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SITS Blockade Induces Multiple Subconductance States in a Large Conductance Chloride Channel

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Abstract. The effect of the chloride channel blocker 4-acetamido-4-isothiocyanatostilbene-2,2-disulfonic acid (SITS) on the gating and amplitude of an endothelial chloride channel was explored using the outside-out configuration of the patch-clamp technique.

Under control conditions the channel displayed two main gating modes: shut and fully open. Transitions to equally spaced subconductance states were rarely observed (less than 10 events/minute).

At low concentrations (<45 μM), SITS increased the number of transitions to the three subconductance states in a concentration-dependent manner, while reducing the number of transitions to the fully open state. This effect was maintained after removing SITS from the bath solution, suggesting that the modifications in the channel induced by SITS were irreversible. All four conducting states had similar current-voltage relationships.

At higher concentrations (>45 μM), SITS reduced the amplitude of all conducting states (three subconductances and fully open). This effect was fully reversible upon SITS removal from the bath solution. A half-inhibitory concentration (IC_{50}) of 55.6 ± 2.7 μM (+60 mV) and 66.7 ± 2.2 (−60 mV) was obtained from the fitting to a Langmuir function.

All these results are compatible with the existence of two SITS binding sites in the chloride channel: one of high affinity responsible for the increment in the number of transitions to subconductance states, and one low affinity binding site involved in the reduction of the amplitude of all conducting states.

Key words: SITS — Chloride channel — Subconductance states — Endothelial cells

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Introduction

The gating behavior observed in most of the ionic channels studied thus far involves a binary switch between two states: open and shut. However, some channels deviate from this pattern showing complex transitions to conducting states that are smaller than the fully open state (subconductance states).

Subconductance states are defined as the transitions of a single channel to amplitudes different from the fully open state. The mechanisms by which ionic channels can display subconductance states remain unsolved (Fox, 1987; Laver & Gage, 1997). However, in some channels the subconductance states can be induced by several experimental maneuvers such as changes in pH (Zheng *et al.,* 1993), voltage (Kazachenko & Geletyuk, 1984) or the use of channel blockers (Schild, Ravindran & Moczydlowski, 1991).

The fact that subconductance states can be induced by these experimental maneuvers brings the opportunity of studying the mechanisms and possible modifications that the single channel may undergo in order to display several conducting states.

In the present study, the chloride channel blocker SITS was utilized to affect the gating and amplitude of a chloride channel, resulting in the appearance of four, equally spaced subconductance states that were rarely observed in the channel not exposed to SITS.

The results presented here indicate that SITS favors the appearance of subconductance states by interacting with a high affinity site in the channel. This interaction is poorly reversible suggesting that SITS, like other disulfonic stilbene derivatives, may be interacting with amino groups in the channel sequence. Higher SITS concentrations reduced the amplitude of all conducting states in a concentration-dependent manner. The reduction in the amplitude of all conducting states was fitted

