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Stilbene Disulfonates Block ATP-sensitive K^+ Channels in Guinea Pig Ventricular Myocytes

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Abstract. Effects of stilbene disulfonates on single K_{ATP} channel currents were investigated in inside-out and outside-out membrane patches from guinea pig ventricular myocytes. All drugs tested, 4,4′-diisothiocyanatostilbene,2,2′-disulfonic acid (DIDS), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), and 4,4′-diaminostilbene-2,2′-disulfonic acid (DADS), inhibited the K_{ATP} channel when they were applied to the intracellular, but not extracellular side of the membrane patch. Inhibitory actions of DIDS and SITS were irreversible, whereas those induced by DNDS and DADS were reversible. K_{ATP} channel inhibition was concentration dependent with an order of potency of DIDS > SITS ≈ DNDS > DADS; the Hill coefficient was close to unity for each drug. No change in channel conductance was observed during exposure to DIDS or DNDS; however, channel kinetics was altered. Distribution of the open time within bursts and that between bursts could be described by a single exponential relation in the absence and presence of DIDS or DNDS. The time constant of the open time within bursts was not altered, but that between bursts was decreased by DIDS (from 40.0 ± 8.1 to 29.8 ± 6.7 msec, $P < 0.05$) and by DNDS (from 43.1 ± 9.3 to 31.9 ± 7.1 msec, $P < 0.05$). Distributions of closed time within bursts were also fitted to a single exponential function both in the absence and presence of drugs, while those of the closed time between bursts were fitted to a single exponential function in the absence of drugs, but a double exponential function was required in the presence of drugs. The rates of onset and development of channel inhibition by DIDS and DNDS appeared to be concentration dependent; a longer time was required to reach a new steady-state of channel activity as drug concentration was decreased. Inhibition by DIDS or DNDS was regulated by intracellular pH; inhibition was greater during acidic conditions. For DIDS (0.1 mM), the open probability (P_o) expressed as a fraction of the value before drug application was 42.9 ± 8.3% at pH 7.4 and 8.2 ± 6.6% at pH 6.5 ($P < 0.01$); corresponding values for DNDS (1 mM) were 39.6 ± 17.6 and 8.9 ± 5.8%, respectively ($P < 0.01$). From these data, we conclude that stilbene disulfonates block the K_{ATP} channel by binding to their target site with one-to-one stoichiometry. Similar to glibenclamide, the binding of stilbene disulfonates may reflect interpolation in an "intermediate lipid compartment" between the cytosolic drug and the site of drug action.

Key words: K_{ATP} channel — Stilbene disulfonate — Patch clamp — Ventricular myocyte

Introduction

Potassium channels modulated by changes in intracellular ATP concentration (K_{ATP} channels) have been described in a variety of cell types including cardiac (Noma, 1983; Trube & Hescheler, 1984) and skeletal muscle (Spruce, Standen & Stanfield, 1985), pancreatic β-cells (Cook & Hales, 1984; Rorsman

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& Trube, 1985), neurons (Ashford et al., 1988), vascular smooth muscle (Standen et al., 1989), and epithelium (Wang, Schwab & Giebisch, 1990). Because of its wide distribution in various tissues and possible physiological importance (Ashcroft, 1988), substantial efforts have been made to clarify the pharmacological modulation of this channel to understand its functional structure and to improve strategy for pharmacological treatment of various diseases. Activation of the K_{ATP} channel with "K^+ channel openers," such as cromakalim, pinacidil, and nicorandil, may hyperpolarize the membrane and decrease tonic tension in smooth muscle, thereby ameliorating hypertension, angina pectoris, or bronchial asthma (Cook, 1988; Edwards & Weston, 1990). In cardiac muscle, opening of the K_{ATP} channel during ischemia results in decreased irreversible cell death, enhancement of the recovery of contractility, and diminution in arrhythmia after re-establishment of coronary artery flow. However, shortening of the transmembrane action potential due to an increase in the membrane K^+ conductance during ischemia may favor occurrence of re-entrant arrhythmia. Thus, agents which inhibit the K_{ATP} channel, such as sulfonylureas, may be useful in treating ischemia-related arrhythmias in the heart (Wolleben, Sanguinetti & Siegel, 1989; Kantor et al., 1990).

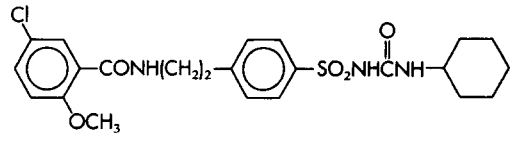
Stilbene disulfonates have been widely used as an anion transport inhibitor and an anion channel blocker (Maddy, 1964; Frölich, 1982; Woll et al., 1987; Kokubun, Saigusa & 1991; Groschner & Kukovetz, 1992). These agents have properties similar to those of sulfonylureas insofar as chemical structure; both drug groups have benzene ring(s) and sulfonic residue(s) (Fig. 1) (Cabantchik, Knauf & Rothstein, 1978). Thus, we designed this study to explore whether or not stilbene disulfonates interact with the K_{ATP} channel and, if so, to characterize the interaction and improve understanding of the pharmacological modulation and functional structure of this channel. Indeed, we found that stilbene disulfonates block the cardiac K_{ATP} channel, and kinetic analyses showed that the mode of the K_{ATP} channel inhibition by these agents is different from their actions on anion channels, but similar in some ways to K_{ATP} channel inhibition by the sulfonylurea, glibenclamide.

Materials and Methods

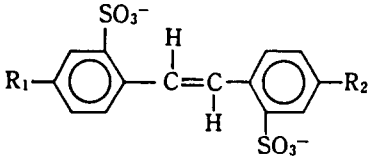
Cell Preparation

Single myocytes were isolated enzymatically from guinea-pig ventricles, as described previously (Furukawa et al., 1993).

glibenclamide



disulfonic stilbene derivatives



R_1	R_2	Name
−NCS	−NCS	DIDS
−NH·CO·CH_3	−NCS	SITS
$-NO_2$	$-NO_2$	DNDS
$-NH_2$	$-NH_2$	DADS

Fig. 1. Chemical structures of sulfonylurea drug glibenclamide (upper panel) and stilbene disulfonates, DIDS, SITS, DNDS, and DADS.

Solutions

In inside-out patch-clamp experiments, the bathing solution (artificial intracellular medium, ATP-free) contained (mM): KCl 142; *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 5; ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) 5; glucose 5.5; pH was adjusted to 7.4 with KOH. Concentration of free Ca^{2+} in the bathing solution was estimated as 1×10^{-10} M and that of free Mg^{2+} was 1×10^{-6} M from the apparent dissociation constants (Fabiato & Fabiato, 1979). When bathing solution of pH 6.5 was used, HEPES was replaced with an equimolar (5 mM) piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES). The composition of the pipette solution (extracellular medium) was (mM): KCl 142, $CaCl_2$ 1.8, $MgCl_2$ 0.53, HEPES 5, glucose, 5.5; pH was adjusted to 7.4 with KOH. In outside-out patch-clamp experiments, the compositions of the bathing and pipette solutions were the reverse of those used in inside-out patch-clamp experiments.

All stilbene disulfonic compounds tested, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS; Sigma, St. Louis, MO) 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS; Sigma), 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS; Wako Chemical, Osaka, Japan), and 4,4'-diaminostilbene-2,2'-disulfonic acid (DADS; Wako Chemical) were prepared daily at concentrations indicated in the text. The drugs were dissolved in bath solution and protected from light with aluminum foil and used within three hours. ATP (magnesium salt; Sigma) was dissolved in the bath solution at a concentration of 50 μM on each experimental day, and was also used within three hours to minimize possible deterioration. The bath medium was replaced completely within 30 sec when solutions were changed. All experiments were done at a room temperature of 22–24°C.

