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Modulation and block of the plasma membrane anion channel of guard cells by stilbene derivatives

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Abstract. An anion channel in the plasma membrane of guard cells (GCAC1) provides a regulatory element for the voltage-dependent release of anions during stomatal closure (Keller et al. 1989) as well as excitability (Hedrich et al. 1990). Recognition sites for plant growth hormones on the extracellular surface of GCAC1 further indicate that this channel may also serve as a transduction element in hormone signaling (Marten et al. 1991 a). Stilbene derivatives were used to study the inhibitor-structure channel-function relationship of GCAC1. We have analyzed the activity, voltage-gate and kinetics of this channel as affected by stilbenes. The stilbene derivatives SITS and DNDS caused a shift in activation potential and a decrease in the peak current amplitude. Channel block through the action of DIDS, on the other hand, was not accompanied by a shift in voltage-dependence. Differences in the dose-dependence of the two effects give clues to the presence of channel sites responsible for gate-shifting and block. The ability to inhibit anion currents (K_d) increased in the sequence: SITS (4 μM) < DNDS (0.5 μM) < DIDS (0.2 μM). All inhibitors reversibly blocked the anion channel from the extracellular side. Channel block on the level of single anion-channels is characterized by a reduction of long open-transitions into flickering bursts and a decrease in channel amplitude.

Key words: Anion channel – Guard cell – Stilbene derivatives – Voltage dependence – Gating – Kinetics

Introduction

Transport of anions across the plasma membrane and the organelle membranes of higher plants plays a major role in growth, osmoregulation, nitrogen metabolism, photosynthesis and respiration.

Since the use of the patch-clamp technique in plant membrane physiology (see Hedrich and Schroeder 1989 for review) anion transport processes have been resolved on the level of single anion channels (Coleman 1985; Schönknecht et al. 1988; Lew 1991; Laver 1991).

Recent studies have shown that anion-selective channels (named GCAC1 for guard cell anion channel; Marten et al. 1992; Hedrich and Jeromin 1992) may provide a key mechanism in the down-regulation of turgor and volume during stomatal closure. This anion channel conducts physiologically important anions with the following sequence of permeabilities $NO_3^- > I^- > Br^- > Cl^- \gg$ malate (Hedrich and Marten 1993). Glutamate and gluconate, on the other hand, are not able to pass the selectivity filter of GCAC1 (Hedrich et al. 1990).

The state of activity triggered by a Ca^{2+}- and nucleotide-dependent process determines both the amplitude and time-course of anion release. Depolarization of the membrane potential into a voltage window, which elicits the opening of anion and K^+ channels, causes efflux of K^+ salts.

The action of extracellular auxins, however, shifts the range of GCAC1 activity towards more hyperpolarized potentials (Marten et al. 1991 a). This shift causes the separation of the working ranges of anion and K^+ release channels. Thus voltage ranges dominated by one channel or the other and activation-inactivation cycles during prolonged voltage-stimulation of GCAC1 (Hedrich et al. 1990) seem to provide the molecular basis for excitability in guard cells (Thiel et al. 1992).

We have used stilbene derivatives [1], a class of well known anion channel blockers in many systems (Greger

Abbreviations: DIDS: 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid; SITS: 4-Acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid; DNDS: 4,4′-dinitrostilbene-2,2′-disulfonic acid; NPPB: 5-Nitro-2-(3-phenylpropylamio)benzoic acid; IAA-94: [(6,7-Dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5yl)oxy] acetic acid; A-9-C: Anthracene-9-carboxylic acid; TEA: Tetraethylammonium

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[1] Part of this work was published in abstract form at the meeting of the ASPP in 1991 (Marten et al. 1991 b)