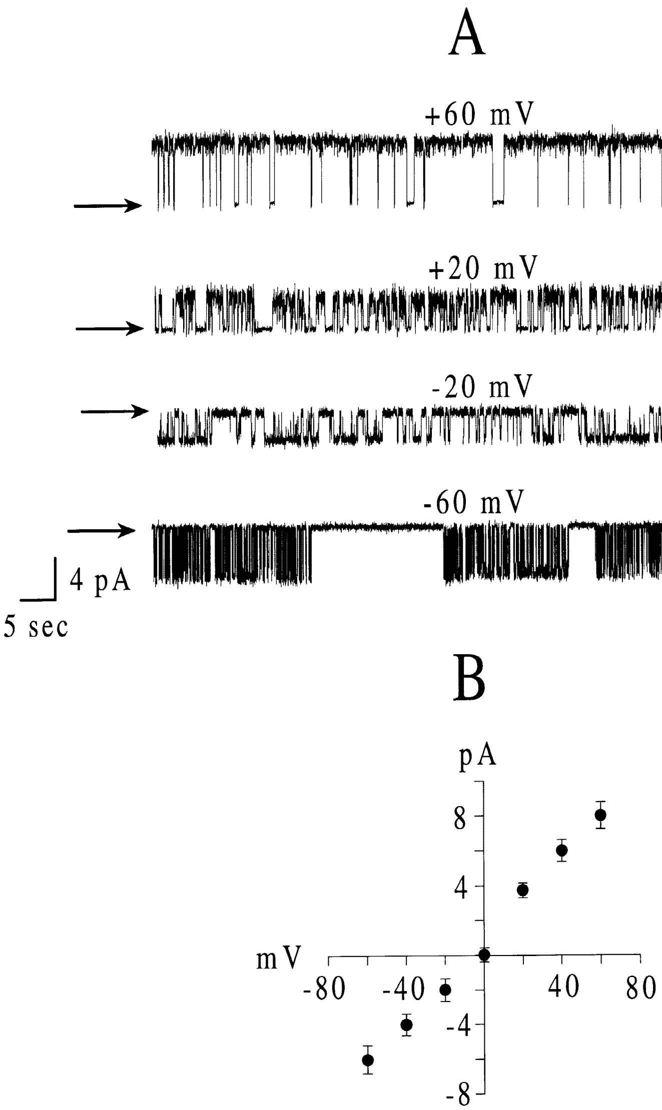

Fig. 1. A large conductance chloride channel from vascular endothelium. (Panel *A*) Representative single channel recordings obtained at the voltages of +60, −60, +20 and −20 mV. Horizontal arrows point to the shut state (zero current level). Channel activity filtered at 3 kHz for illustrative purposes. The scale shows 5 seconds and 4 pA. (Panel *B*) Current-voltage relationship obtained from 7 independent single channel experiments. Symbols represent the mean ± SD.

by a Langmuir function from which an apparent half-inhibitory concentration was obtained.

This interaction was fully reversible after removing SITS from the bath solution. The effect obtained with higher SITS concentrations suggests the presence of a low affinity site, which affects single channel permeation, probably by interacting with amino acids near the pore.

Materials and Methods

Reagents and Solutions

All salts used in this study and 4-acetamido-4-isothiocyanatostilbene-2,2-disulfonic acid (SITS) were purchased from Sigma Chemical (St. Louis, MO). The Bath (extracellular) and pipette (intracellular) solutions contained 100 mM N-methyl-D-Glucamine, 50 mM mannitol and 20 mM N-[2-hydroxuethyl]-piperazine-N′-[2-ethanelsulfonic acid] (HEPES, Sigma Chemical). The pH was adjusted to 7.2 with HCl.

Cell Culture

Bovine aortic endothelial cells (BAEC) were purchased from the American Type Culture Collection (ATCC) and maintained in culture at 37°C in a humidity controlled incubator with 5% CO_2. The media used is Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin. When monolayers reached confluency the cells were mechanically dispersed with a plastic pipette. Cells were then placed on 35 mm Petri dishes and mounted on the stage of an inverted microscope (NIKON). Only passages 5–15 were used for the experiments to be described.

Single Channel Recording

The outside-out configuration of the patch-clamp technique (Hamill *et al.,* 1981) was utilized to study single chloride channels obtained from