Recording Methods

Recordings of the single channel current were made by using a patch-clamp amplifier (Axopatch-1C, Axon Instruments, Burlingame, CA). The current signals were amplified to 0.2 V/pA and stored on a video cassette recorder (HR-S 7000, Victor, Tokyo, Japan) via a PCM converter system (RP-882, NF Instruments, Yokohama, Japan) at a conversion rate of 40 kHz. The recorded signals were filtered off-line through an 8-pole Bessel low-pass filter (48 dB/octave, FV-665, NF Inst.) at a −3 dB frequency (f_c) and digitized at 1–10 kHz onto the disk of a personal computer (IBM-PC/AT) using an analog-to-digital converter (CED 502, Cambridge Electronic Design, Cambridge, UK), or recorded through an omnicorder (model 8M14, NEC San-ei Instruments, Tokyo, Japan).

Data Analysis

A "50% threshold" criterion was used to detect events with the help of manual confirmation. The open probability (P_o) was calculated using the equation:

$$P_o = \left(\sum_{j=1}^{N} t_i j\right)/(T_d N) \quad (1)$$

where t_i is the time spent at current levels corresponding to j = 0, 1, 2, . . . , N channels in the open state. T_d is the duration of the recording and N is the number of channels active in the patch. The unitary current amplitude of the K_{ATP} channel was measured from a histogram formed by using all-points amplitude. Thereafter, the histogram was expressed by a sum of several Gaussian distributions with mean and variance (Bhattacharya 1967). The difference of the means of two adjacent Gaussian peaks was taken as a measure of the unitary current amplitude. Open and closed times were measured from records where only a single K_{ATP} channel was activated. The distribution of open time was obtained by measuring the lifetime of open events within bursts and between bursts separately (Fan, Nakayama & Hiraoka, 1990). Measurement of the open time within bursts was made from continuous recordings lasting more than 60 sec and the cut-off frequency of the filter (f_c) was set at 10 kHz to increase the resolution, whereas measurements of the open time between bursts was made from continuous recordings lasting more than 5 min and the f_c was set at 0.1 kHz to decrease the errors in detection caused by flickering. The distribution of closed time was obtained in a similar way. A simplex method of least-squares analysis (Nelder & Mead, 1965) was applied to fit a probability density function to open or closed times with a form of single or double exponential functions.

Data are presented as mean ± SD. Student's paired or unpaired t-test was used to calculate statistical significance, where appropriate. A P value less than 0.05 was considered significant.

Results

Inhibition of the K_{ATP} Channel by Stilbene Disulfonates

Figure 2 shows representative recordings demonstrating the effects of various stilbene disulfonates (1 mM) on K_{ATP} channels in inside-out membrane patches; membrane potential was set at −60 mV. Addition of DIDS (trace *a*), SITS (trace *b*), DNDS (trace *c*), and DADS (trace *d*) to the bath (internal surface) solution inhibited channel openings (Fig. 2). For DIDS and SITS, the inhibitory effect was irreversible after the drug was removed from the bath solution, whereas the inhibitory effect by DNDS and DADS appeared to be, at least partially, reversible by 20 min washout.

Inhibition of K_{ATP} channels by intracellularly applied stilbene disulfonates was concentration dependent (Fig. 3). P_o expressed as a fraction of the value before application of drugs was plotted against a logarithm of the concentration of drugs. The data were fitted by a least-squares analysis according to the Hill equation (Eq. (2)):

$$\text{Normalized } P_o = 1/\{1 + ([\text{drug}]/k)^n\} \quad (2)$$

where k is [drug] causing half-maximal inhibition, and n is the Hill coefficient. There was no significant difference in n among drugs; n was close to unity in all cases, and was 0.83 for DIDS, 0.84 for SITS, and 0.97 for DNDS. The order of potency in inhibiting channel activity was DIDS > SITS ≈ DNDS > DADS; the k value was 71 μM for DIDS, 393 μM for SITS, and 593 μM for DNDS. The n and k values for DADS were not obtained since a maximal concentration of DADS soluble in the solution (10 mM) did not suppress channel inhibition completely.

The stilbene disulfonates, DIDS, SITS, DNDS, or DADS at concentrations up to 10 mM also were applied to the bath solution in outside-out membrane patches (extracellular side of membrane). Extracellularly applied stilbene disulfonates did not decrease the activity of K_{ATP} channels (Fig. 3).

Effects of Membrane Potential on K_{ATP} Channel Inhibition

We carried out various experiments to clarify the inhibitory action of the stilbene disulfonates. Their actions on K_{ATP} channels may be considered in terms of their reversibility of effects; DIDS and SITS form one group in which inhibitory effects are irreversible, while DNDS and DADS form another group in which inhibitory effects are, at least, partly reversible. In this and following analyses, we chose one drug from each group, DIDS and DNDS. We first determined whether the magnitude of blocking effect by stilbene disulfonates is dependent on membrane potential. The P_o values at membrane potentials of −60, −30, +30, and +60 mV were measured during exposure to 0.1 mM DIDS or 1 mM DNDS, and were expressed as a fraction of control values (Fig.

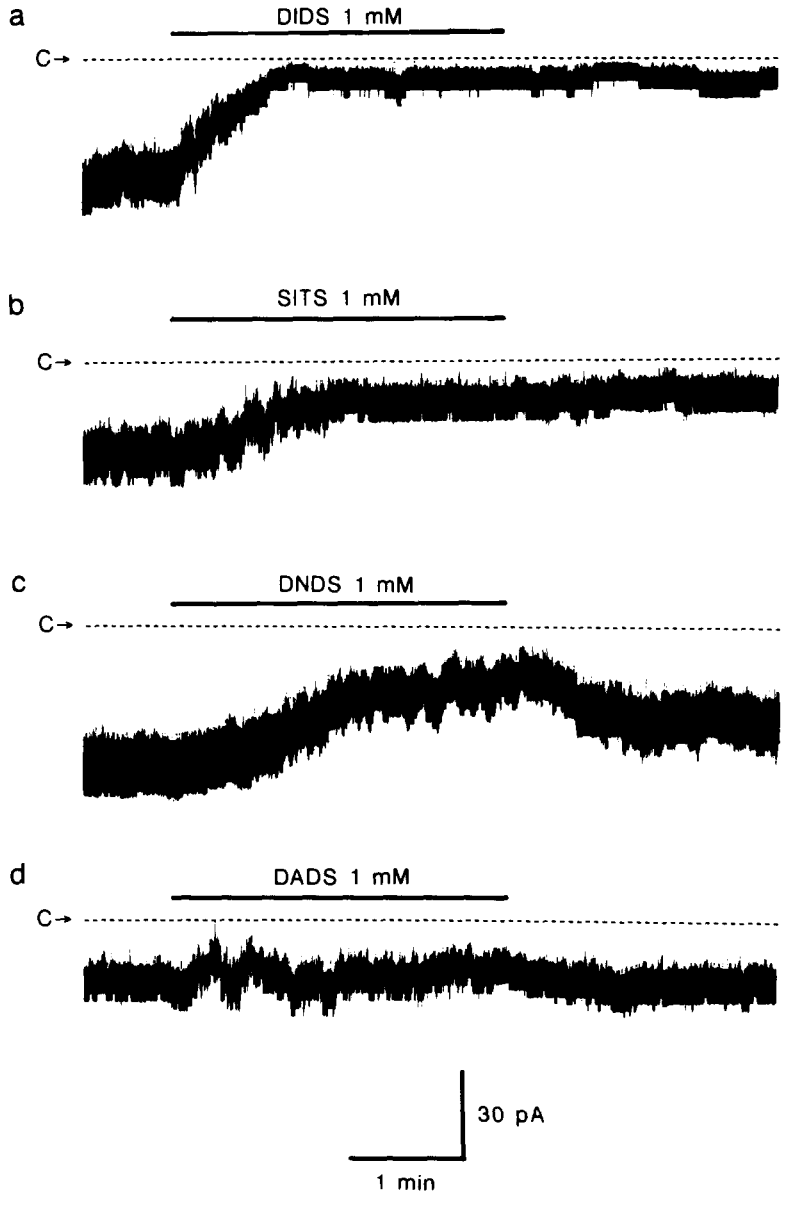


Fig. 2. Inhibition of the K_{ATP} channel current by stilbene disulfonates, DIDS (panel *a*), SITS (panel *b*), DNDS (panel *c*), and DADS (panel *d*). Records were made from cell-free inside-out membrane patches containing multiple K_{ATP} channels. The membrane potential was held at -60 mV. *C* represents closed level for K_{ATP} channels. Inward currents are shown as downward deflections. Current records were filtered at f_c = 1 kHz for display. (*B*) P_o measured from a 10 sec-

4). The magnitude of inhibition of K_{ATP} channels was not significantly different among the membrane potentials tested.