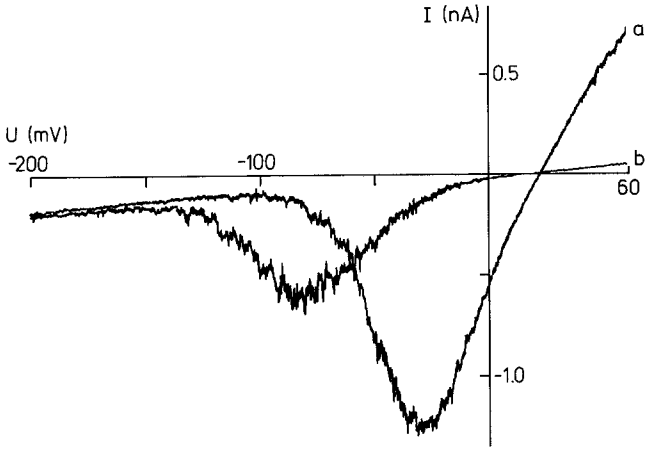

Fig. 1. Modulation of the plasma membrane anion channel of guard cells by 10 μM DNDS. DNDS-induced inhibition of inward anion current, modulation of the peak amplitude of anion efflux and shift in activation potential. Current-voltage relation of whole-cell anion fluxes in the absence **a** and 40 s **b** after application of 10 μM DNDS resulting from 1 s voltage-ramps from −200 to +60 mV

1992), to elucidate their effects on sites within GCAC1 responsible for anion permeation and voltage-dependence.

Materials and methods

Protoplast isolation and patch-clamp recordings

Guard cell protoplasts were enzymatically isolated from 2–3 week old leaves of the broad bean, *Vicia faba* (Hedrich et al. 1990). Patch pipettes were sealed against the plasma membrane to study ion fluxes in the whole-cell configuration and in outside-out patches as described by Hedrich et al. (1990). Current measurements were made with an EPC-7 patch-clamp amplifier (List-electronic Darmstadt, FRG), low-pass filtered at 1 kHz (or 10 kHz in Fig. 4C) with an eight-pole Bessel filter. Data were digitized (VR10, Instrutech Corp., Elmont, NY, USA), stored on video tape and analyzed using patch-clamp software of Instrutech Corp. on an Atari Mega ST4.

Solutions

Solutions for the experiments shown in Figs. 1–4 were composed of 40 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM MES/TRIS pH 5.6 in the bathing medium and 150 mM KCl or TEACl, 2 $MgCl_2$, 10 mM MgATP and 10 mM Na_2GTP, 0.1 mM EGTA, 10 mM HEPES/TRIS pH 7.2 in the pipette (cytoplasm).

In the experiments shown in Fig. 5 guard cell protoplasts were exposed to 30 mM KCl (or 40 mM $CaCl_2$ to activate anion channels), 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM MES/TRIS pH 5.6 in the bath and to 150 mM KCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 10 mM MgATP and 10 mM Na_2GTP, 10 mM HEPES/TRIS pH 7.2 in the pipette. Stilbene derivatives DIDS, SITS and DNDS were dissolved in distilled water.