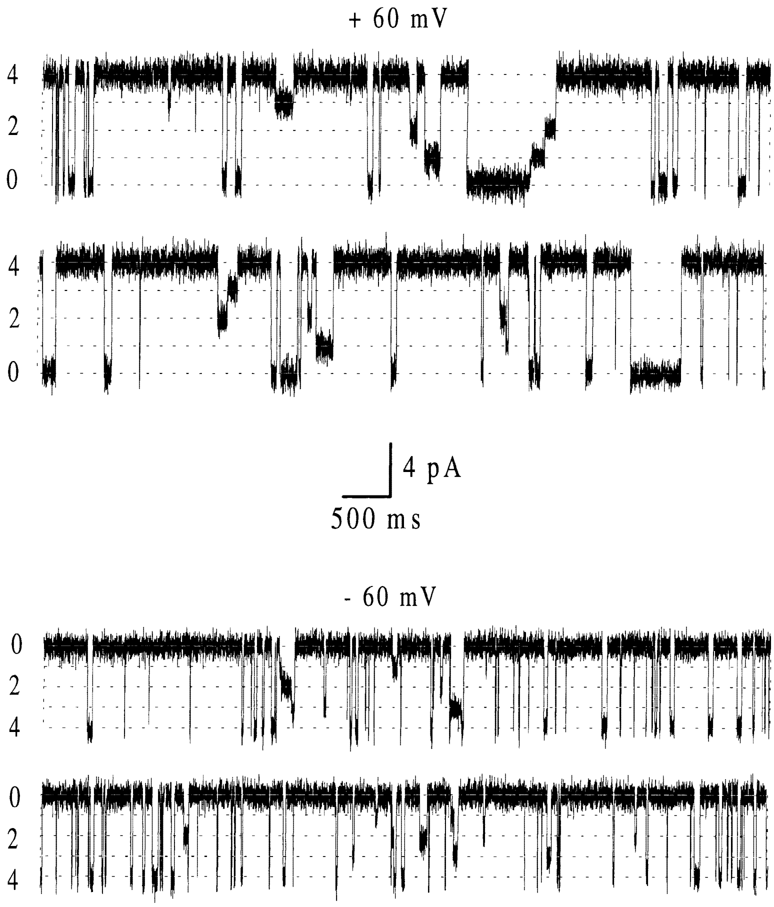

Fig. 2. The chloride channel displays spontaneous subconductance states. Representative examples of single channel activity obtained at +60 and −60 mV showing the scarce, spontaneous transitions to subconductance states. The data were filtered at 5 kHz and digitized at 10 kHz. Channel activity filtered at 3 kHz for illustrative purposes. Dotted lines indicate the different amplitudes and are also numbered accordingly. At +60 mV upward deflections indicate channel openings, the opposite for −60 mV. The scale shows 500 msec and 4 pA.

single of bovine aortic endothelial cells (BAEC). Patch pipettes were made of 7052 or 8161 glass (Garner Glass), pulled and fire polished to obtain pipette resistances in the range of 6–8 MΩ. The reference electrode was an Ag-AgCl plug connected to the bath solution via a 150 mM KCl agar bridge. The amplifier was the Axopatch 200A (Axon Instruments, Foster City, CA) connected to the Digidata 1200 analog-digital interface (Axon Instruments). Single channel recordings were initially stored on tape using a VCR (Vetter Instruments, CA). Selected traces of single channel data were digitized on a pentium computer for analysis. Data were filtered at 5 kHz with an 8-pole Bessel filter (Frequency Devices) and digitized at 10 kHz. A Picospritzer II (General Valve) was utilized to deliver SITS to the patch pipette. In all the experiments to be described n = number of experiments performed for each condition.

Data Analysis

Single channel recordings were analyzed using E.P. Professional software developed by B. Steiner and L. Vaca (Department of Molecular Physiology and Biophysics, Baylor College of Medicine). The algorithm first performs total point amplitude histograms from which the user can select amplitude levels. The amplitude for each level is obtained from a nonlinear Gaussian fit of the all points amplitude histograms. Transitions to and from any given amplitude level follow the half amplitude criterion.

Channel analysis was obtained from 2–3 min of continuous single channel recordings. Patches of membrane with more than one channel were discarded from the analysis. The presence of a single channel was verified in control conditions before the addition of SITS. Under these conditions the channel displayed only two gating modes: fully open and shut, subconductance states were rarely observed (less than 10 events/min). Under these conditions it was possible to determine if more than one channel was present in the membrane patch.

SigmaPlot (Jandel) was utilized to fit the channel amplitudes at different SITS concentrations to a Langmuir function of the type:

$$Y = Ymax/(1 + IC_{50}/[SITS])$$

Where Y = channel subconductance state amplitude, $Ymax$ = asymptotic value of the curve, [SITS] = the SITS concentration tested for each experiment and IC_{50} = half-inhibitory concentration.

Results

A Large Conductance Chloride Channel with Two Gating Modes: Fully Open and Shut

We have previously reported the presence of a large conductance, voltage-gated chloride channel from bovine aortic endothelial cells (BAEC) which is modulated by the cAMP-dependent protein kinase (Vaca & Kunze, 1993). The channel is blocked by micromolar concen-