Effects of Stilbene Disulfonates on the Unitary Current Amplitude

Figure 5*A* shows a representative recording of patch current containing a single K_{ATP} channel at a membrane potential of −60 mV and an all-points amplitude histograms in the control state (panel *a*) and during exposure to 0.1 mM DIDS (panel *b*). Both in the control state and during exposure to DIDS, the amplitude histogram had two Gaussian peaks; one at 0 pA (closed level) and the other at an amplitude slightly less than −4 pA (open level). During exposure to 0.1 mM DIDS, although the height of the peak corresponding to the open level was decreased, the difference of the means of the two Gaussian peaks was not changed (in this example, 3.80 pA in the control state and 3.89 pA during exposure to 0.1 mM DIDS). Figure 5*B* shows averaged *I-V* relationships measured during control and exposure to 0.1 mM DIDS from six patches (panel *a*) and 1 mM DNDS from five patches (panel *b*). In the absence and presence of drugs, *I-V* curves showed a linear relation at potentials negative to 0 mV and showed moderate inward rectification at potentials positive to 0 mV. Neither the slope conductances at negative potentials nor the magnitude of inward rectification at positive potentials was significantly different be-

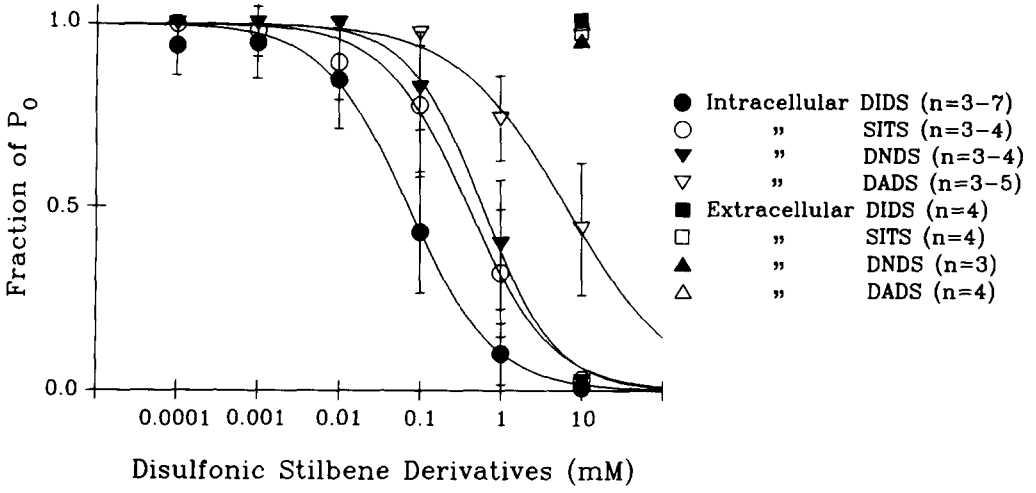


Fig. 3. Concentration-response relationships: P_o (open probability) is plotted *vs.* the concentration of stilbene disulfonates which inhibit the K_{ATP} channel current. P_o during exposure to drugs was normalized to the value before exposure to drugs. The unbroken lines were obtained by fitting of the Hill equation (Eq. (2) in the text) to the data.

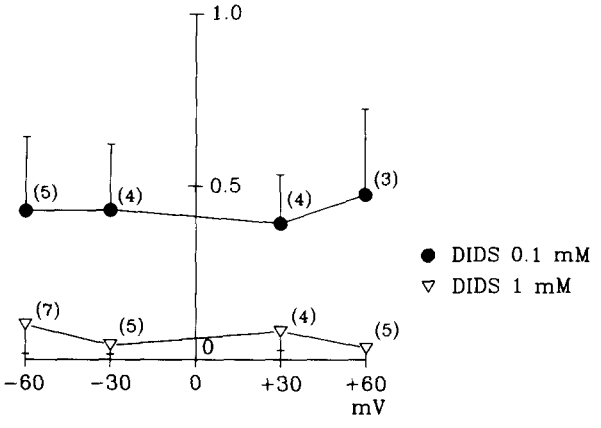


Fig. 4. Relationships between the magnitude of DIDS-induced K_{ATP} channel inhibition and membrane potential. P_o during exposure to 0.1 mM DIDS (filled circles) or 1 mM DNDS (open inverted triangles) is expressed as a fraction of the value before application of drugs, and is plotted against the membrane potential. The numbers in parentheses next to data points indicate the number of experiments.

tween the control and during exposure to drugs. For 0.1 mM DIDS, the slope conductance was 64.0 ± 0.9 pS in the control state and 64.6 ± 1.0 pS during exposure to drug (NS), and the corresponding values for unitary current amplitude at +100 mV were 4.04 ± 0.13 and 4.03 ± 0.16 pA, respectively (NS). For 1 mM DNDS, control slope conductance was 64.8 ± 0.9 and 64.3 ± 1.0 pS during exposure to drug (NS), and the corresponding values for unitary current amplitude at +100 mV were 4.06 ± 0.13 and 4.00 ± 0.16 pA, respectively (NS).

Modulation of the K_{ATP} Channel Kinetics by Drugs

Figure 6 displays a 16 sec continuous recording of a single K_{ATP} channel in the inside-out patch configuration at −60 mV during control (panel *a*) and exposure to 0.1 mM DIDS. During exposure to DIDS, long closed times between bursts (a maximum of around 10 sec in this tracing) were observed. The open and closed time distributions were analyzed to estimate effects of DIDS on the kinetic properties of K_{ATP} channel currents. Figure 7 shows the histogram for open time within bursts measured at an f_c of 10 kHz (panel *a*) and that for open time between bursts measured at an f_c of 0.1 kHz (panel *b*) in the control state (Fig. 7*A*) and during exposure to 0.1 mM DIDS (Fig. 7*B*). Both distributions of open time within bursts and open time between bursts were best fitted to a single exponential function. The time constant for the histogram of open-time within bursts (τ_{O1} in panel *a*) was not changed by exposure to DIDS (1.41 msec in the control state *vs.* 1.41 msec during exposure to DIDS), while that of open time between bursts (τ_{O2} in panel *b*) was slightly shortened by exposure to DIDS (42.6 msec in the control state *vs.* 36.5 msec during exposure to DIDS).