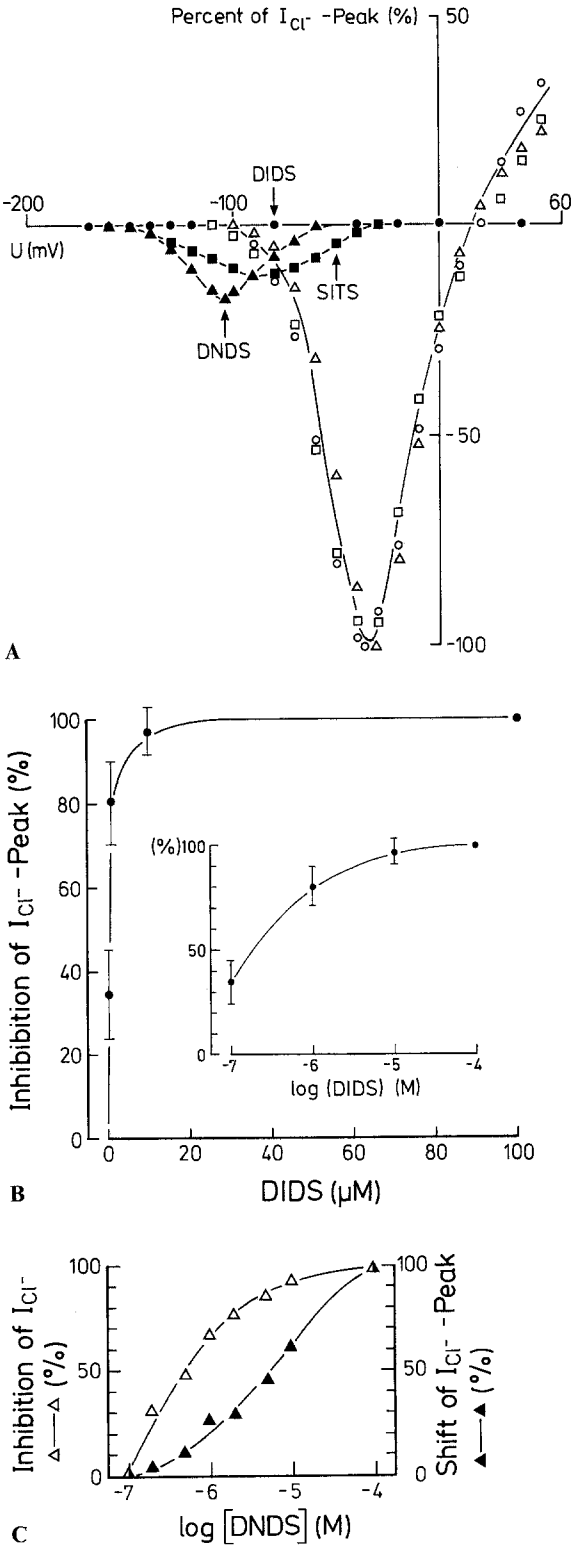

Fig. 2 A–C. Modulation of the plasma membrane anion channel of guard cells by various stilbene derivatives. **A** Alteration of peak amplitude and shift in activation potential of inward current. Current-voltage relation of whole-cell anion fluxes in the absence of stilbene (*open symbols*) and during 100 μM DIDS (*closed circles*), SITS (*closed squares*), and DNDS (*closed triangles*) treatment extracted from voltage-ramps (see legend to Fig. 1). **B** Concentration-dependence of DIDS-induced block of inward anion channels at the peak current potential. **C** Superposition of concentration-dependence of DNDS-induced block and shift of GCAC1 activity. Currents were normalized with respect to the inhibition and gate-shifting in the presence of 100 μM DNDS