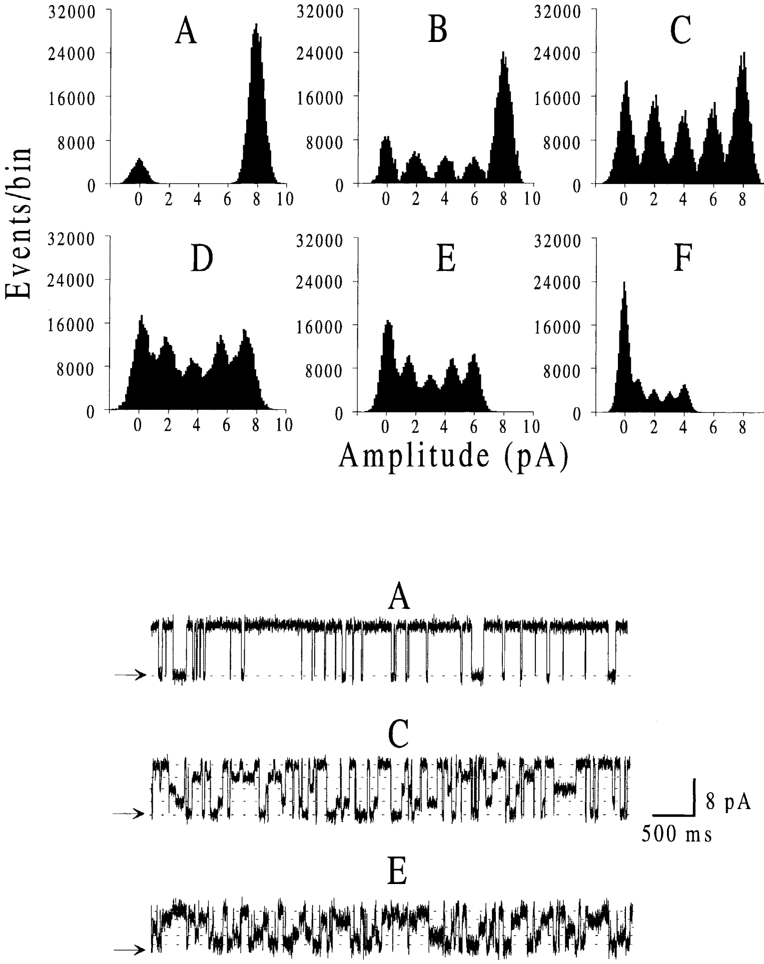

Fig. 3. Effect of SITS on the subconductance states at +60 mV. (Upper panel) Total points amplitude histograms from 50–60 sec of continuous channel activity in (*A*) control conditions and (*B*) 15 μM, (*C*) 30 μM, (*D*) 45 μM, (*E*) 60 μM and (*F*) 75 μM SITS. The binwidth is 0.1 pA. (Lower panel) Representative records with single channel activity under (*A*) control conditions, (*C*) 30 μM and (*E*) 60 μM SITS. Channel activity filtered at 3 kHz for illustrative purposes. The vertical arrows point to the shut state (zero current level). Dotted lines indicate the different amplitudes. The scale shows 500 msec and 8 pA. The holding potential was +60 mV.

trations of SITS (Vaca & Kunze, 1993). The present study characterizes the effects of SITS on channel gating and amplitude, utilizing the outside-out configuration of the patch-clamp technique.

Figure 1 illustrates the gating of a single chloride channel under control conditions (in the absence of SITS). Figure 1*A* shows representative recordings obtained at four different voltages. Figure 1*B* illustrates the current-voltage relationships obtained from 7 independent experiments like those shown in Fig. 1*A*.

Under these conditions the channel displayed one main conducting mode: the fully open state. However, spontaneous transitions to subconductance states were rarely observed. Figure 2 illustrates selective recordings obtained at two voltages in which transitions to subconductance states were present. Transitions to four equally spaced amplitudes were rarely observed (Fig. 2). These transitions were infrequent (less than 10 events/min) and occurred in bursts separated by several minutes of channel activity with transitions between the fully open and the shut state.

Voltage did not appear to influence the frequency or time of appearance of the subconductance states (range tested −80 to +80 mV), however this was difficult to evaluate due to the low occurrence of such events.

The Frequency of Subconductance States is Increased by SITS

Exposing the channel to several concentrations of SITS significantly increased the number of transitions to subconductance states (Fig. 3). When explored at +60 mV, the appearance of subconductance states was observed with SITS concentrations of 15 μM and higher (Fig. 3*B*–*F*). This effect was observed between 20–30 sec after applying SITS to the outside surface of the channel. Concentrations of less than 10 μM had no measurable effect on the channel, at least, for the duration of the experiments (2–3 minutes of continuous recording; *data not shown*).

From the number of subconductance events observed in the total points amplitude histograms, it appears that increasing the SITS concentration from 15 μM