Figure 8 shows the histogram for closed time within bursts measured at an f_c of 10 kHz (panel *a*) and that for closed time between bursts measured at an f_c of 0.1 kHz (panel *b*) during control (Fig. 8*A*) and exposure to 0.1 mM DIDS (Fig. 8*B*). Both during control and exposure to DIDS, the distribution of closed time within bursts was best fitted to a single exponential function, and the time constant was not

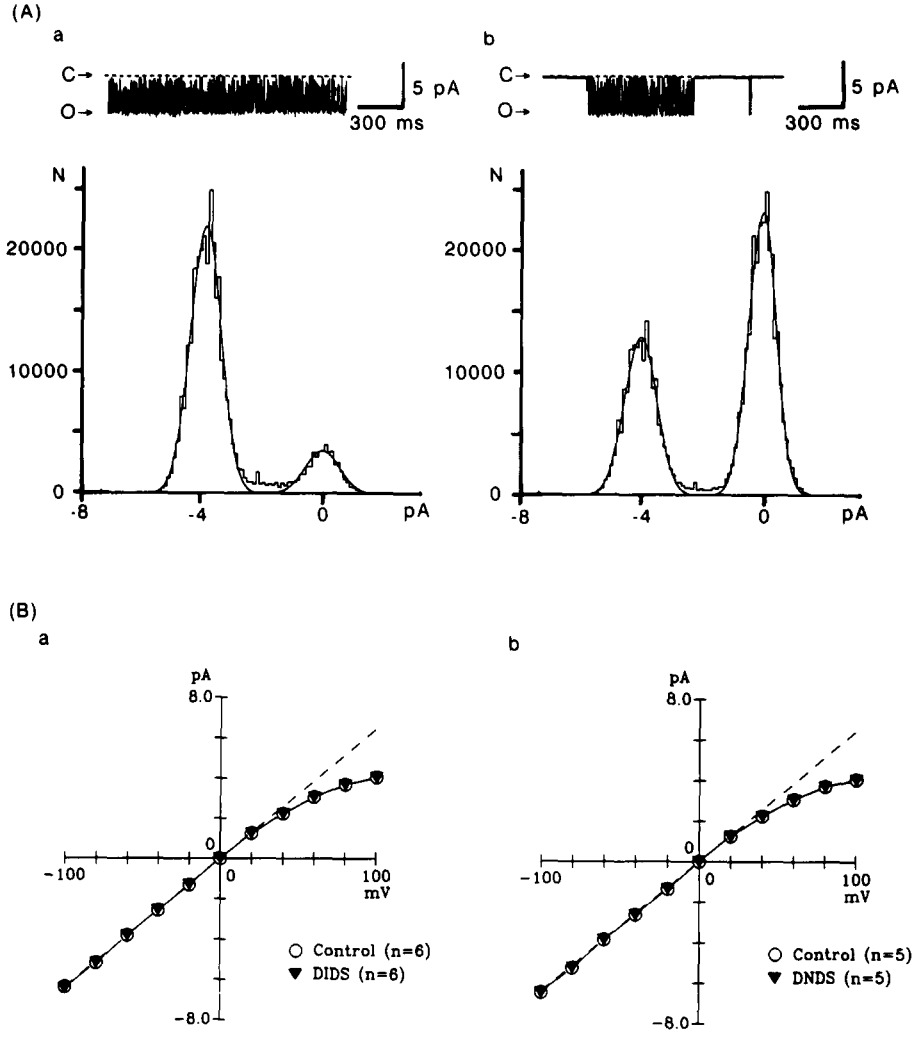


Fig. 5. The effects of DIDS and DNDS on the unitary conductance of the K_{ATP} channel. (*A*) A representative recording of a single K_{ATP} channel current (upper panel) and an all-amplitudes histogram before (panel *a*) and during (panel *b*) exposure to 0.1 mM DIDS. Records were made from cell-free inside-out membrane patches. The membrane potential was held at −60 mV. *C* represents the closed level for K_{ATP} channels, and *O* represents an open state level; inward currents are downward deflections. The current records were filtered at f_c = 1 kHz for display. (*B*) *I-V* relationships of the K_{ATP} channel in the control state and during exposure to 0.1 mM DIDS (panel *a*) or 1 mM DNDS (panel *b*). The SD value is very much smaller than each data point, and is not visible on this graph.

altered by exposure to DIDS (0.22 msec during control *vs.* 0.22 msec during exposure to DIDS). On the other hand, the distribution of closed time between bursts was best fitted to a single exponential function during control, while a double exponential function was required during exposure to DIDS. These data on modulation of channel kinetics are summarized in the Table. It appears from these data that the inhibition of the channel by DIDS or DNDS is due to the occurrence of very long closed times between bursts (blocked state) during exposure to drugs and, in addition, to a slight shortening of the open time between bursts.

Specificity of Channel Blocking Effect

To test whether the blocking effect by stilbene disulfonates was specific to the K_{ATP} channel relative to its action on the inward rectifier K^+ channel, DIDS or DNDS (1 mM) was applied to the intracellular side of an inside-out membrane patch containing a K_{ATP} channel and an inward rectifier K^+ channel (Fig. 9). In the control state (*A*), the tracing of patch current shows four current levels; the closed state level (*C*), the level in which only the inward rectifier K^+ channel was open (*O1*), the level in which only the K_{ATP} channel was open (*O2*), and the level in

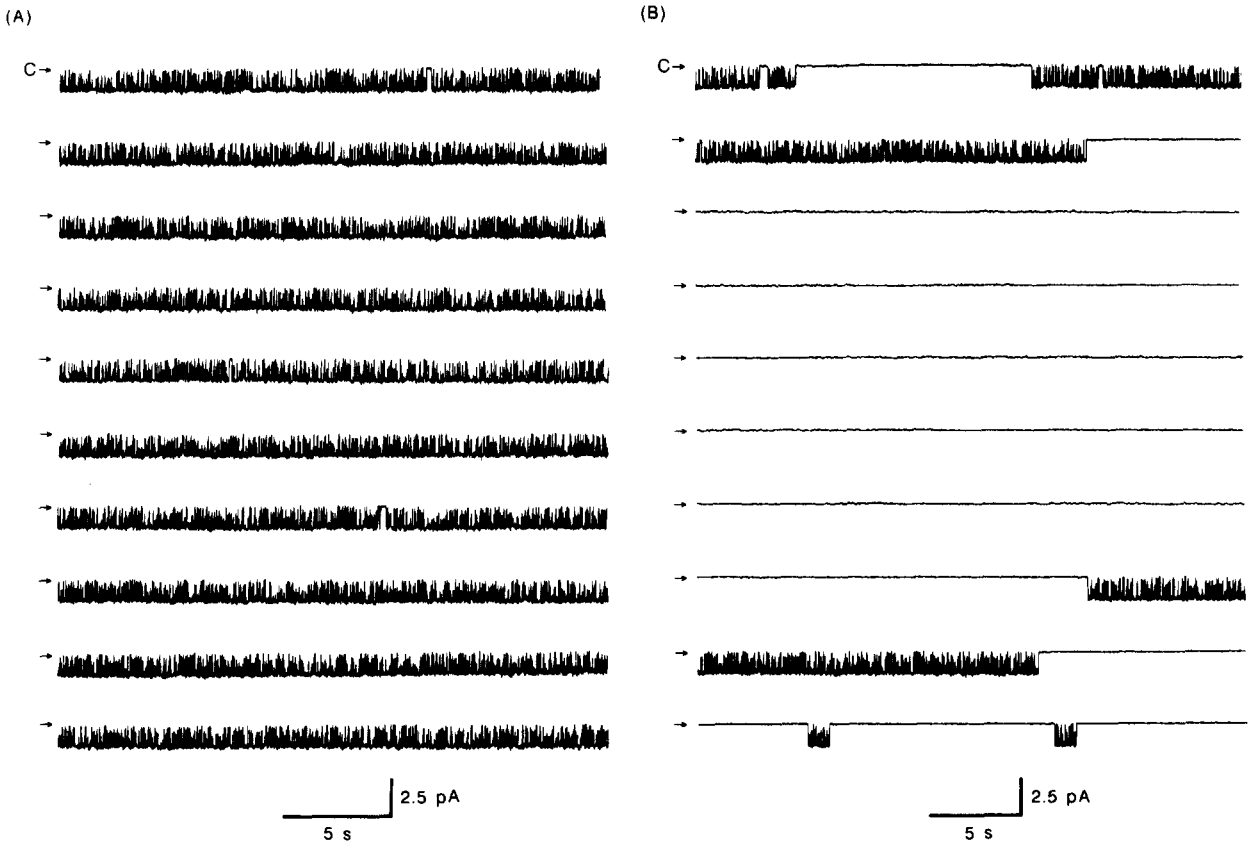


Fig. 6. A continuous recording of membrane current in an inside-out membrane patch containing a single K_{ATP} channel in the control state (*A*) and during exposure to 0.1 mM DIDS (*B*). The membrane potential was held at −60 mV. *C* represents closed level for the K_{ATP} channel. Inward currents are shown as downward deflections. The current records were filtered at f_c = 1 kHz for display.