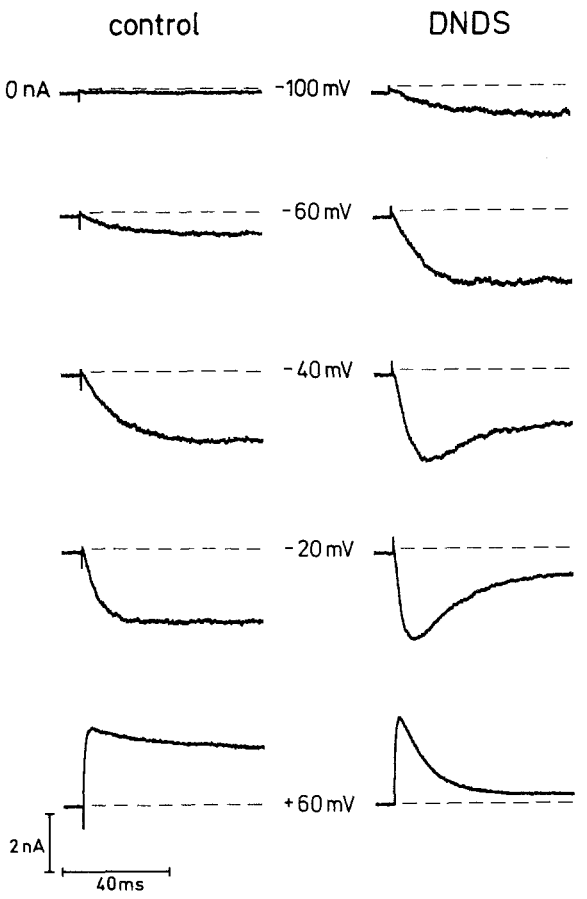

Fig. 3. Modulation of the voltage-dependent kinetics of the anion channel in control cells and cells treated with the stilbene derivative DNDS. Time course of activation and inactivation in the absence (*left panel*) and presence (*right panel*) of 1 μM DNDS were obtained by 70 ms voltage-steps to depolarizing potentials from a resting level of −200 mV

Results

Modulation of voltage-dependence

In order to study inhibitor-induced alterations in plasma membrane anion fluxes we applied the patch-clamp technique (Hamill et al. 1981) to guard cell protoplasts (Schroeder et al. 1984). In the whole-cell configuration voltage-dependent anion channels were activated by nucleotides and calcium (Hedrich et al. 1990). Under these conditions outward and inward chloride fluxes were the predominant ionic conductances (as confirmed by the experiments shown in Fig. 1). Chloride fluxes were activated near −100 mV and peaked at −30 to −40 mV. Inward currents (chloride efflux) decline through a decrease in the electro-chemical driving force when stepping the membrane potential to more depolarized values than −30 mV. They reversed at the Nernst potential for chloride ($E_{Cl^-} = +16$ mV; Fig 1, see also Hedrich et al. 1990).

When SITS and DNDS were applied to the bathing solution both the activation potential and current amplitude were altered (Figs. 1, 2). Inhibition of outward current (chloride influx) was observed before peak inward current was inhibited (voltage-dependent block) and its activation potential shifted to more hyperpolarized potentials. The modulation of the anion channel was fully reversed when the stilbene derivatives were removed from the extracellular medium.

In order to determine whether the interaction between the stilbene derivatives and the channel protein is characterized by side-specificity the cytoplasm was equilibrated with DIDS, SITS or DNDS through the patch pipette. Under these conditions anion currents were not affected, indicating that the binding-site of these inhibitors is located at the external face of the channel. Our observation that intracellular application of 100 μM concentration of stilbene derivatives did not affect the channel is in agreement with the side-specific inhibition of anion channels from epithelia and the electric organ of *Torpedo* (for review see Frizzel and Halm 1991).

Within the group of the stilbene derivatives DIDS, with a half-inhibition concentration of 0.2 μM (Fig. 2B), was most effective:

SITS (4 μM) < DNDS (0.5 μM) < DIDS (0.2 μM).

It should be noted, that K_d-values were determined at pH 5.6 and in the presence of 84 mM chloride in the bath. Thus we can not exclude the possibility that more alkaline pH values and low chloride concentrations in the bath could alter the blocker efficiency on GCAC1.

In the concentration range between 0.1 and 100 μM DIDS (Fig. 2B) we did not observe a shift in activation potential. When DIDS was applied to the closed channels at hyperpolarized membrane potential, prior to the activating voltage-pulse e.g. to −40 mV, currents were not elicited during stimulation. This indicates either similar kinetics of block and channel opening or an inhibition through DIDS-binding to the exterior of the channel pore (see also Miller and White 1984).

Different sites on GCAC1 for voltage-gating and block

Dose-response analysis was performed to determine whether one or two ligand binding sites are involved in gate-shifting and block of the anion channel. Figure 2C shows the saturation of shift and block as a function of DNDS concentration. The difference between the two saturation curves may represent two modes of interaction with one putative binding site or two sites on the extracellular surface of GCAC1. Similar observations on the sodium channel by Armstrong and Cota (1991) have been explained by the assumption of two sites, one where the ligand interacts with the open mouth of the channel (for flickering block see also Fig. 4A) and the other where the adsorption of the ligand directly alters the intramembraneous field.