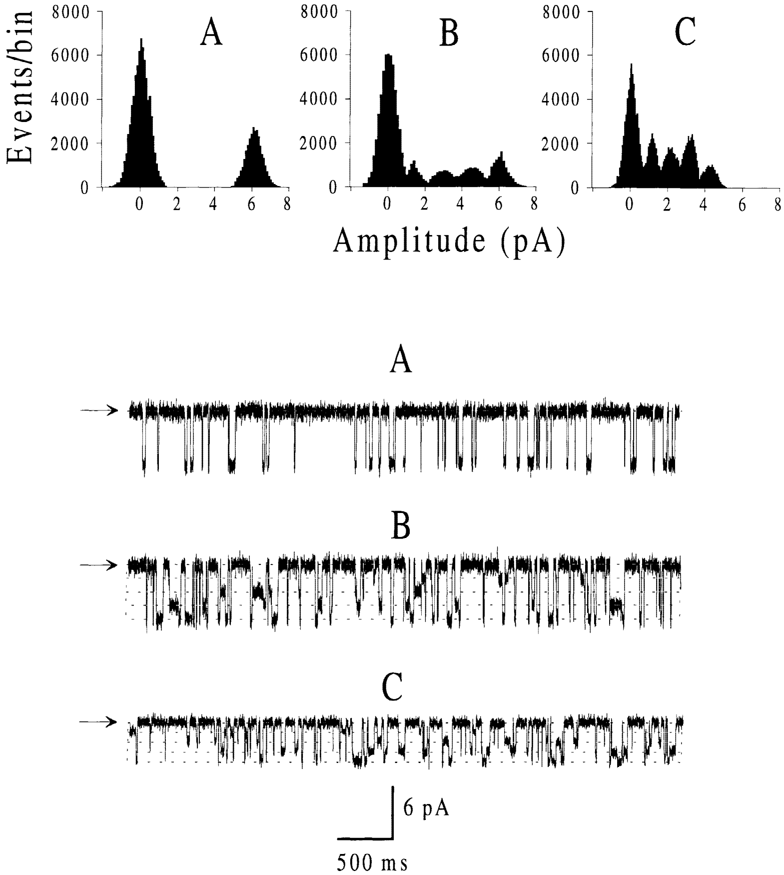

Fig. 4. Effect of SITS on the subconductance states at −60 mV. (Upper panel) Total points amplitude histograms from 50–60 sec of continuous channel activity in (*A*) control, (*B*) 30 μM and (*C*) 60 μM. The binwidth is 0.1 pA. (Lower panel) Examples of channel activity obtained under the conditions indicated by the labels (control, 30 μM SITS and 60 μM SITS). Channel activity filtered at 3 kHz for illustrative purposes except for (*C*) where channel activity was filtered at 1 kHz. The vertical arrows point to the shut state (zero current level). Dotted lines indicate the different amplitudes. The scale shows 500 msec and 6 pA. The holding potential was −60 mV.

(Fig. 3*B*) to 30 μM (Fig. 3*C*) increased also the number of transitions to three subconductance states while reducing the number of transitions to the fully open state.

Increasing the concentration of SITS above 30 μM resulted in the gradual reduction of the amplitude of all conducting states (Fig. 3*D–F*). With 75 μM SITS the amplitude of all conducting states was reduced by 50 ± 5% (Fig. 2*F*) as compared to the amplitudes obtained at 15 μM SITS (Fig. 2*B*). At SITS concentrations of 60 μM or higher, the number of transitions to the shut state was significantly increased (compare Fig. 3*E* and *F,* zero current level).

SITS induced 4 equally spaced subconductance states at both positive and negative potentials. Figure 4 illustrates the effect of two SITS concentrations on the total points amplitude histograms of a single chloride channel obtained at −60 mV. Comparing the number of events obtained with 30 μM SITS (Fig. 4*B*) with those obtained with 60 μM SITS (Fig. 4*C*) indicates that increasing the SITS concentration augmented the number of transitions to subconductance states. At negative potentials, increasing SITS above 30 μM also reduced the amplitude of all conducting states (compare Fig. 4*B* and *C*).

As illustrated in Fig. 5, SITS increased in a concentration-dependent manner the number of transitions to the three subconductance states while reducing the transitions to the fully open state. This effect was evident at both negative (−60) and positive (+60) potentials.

These results strongly suggest the presence of two sites of action for SITS, one of high affinity responsible for the appearance of the subconductance states, and one of low affinity involved in the reduction of the amplitude of all conducting states. Therefore, these results are compatible with two types of blockade: one slow (induction of subconductance states) and one fast (fast flickering block responsible for the reduction of the subconductance states amplitudes).

All conducting states obtained at two different SITS concentrations showed linear current-voltage relationships (Fig. 6*A–B*) similar to the current-voltage relationship obtained with the channel under control conditions (not exposed to SITS, Fig. 1*B*).

Effect of SITS on the Amplitude of the Subconductance States

High SITS concentrations resulted in the reduction of the amplitude of all conducting states as illustrated in the total points amplitude histograms of Figs. 3 and 4.

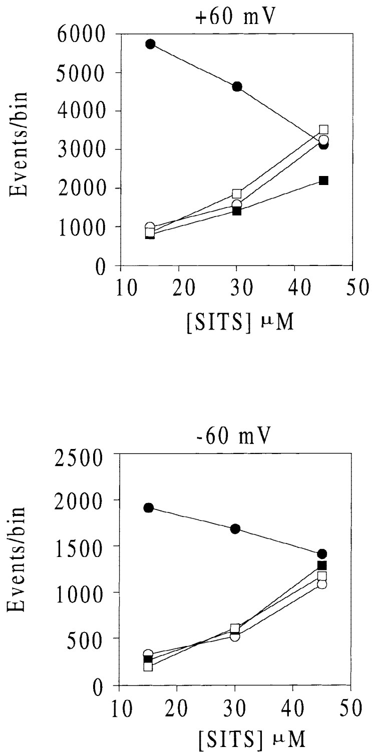

Fig. 5. SITS increases the number of transitions to subconductance states. Representative experiment showing the amplitudes of the three subconductance states and the fully open state as a function of the SITS concentration obtained at +60 mV (Upper panel) and −60 mV (Lower panel) for the fully open (filled circles), the third (open circles), second (filled squares) and first (open squares) subconductance amplitudes. The subconductance states amplitudes are ordered in decreasing order. Amplitudes obtained after fitting to Gaussian functions the total points amplitude histograms like those shown in Figs. 3 and 4. Histograms obtained from 40 sec of continuous single channel activity.