which both the inward rectifier K^+ channel and the K_{ATP} channel were open (*O3*). Accordingly, the amplitude histogram also had four Gaussian peaks. Similarly, during exposure to DIDS (*B*), the tracing of patch current shows four current levels (*C*, *O1*, *O2*, and O3), and the amplitude histogram has four Gaussian peaks. During exposure to DIDS, although the activity of the K_{ATP} channel (current levels of *O2* plus *O3*) was decreased (in this case, P_o was decreased from 0.84 to 0.05), the activity of the inward rectifier K^+ channel (current levels of *O1* plus *O3*) was not affected (in this case, P_o was changed from 0.91 to 0.95). Similar experiments were performed using DIDS (1 mM) in six membrane patches containing both channel types and using DNDS (1 mM) in four such membrane patches. The mean P_o of the inward rectifier K^+ channel was 0.92 ± 0.08 in the control state and 0.94 ± 0.09 during exposure to DIDS (NS); the corresponding values for DNDS were 0.93 ± 0.09 and 0.92 ± 0.10, respectively (NS). Thus, the blocking effect of 1 mM DIDS or DNDS was specific for the K_{ATP} channel *vs.* the inward rectifier K^+ channel.

Time Course of Inhibition

As shown in Fig. 2, the rates of onset and development of K_{ATP} channel inhibition by stilbene disulfonates were slow and gradual. We determined whether the rates of onset and development were concentration dependent in a fashion similar to glibenclamide (Findlay, 1992*a*) (Fig. 10). Representative tracings displaying the time course of inhibitory action by 0.1, 1, and 10 mM DIDS are shown in Fig. 10*A*, and averaged time courses for DIDS and DNDS in Fig. 10*B*. The time course of the onset of channel inhibition and the time required to reach a steady-state were concentration dependent for both DIDS and DNDS. A longer time was required to reach a new steady-state of channel activity as drug concentration was decreased.

Effect of Intracellular pH on Channel Inhibition

Finally, to further examine the similarity in K_{ATP} channel inhibition by glibenclamide and stilbene dis-

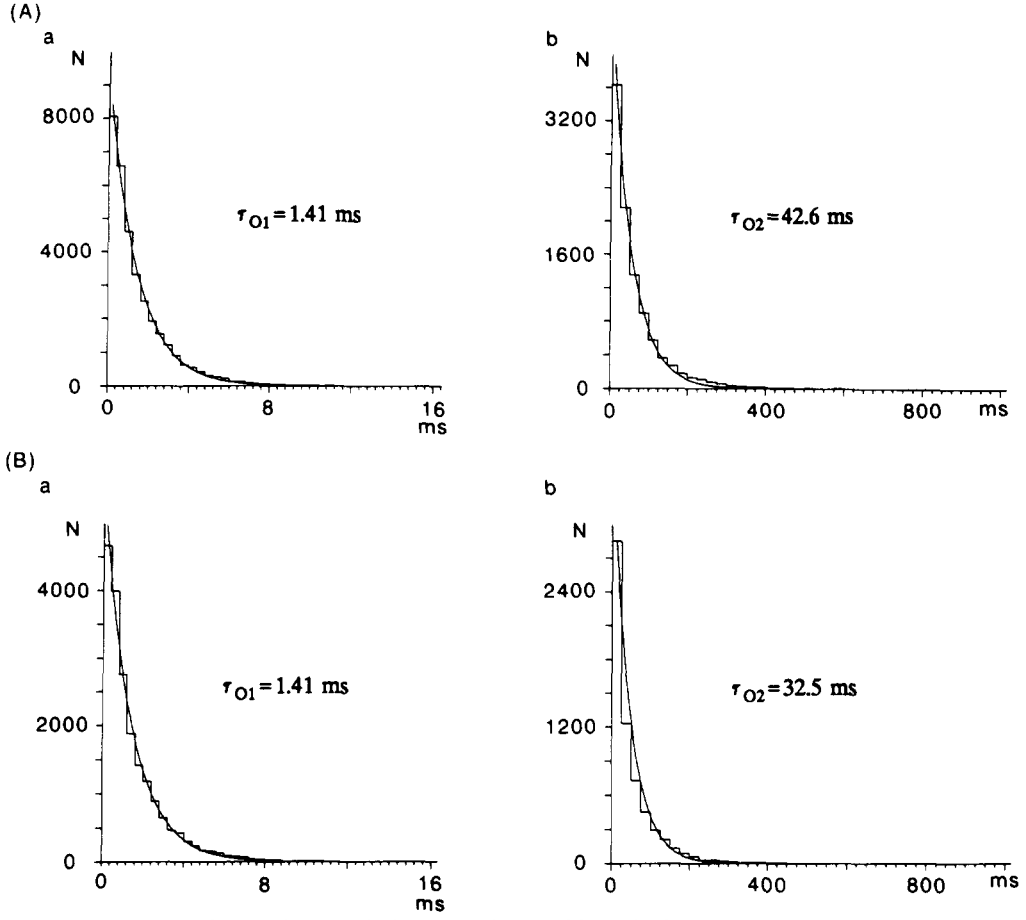


Fig. 7. Histograms of open time within bursts analyzed with an f_c of 10 kHz (panel *a*) and histograms of open time between bursts analyzed with an f_c of 0.1 kHz (panel *b*) in the control state (*A*) and during exposure to 0.1 mM DIDS (*B*). The time constant of open time within bursts (τ_{O1}) and that of open time between bursts (τ_{O2}) are shown for each condition.

ulfonates, we tested the effects of altered intracellular pH on channel inhibition. Figure 11*A* shows representative tracings of the effects of 0.1 mM DIDS at intracellular pH of 7.4 (trace *a*) and 6.5 (trace *b*); the magnitude of channel inhibition is greater at pH 6.5 than pH 7.4. Figure 11*B* shows P_o (expressed as a fraction of the control value) during exposure to 0.1 mM DIDS or 1 mM DNDS at intracellular pH of 7.4 (filled bars) or 6.5 (cross-hatched bars). For both drugs, P_o in the presence of drugs was lower at pH 6.5; for 0.1 mM DIDS, P_o was 42.9 ± 16.6% at pH 7.4 and 8.2 ± 6.6% at pH 6.5 ($P < 0.01$); for 1 mM DNDS, corresponding values were 39.6 ± 17.6% and 8.9 ± 5.8%, respectively ($P < 0.01$).

Discussion

In this study, we demonstrate for the first time that stilbene disulfonates, recognized anion transport and anion channel blockers, inhibit the activity of the K_{ATP} channel. The blocking action on the K_{ATP} channel by these drugs appears to be different from their actions on anion channels and similar in some ways to the blockade of the K_{ATP} channel by glibenclamide.