Alterations in kinetic properties

The modulation of the kinetic properties of the anion channel was studied with a series of voltage pulses (Fig. 3). The activation and inactivation kinetics of con-

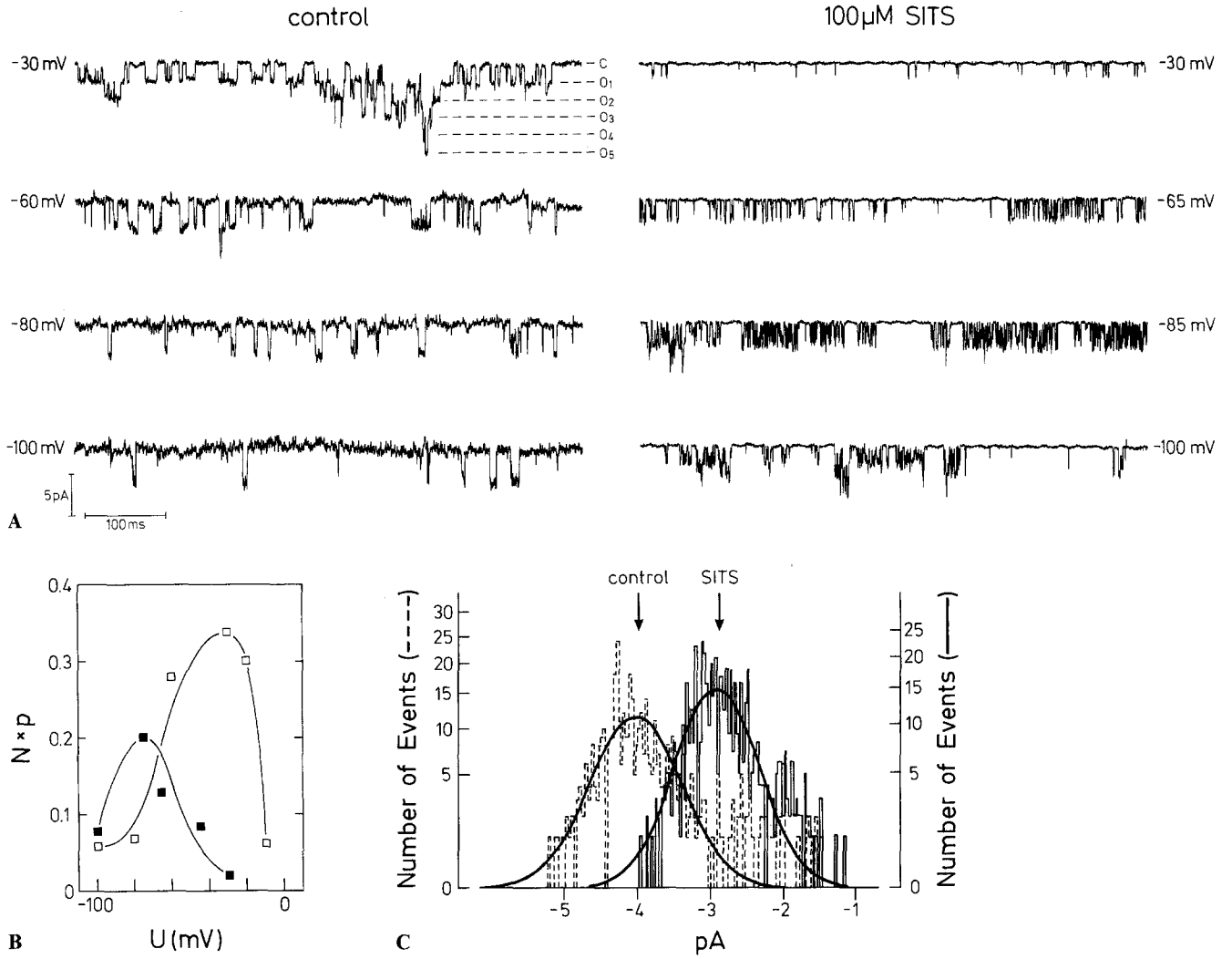

Fig. 4 A–C. Modulation of the properties of single anion-channels by the stilbene derivative SITS. **A** Single-channel activity recorded from an outside-out membrane patch in the absence (*left lane*) and presence of 100 μM SITS (*right lane*) with the membrane potential clamped as indicated. **B** Single-channel activity before (*open symbols*) and after SITS application (*closed symbols*). Single channel activity (N · p) is expressed by the number (N) of channels in the membrane patch and their open-probability (p). **C** Distribution of single-channel amplitudes in the presence and absence of 100 μM SITS at −100 mV

trol cells as well as DNDS treated cells could be described by single exponentials. The rise time of activation decreased with depolarizing voltage pulses. In control cells anion currents activated at −60 mV and −20 mV with time constants of 13.4 ms and 5.2 ms. The rise-times of DNDS inhibited anion currents were, however, reduced ($\tau_{-60\,mV} = 9.2$ ms; $\tau_{-20\,mV} = 3.5$ ms) with respect to the peak current potential of the non-blocked channel. Additionally, a pronounced decay in anion current with respect to control cells ($\tau_{+40\,mV} = 17.0$ ms) was observed in the presence of DNDS ($\tau_{+40\,mV} = 12.2$ ms; Fig. 3).