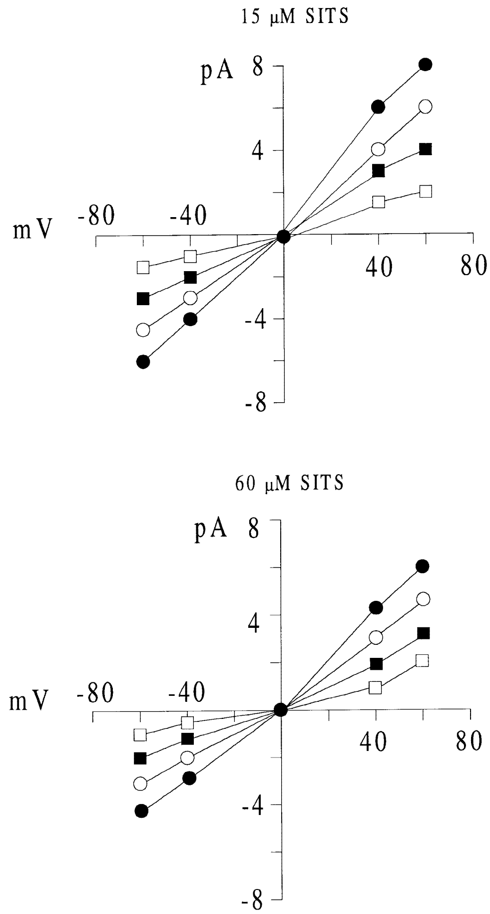

Fig. 6. Current-voltage relationships obtained from the subconductance states. Representative current-voltage relationships for the 4 conducting states (fully open and three subconductance states) obtained with 15 μM SITS (Upper panel) and 30 μM SITS (Lower panel). The data points were obtained after fitting the total points amplitude histograms like those shown on Figs. 3 and 4 to Gaussian functions. Each data point is the mean value for each Gaussian. Filled circles represent the fully open amplitude. The lines are used only to connect the data points.

The reduction in the amplitude of all conducting states was well fitted by a Langmuir function yielding half-inhibitory concentrations of 55.6 ± 2.7 and 66.7 ± 2.2 μM for +60 and −60 mV, respectively (Fig. 7). These results indicate that the effect of high SITS concentrations on the amplitude of all conducting states is slightly voltage dependent.

Reversibility and Irreversibility of the Effects of SITS on Channel Amplitude and Gating

To determine if the effects of SITS on amplitude and gating are reversible, experiments were performed in which several SITS concentrations were tested on the channel and later removed from the bath solution. Figure 8 illustrates one of such experiments. A concentration of 60 μM SITS induced a typical response consisting in appearance of 4 equally spaced subconductance states with amplitudes of about half of what can be obtained with 15 μM SITS. Replacing the bath solution with SITS-free media resulted in the increment in the amplitude of all conducting states. However, removing SITS from the bath solution did not restore the channel to the control condition (only one conducting state, the fully open state). The four subconductance states remained even after replacing the bath solution with 10 times its original volume, suggesting that the modification on the channel induced by SITS was poorly reversible.

These results indicate that the effect of SITS on the amplitude of the subconductance states is fully reversible upon removal of this substance from the bath solution, however, the modifications produced by SITS resulting in the appearance of the subconductance states cannot be reverted.

