affected by external SITS or DIDS at all.

**Discussion**

Disulfonic stilbene derivatives such as SITS and DIDS possess two negative charges. The interaction of these aminoreactive reagents with sites located on the inner surface of the membrane increases the density of negative fixed charges at the inner membrane layer, which shifts the phase boundary potential between the inner membrane surface and the internal bulk solution in a more negative direction. If this shift in the phase boundary potential influenced the K channel, the voltage-dependent characteristics of \( g_K \) would be expected to shift in the
positive direction along the voltage axis. Contrary to our expectations, the results in Figs. 6 and 7 show that the steady $g_K$ appeared at a voltage much more negative than normal after the SITS application. Furthermore, as shown in Figs. 4 and 5, SITS did not significantly shift the rate constants along the voltage axis, but simply made them slower in the whole voltage range. Therefore, we suggest that the changes in the characteristics of $g_K$ described in this article were brought about by direct interactions of the K channel with the reagents rather than by the surface potential change effects.

The results in Fig. 8 demonstrate that $g_K$ inactivates at relatively high temperature (>8°C). Considering that the squid lives at temperature above 10°C (usually at ~15°C), the inactivation may be involved in physiological functions of the K channel, and thus the kinetics of $g_K$ under the physiological circumstances should be expressed by the following equation, which includes an inactivation term, $k$:

$$g_K = g_{K\text{p}} \theta_k.$$  

$p$ is 4 according to Na theory (Hodgkin and Huxley, 1952) and $k$ involves both the fast and the slow components (Inoue, 1981). SITS blocks at least the fast inactivation.

Although K+ accumulation in the periaxonal space produces a decay of $I_K$, it does not contribute significantly to the real time course of $I_K$, at least in its decaying phase at physiological temperatures. This notion is consistent with the results of Adelman et al. (1973) that a greater part of the K+ accumulation effect appears during the rising phase of $I_K$ than during the decaying phase. It has been confirmed that SITS does not affect $E_K$. This suggests that SITS does not change the ionic selectivity of the K channel.

Sokabe et al. (1983) tested the effect of DIDS on single channel currents of K channel of sarcoplasmic reticulum using a system in which sarcoplasmic reticulum vesicles are incorporated into a planar bilayer by fusion. They observed the following phenomena: (a) the single channel conductance was not affected by DIDS, (b) the open time of the channel was elongated, and (c) a high concentration of DIDS locked the channel in its open state. Their observations suggest that the gates of K channels possess affinity sites for the disulfonic stilbene derivatives.

Finally, it is pointed out that the effects of such disulfonic stilbene derivatives as SITS and DIDS on $g_K$ of the squid axon membrane are somewhat similar to the effects on $g_Na$ of one group of drugs, such as veratridine (cf. Benforado, 1968; Ohta et al., 1973; Ulbricht, 1969), batrachotoxin (cf. Catterall et al., 1981; Huang et al., 1979), and grayanotoxin (cf. Narahashi and Seyama, 1971; Seyama and Narahashi, 1973; Seyama, 1978), which are widely used as Na channel openers. In this connection, it is expected that the disulfonic stilbene derivatives can be used as a tool to open K channels. One advantage of using these drugs, rather than K+ depolarization, to open the K channel may be that uncertainties in measurements originating from other voltage-dependent phenomena can be minimized. On the other hand, one disadvantage of using these drugs may be the blocking of the voltage-dependent Cl channel (Inoue, 1985).
This work was supported by a grant-in-aid for Special Research on Molecular Mechanism of Bioelectrical Response (60115007) from the Japanese Ministry of Education, Science and Culture.

Original version received 13 November 1985 and accepted version received 19 May 1986.

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