In various Cl^- channels including those of mammalian vascular smooth muscle cells (Kokubun et al., 1991), endothelial cells (Groschner & Kukovetz, 1992), and amphibian skeletal muscle cells (Woll et al., 1987), reduction of unitary current amplitude by DIDS has been reported; this may reflect extremely rapid and thus unresolved channel flicker as a consequence of open channel block. It is hypothesized that negatively charged sulfonic residues (SO_3^-) compete with Cl^- ions at site(s) of anion binding, inhibiting permeation of anions through the channel pore (Fröhlich, 1982). In the case of the K_{ATP} channel, however, neither DIDS nor DNDS affected the unitary current amplitude at various membrane potentials (Fig. 4). Kinetic analysis revealed that very long channel closures between bursts emerged in the presence of intracellular DIDS (Fig. 6), and, consequently, additional components with a very

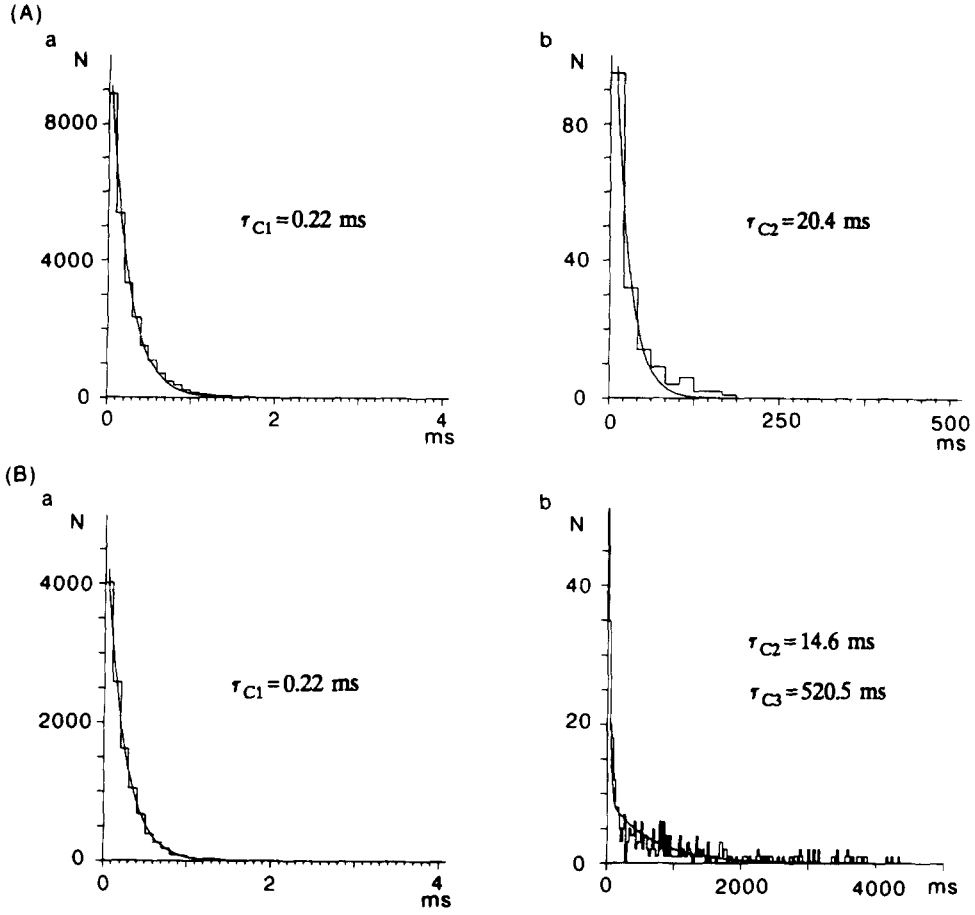


Fig. 8. Histograms of closed time within bursts analyzed with an f_c of 10 kHz (panel *a*) and histograms of closed time between bursts analyzed with an f_c of 0.1 kHz (panel *b*) in the control state (*A*) and during exposure to 0.1 mM DIDS (*B*). The time constant of closed time within bursts (τ_{C1}) and closed time between bursts (τ_{C2} or τ_{C3}) are shown for each condition. Please note that the time scales in histograms for closed time between bursts are different between the control state and during exposure to DIDS.

Table. Effects of DIDS on K_{ATP} channel kinetics

Experimental condition	Open-time (msec)		Closed-time (msec)		
	τ_{O1}	τ_{O2}	τ_{C1}	τ_{C2}	τ_{C3}
Control	1.44 ± 0.22 (n + 7)	40.0 ± 8.1 (n = 4)	0.21 ± 0.02 (n = 7)	16.4 ± 2.9 (n = 4)	
DIDS (0.1 mM)	1.53 ± 0.18 (n = 7)	29.8 ± 6.7 (n = 4)	0.21 ± 0.01 (n = 7)	14.6 ± 3.2 (n = 4)	419.4 ± 123.2 (n = 4)
P value	NS	<0.05	NS	NS	
Control	1.51 ± 0.03 (n = 5)	43.1 ± 9.3 (n = 4)	0.22 ± 0.03 (n = 5)	15.9 ± 3.5 (n = 4)	
DNDS (1 mM)	1.46 ± 0.19 (n = 5)	31.9 ± 7.1 (n = 4)	0.21 ± 0.02 (n = 5)	16.3 ± 4.1 (n = 4)	451.1 ± 119.3 (n = 4)
P value	NS	<0.05	NS	NS	NS

τ_O = time constant for open time; τ_{O1} = τ_O within bursts; τ_{O2} = τ_O between bursts; τ_C = time constant for closed-time; τ_{C1} = τ_C within bursts; τ_{C2} and τ_{C3} = τ_C between bursts.

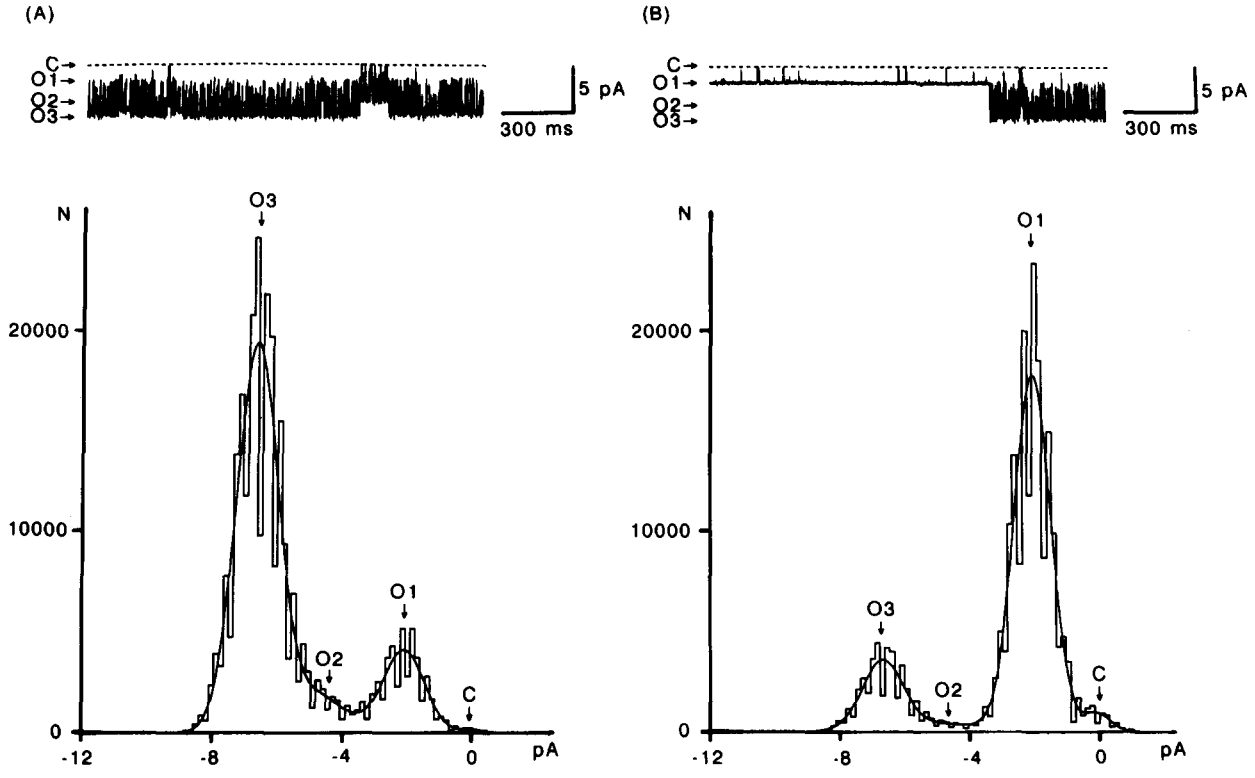


Fig. 9. Specificity of the inhibitory effect of DIDS on the K_{ATP} channel over the inward rectifier K^+ channel. A representative recording of membrane current (upper panel) and an all-points amplitude histogram (lower panel) obtained from an inside-out membrane patch containing a K_{ATP} channel and an inward rectifier K^+ channel in the control state (*A*) and during exposure to 0.1 mM DIDS (*B*). The membrane potential was held at −60 mV. *C* represents closed level for the K_{ATP} channel; *O1* represents the level in which only the inward rectifier K^+ channel is open; *O2* represents the level in which only the K_{ATP} channel is open; and *O3* represents the level in which both the inward rectifier K^+ channel and the K_{ATP} were open. Inward currents are shown as downward deflections. The current records were filtered at f_c=1 kHz for display.