All these results strongly suggest the presence of a

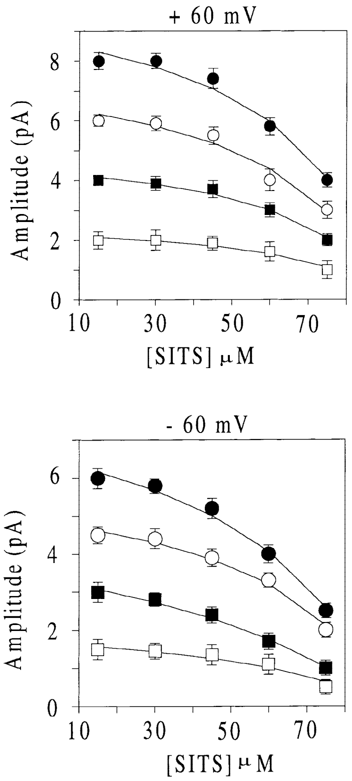

Fig. 7. Effect of SITS on the amplitude of all conducting states. Mean ± SD of the subconductance states amplitudes obtained with 15, 30, 45, 60 and 75 μM SITS at a holding potential of +60 mV (Upper panel) and −60 mV (Lower panel). All values were obtained after fitting the total points amplitude histograms to Gaussian functions. Each data point represents 7 independent observations. The solid lines represent the best fit to a Langmuir function (Materials and Methods). The IC_{50} obtained at +60 mV are 56.39 (filled circles), 58.99 (open circles), 54.39 (filled squares) and 52.71 (open squares) mean ± SD = 55.6 ± 2.7. The IC_{50} obtained at −60 mV are 64.42 (filled circles), 66.46 (open circles), 69.81 (filled squares), 66.30 (open squares) mean ± SD = 66.7 ± 2.2.

high affinity site for SITS responsible for inducing multiple subconductance states on this chloride channel, these channel modifications appear to be irreversible. A second, low affinity site for SITS may be responsible for the reduction in the amplitude of all conducting states; this effect is fully reversible after removing SITS from the bath solution.

Disulfonic stilbene derivatives (such as SITS) are amino reagents, thus, one possible explanation for these results is that SITS may be interacting with amino groups in the channel which results in modifications that favor the appearance of the subconductance states.

Discussion

The results obtained in the present study illustrate the effect of SITS on the amplitude and gating of a large conductance endothelial chloride channel. Previous studies have shown that this channel is activated upon β-adrenergic stimulation of this endothelium, which results in phosphorylation of the channel and a change in its voltage dependence (Vaca & Kunze, 1993).

SITS Produced Complex Changes in Channel Gating and Amplitude

In the absence of SITS, transitions to subconductance states were rarely observed at all the voltages explored (less than 10 events/minute). Under this condition, the channel displayed two main states: shut and fully open at both depolarizing and hyperpolarizing potentials. The scarce spontaneous transitions to subconductance states observed under these circumstances suggest that the channel is capable of going into these subconductance modes, but they might not be favorable or stable. The addition of SITS may stabilize these subconductance modes increasing the probability of occurrence, favoring in this way its detection and analysis.

In fact, SITS increased in a concentration-dependent manner, the amplitude of the subconductance states in the total points amplitude histograms (Figs. 3–5). SITS reduced the number of transitions to the fully open amplitude (Fig. 5).

SITS affected the channel amplitude in two ways: at low concentrations (<45 μM) SITS induced the appearance of 3 equally spaced amplitudes in addition to the fully open amplitude. SITS concentrations of 60 μM and higher reduced the amplitude of all conducting states (fully open and subconductance states) to a point where, at SITS concentrations of 70 μM or higher, it was difficult to identify all the different amplitudes.

These results are compatible with two sites of action for SITS: a high affinity site (in the low μM range) is responsible for increasing the number of transitions to subconductance states, a low affinity site (in the high μM range) is involved in the reduction of the amplitude of all conducting states. SITS appears to bind irreversibly to the high affinity site, while the binding to the low affinity site appears to be reversible, based on the washout experiments performed (Fig. 8).

The Blockade of Some Channels Induces Subconductance States

The present report is not the first case in the literature of a channel blocker that favors the appearance of subconductance states. Previously published studies have shown that in cardiac sodium channels, zinc blockade induces a subconductance state at both negative and positive potentials (Schild *et al.*, 1991). In this study, a linear relationship between reciprocal fully open state lifetime and zinc concentration was observed. In the study presented