long time constant (blocked state) appeared on the histogram of closed times between bursts (Fig. 8). Thus, in the absence of stilbene disulfonates, channel kinetics could be accounted for by a scheme with one open state and two closed states, whereas one open state and three closed states are required to account for the channel kinetics in the presence of drugs, as follows:

$$C_3 \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} C_2 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} O \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} C_1$$

where the rate constant k_3 is very slow compared to k_2 or k_1. There is general agreement that at least one open state and two closed states are required to account for K_{ATP} channel kinetics (Kakei & Noma, 1984; Trube and Hescheler, 1984; Spruce et al., 1987). Although we previously reported a kinetic scheme with three closed states, this was derived from data obtained using a low concentration of intracellular ATP (Fan et al., 1990). Thus, the main effect of stilbene disulfonates is to shift channels to the blocked state (C_3), and the dissociation rate from this state appears to be very slow. Additionally, the open time between bursts was slightly shortened, suggesting that the transition rate from the open state (O) to the closed state $C_2(k_{-2})$ was accelerated, which may reflect the appearance of transition from C_2 to C_3. The Hill coefficients obtained for inhibition by intracellularly applied stilbene disulfonates were close to unity (0.83 for DIDS, 0.84 for SITS, and 0.96 for DNDS); we suggest that these drugs interact with their target in a one-to-one stoichiometry. Thus, binding of a single molecule of stilbene disulfonates may alter K_{ATP} channel kinetics as described above.

High intracellular concentrations of DIDS irreversibly block the Cl^- channel of pig aortic endothelial cells, and this action of DIDS has been considered a nonspecific effect (Groschner & Kukovetz, 1992). Since the inhibitory actions of DIDS and SITS on the K_{ATP} channel were irreversible (Fig. 2), one might argue that DIDS- or SITS-induced inhibition of the K_{ATP} channel is also a nonspecific action of

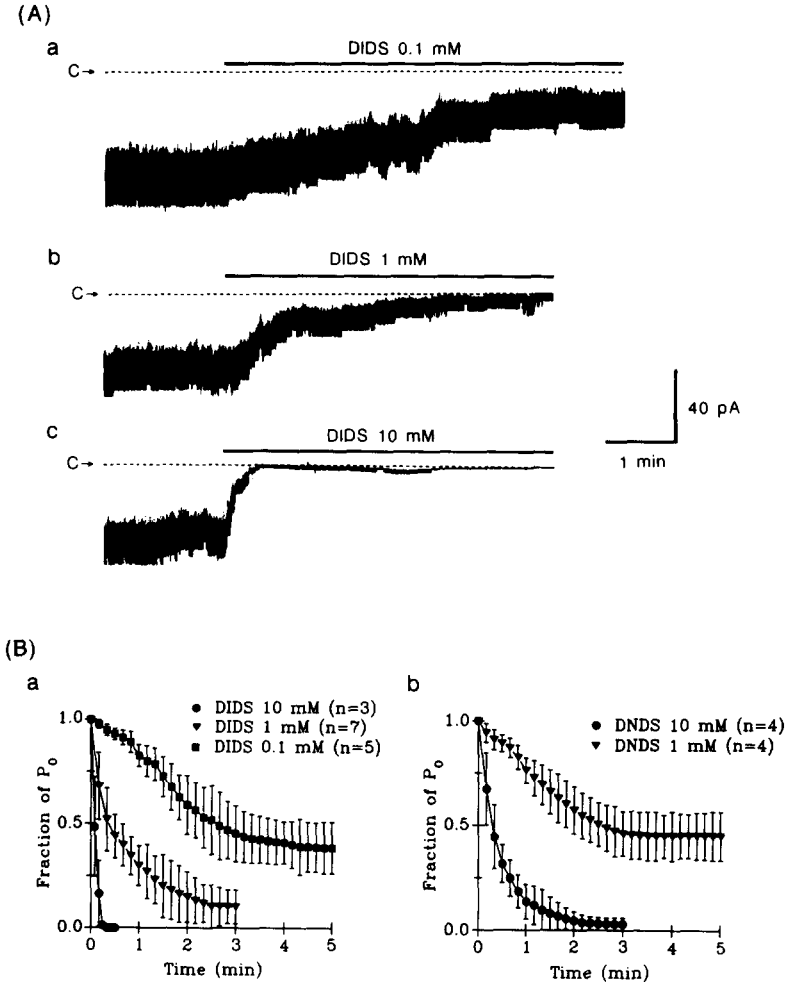


Fig. 10. Time course of the effect of DIDS or DNDS on the K_{ATP} channel current. (*A*) Representative recordings displaying the time course of the effect of 0.1 mM DIDS (trace *a*), 1 mM DIDS (trace *b*), and 10 mM DIDS (trace *c*) on a K_{ATP} channel current recorded in cell-free inside-out membrane patches containing multiple K_{ATP} channels. The membrane potential was held at −60 mV. *C* represents closed level for K_{ATP} channels. Inward currents are shown as downward deflections. Current records were filtered at f_c = 1 kHz for display. (*B*) P_o measured from a 10 sec-long recording of a K_{ATP} channel current at every 10 sec after the application of drug (time zero) was averaged and plotted against time after drug application. Panel *a* displays data for DIDS, and panel *b* for DNDS.

these drugs. However, this possibility seems unlikely because the inhibitory action by stilbene disulfonates showed clear concentration dependency, and occurred at relatively low concentrations (Fig. 3). For example, IC_{50} for DIDS block (71 μM) is not high compared to the value reported for its blocking action on endothelial Cl^- channel (about 100 μM) (Groschner & Kukovetz, 1992). Moreover, the inhibitory action of DIDS was specific for the K_{ATP} channel *vs.* the inward-rectifier K^+ channel, and the inhibitory actions displayed by other stilbene disulfonates, DNDS and DADS, were reversible. Thus, we consider the inhibition of the K_{ATP} channel to be a specific action of stilbene disulfonates. The difference in reversibility of inhibitory actions between DIDS/SITS and DNDS/DADS may be explained by the presence of isothiocyano structure (−NCS) in the former agents. The inhibitory action on the anion transporter of erythrocyte membranes is also irreversible for DIDS and SITS, but not for DNDS or DADS, and this difference has been explained by the presence of the isothiocyano structure (−NCS) in DIDS and SITS (Cabantchik et al., 1978).