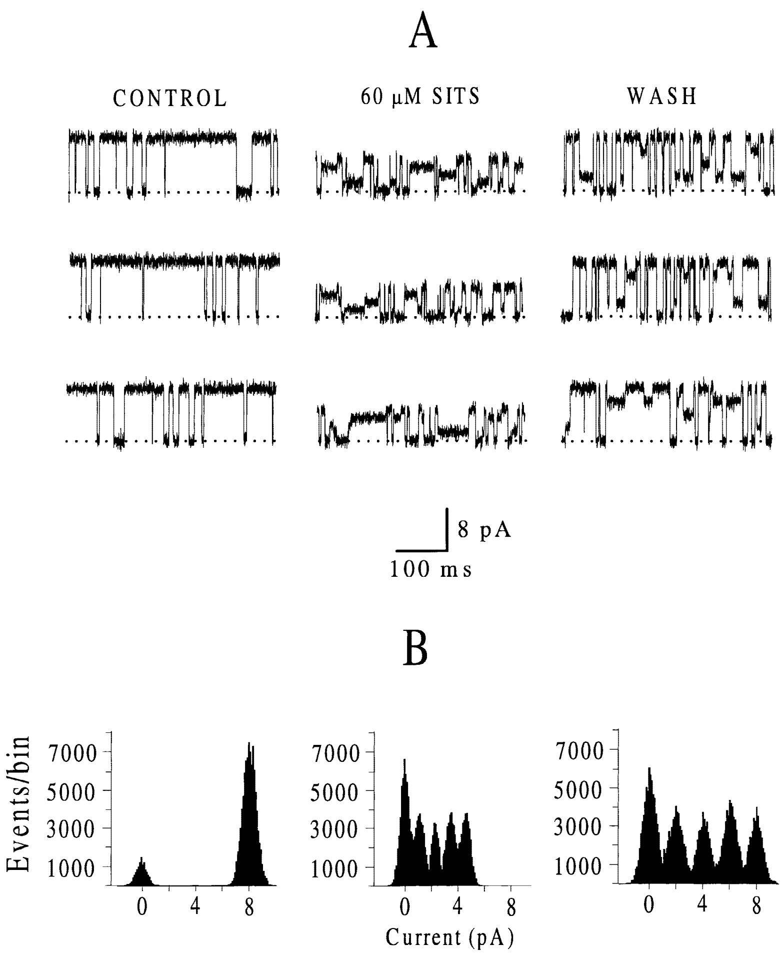

Fig. 8. Reversibility of the effect of SITS on channel amplitude. (Panel *A*) Representative examples of channel activity obtained under control conditions, after applying 60 μM SITS and 3 min after perfusing continuously the patch of membrane with SITS-free solution (wash). The data were filtered at 5 kHz and digitized at 10 kHz, except for the SITS panel where single channel activity was filtered at 3 kHz and digitized at 6 kHz. The scale shows 100 msec and 8 pA. (Panel *B*) Total points amplitude histograms obtained from the single channel activity illustrated in Panel *A*. The binwidth is 0.1 pA.

here, low concentrations of SITS increased the number of openings to subconductance states.

Other experimental maneuvers can induce the appearance of subconductance states in a wide variety of channels. For instance, cytoplasmic acidosis induces multiple subconductance states in ATP-sensitive potassium channels (Zheng *et al.,* 1993). In this study, pH of 5 or less induced two subconductance states, in addition to the fully open state. In a different study, cAMP induces a subconductance state in a dopamine-modulated potassium channel from striatal neurons (Grief, Lin & Freedman, 1995). The mechanisms by which these experimental maneuvers can induce subconductance states are not fully understood yet and have been extensively discussed elsewhere (Laver & Gage, 1997).

A Multi-barreled Chloride Channel?

Two main models have been extensively used to explain the existence of subconductance states in ionic channels (Berry & Edmonds, 1993; Laver & Gage, 1997). The series model postulates the presence of a single conducting path in the channel, which may undergo different conformational transitions, each one responsible for one subconductance amplitude. On the other hand, the parallel model postulates a multi-barreled channel where each barrel is responsible for a subconductance level.

Coupling of pores has been invoked in particular to explain equally spaced subconductance states in a wide variety of cation and anion channels (Miller, 1982; Kazachenko & Geletyuk, 1984; Krouse, Schneider & Gage, 1986; Hunter & Giebisch, 1987; Vaca & Kunze, 1992).

The best-characterized chloride channel formed of multiple pores is the CLC-0 from the *Torpedo* electric organ (Miller, 1982). In a recent study, the independence of the fast gating mode of the doubled-barreled CLC-0 channel was investigated (Ludewig *et al.,* 1997). A correlation analysis indicates that the gating mechanism operates independently on each pore. This type of analysis was possible thanks to the use of mutant channels with smaller conductances. By mixing mutants and wild-type channels to form doubled-barreled chimeras it was possible to identify each pore by its conductance. Unfortunately such analysis could not be performed on the endothelial chloride channel, since this channel has not been cloned thus far; therefore, identifying each subconductance state is not possible at the present time.

In the present study, all the subconductance states induced by SITS showed similar voltage-dependence and current-voltage relationships. Furthermore, increasing the SITS concentrations affected all the conducting states in a similar way while preserving their voltage-dependence and gating properties.

Although these results are not sufficient to clearly distinguish between a series and a parallel model, the accumulation of experimental evidence in combination with molecular biology techniques may help to elucidate in the future, the mechanisms by which some channels display subconducting states.

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