The stilbene disulfonates appear to induce K_{ATP} channel inhibition in a manner similar in some ways to the blockade of the K_{ATP} channel by the sulfonylurea drug, glibenclamide (Findlay, 1992*a*, *b*). The onset and development of inhibitory action are relatively slow, and dependent on the concentration of cytosolic DIDS or DNDS (Fig. 10). To account for the slow rates of onset and development of K_{ATP} channel inhibition by glibenclamide, Findlay (1992*a*) proposed a "membrane lipid compartment" model, in which the existence of an "intermediate compartment," interpolated between the cytosolic drug source and the site of drug action is hypothesized. To examine whether this model could be applied to K_{ATP} channel inhibition by stilbene disulfonates, we tested the effects of reduced intracellular pH on drug-induced channel inhibition. If the slow onset of channel inhibition by stilbene disulfonates could be accounted for by a "membrane lipid compartment" model, then channel inhibition should be affected by changes in intracellular pH which alter the fraction of the uncharged, lipid soluble form of the drugs. As expected, the magnitude of inhibitory action was greater at pH 6.5, when the uncharged, lipid soluble form predominates. Thus, stilbene dis-

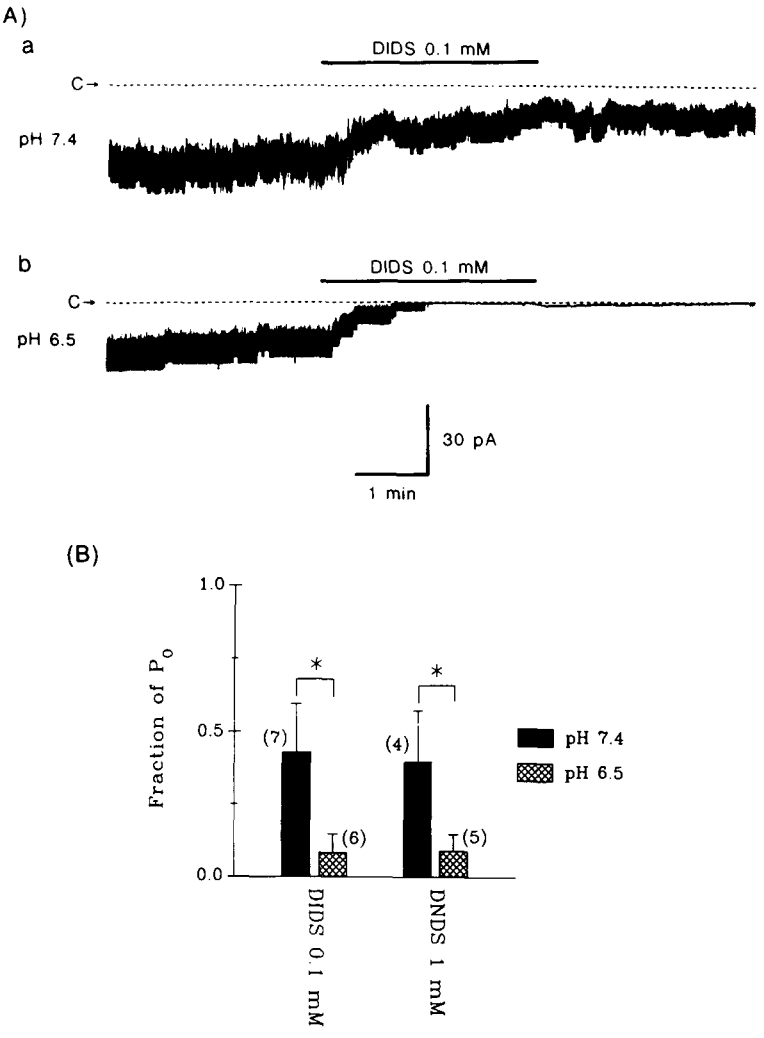


Fig. 11. Effects of changes in intracellular pH on the K_{ATP} channel inhibition by DIDS or DNDS. (*A*) Representative recordings showing the effect of 0.1 mM DIDS at pH 7.4 (trace *a*) or at pH 6.5 (trace *b*). Records were made from cell-free, inside-out membrane patches containing multiple K_{ATP} channels. The membrane potential was held at -60 mV. *C* represents closed level for K_{ATP} channels; inward currents are downward deflections. Current records were filtered at f_c=1 kHz for display. (*B*) P_o during exposure to 0.1 mM DIDS or 1 mM DNDS was averaged for pH 7.4 (filled bars) or for pH 6.5 (cross-hatched bars), and was expressed as a fraction of the value before application of drugs. Asterisks represent the *P* values of less than 0.01.

ulfonates drugs may have to be absorbed into membrane lipids before reacting with the target site; the rates of drug absorption into and exit from membrane lipids may be an important factor involved in their K_{ATP} channel inhibition. One may question why extracellularly applied stilbene disulfonates do not inhibit the K_{ATP} channel if drugs access their target sites through membrane lipids. It is possible that the highest concentration of drugs we tested, which also was the maximum dissolvable concentration, was not sufficient to induce the inhibitory action from the extracellular side of the membrane patch. Alternatively, stilbene disulfonates are amphipathic having both hydrophilic and lipophilic components in their chemical structures. Thus, although drug absorption into membrane lipids may be necessary to demonstrate their effect, the entire structure of the drug may not penetrate into the lipid layer; thus, the inhibitory effect is different between intracellular and extracellular application.

The similarity in channel inhibition by sulfonylurea and stilbene disulfonates could be accounted for by their common chemical structure, either the benzene ring or sulfonic residue. We recently reported that aromatic aldehydes and aromatic ketones open cardiac K_{ATP} channels (Fan et al., 1992). Since both aromatic aldehydes and aromatic ketones also have a benzene ring in their structure, it appears contradictory that the benzene ring has a blocking action in some compounds (sulfonylureas and stilbene disulfonates) and an activating action in other compounds (aromatic aldehydes and aromatic ketones). Thus, the benzene ring may not be important for drug inhibition on the K_{ATP} channel, but may be necessary to account for the slow onset of drug action because the other common structure, sulfonic residue, is hydrophilic and thus cannot account for this characteristic of channel inhibition. It is interesting to note that the inhibitory action by glibenclamide, a sulfonylurea drug with two benzene rings,

and by stilbene disulfonates which also have two benzene rings, show slow onset of drug action. In contrast, the inhibitory action by tolobutamide, a sulfonylurea drug with only one benzene ring, or the activation action by aromatic aldehydes or aromatic ketones that have also one benzene ring, do not show slow onset of action (Findlay 1992*a*, *b*; Fan et al., 1992). Thus, we hypothesize that two benzene rings may underlie the slow drug action. The other common structure for sulfonylureas and stilbene disulfonates, sulfonic residue, may be responsible for closure of the K_{ATP} channel. On the other hand, the common chemical structures between glibenclamide and stilbene disulfonates may be coincidental and may not be related to their similar mode of inhibitory action. Further studies are required to elucidate why glibenclamide and stilbene disulfonates exhibit similar inhibitory action on the K_{ATP} channel.

Stilbene disulfonates block Cl^- channels (Maddy, 1964; Frölich, 1982; Woll et al., 1987; Kokubun et al., 1991; Groschner & Kukovetz, 1992) and, as shown by us, inhibit K_{ATP} channels. Sulfonylureas, K_{ATP} channel blockers, also inhibit cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- currents (Sheppard & Welsh, 1992). These data suggest that K_{ATP} and CFTR Cl^- channels have similar pharmacological characteristics. These channels are also similar in their intracellular regulation. Both the K_{ATP} channel and the CFTR Cl^- channel require intracellular ATP to maintain their channel activity, and hydrolysis of ATP, other than phosphorylation by cAMP-dependent protein kinase, appears to be needed for this action (Anderson et al., 1991; Furukawa et al., 1993). Thus, it is intriguing to speculate that the K_{ATP} channel and the CFTR Cl^- channel have some similar characteristics in channel structure. The primary structure of an inwardly rectifying ATP-regulated K^+ channel was recently reported (Ho et al., 1993); however, this channel may not be identical to the K_{ATP} channel because it was not inhibited by intracellular ATP and the sulfonylureas do not affect its activity. Thus, the comparison of channel structure between the K_{ATP} channel and the CFTR Cl^- channel has to await the information of the primary structure of the K_{ATP} channel.

Finally, we note that the inhibitory action of stilbene disulfonates on the K_{ATP} channel may not presently be applicable in the clinical treatment of ischemia-related cardiac arrhythmias since they are ineffective K_{ATP} channel blockers when applied extracellularly and they clearly inhibit anion transporters and anion channels. Nevertheless, we believe that the information obtained in this study provides new clues to better understand the pharmacological modulation and the functional structure of the cardiac K_{ATP} channel.

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