Modulation of gating-characteristics

In our previous work, single-channel analysis demonstrated that whole-cell anion currents are carried by 32–40 pS channels. Deactivation of anion currents at hyperpolarized potentials was shown to be the result of an increase in the closed-times of the anion channel (Hedrich et al. 1990).

In order to determine the type of block imposed by the inhibitors, outside-out patches were exposed to inhibitor-containing media (Fig. 4A). A common observation with stilbene inhibitors is their capability to transform long open-intervals of the channel into flickering bursts, reminiscent of the "intermediate block" described for some animal anion channel inhibitors (Gögelein 1988). Figure 4A shows fluctuations in open-closed transitions of up to five anion channels in the absence (left panel) and presence of 100 μM SITS (right panel). Upon the application of the effector the peak activity, expressed by the number (N) of channels multiplied by the open-probability (p), shifted towards more hyperpolarized potentials (Fig. 4B) as shown for the macroscopic anion currents (Fig. 2). Besides the shift in voltage-dependence SITS reduced the unit conductance by about 23% (Fig. 4C), characteristic for a "very fast block" (Hille 1992).

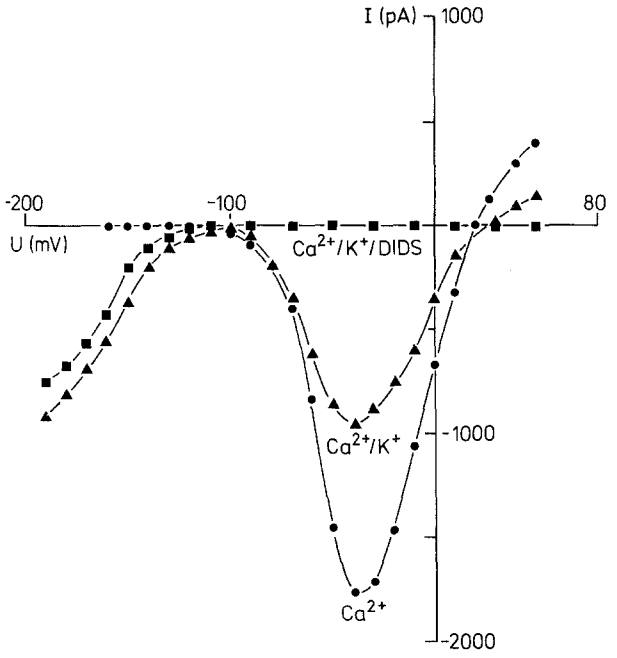

Fig. 5. Simultaneously recording of voltage-dependent potassium and anion channels in the absence (*triangles and circles*) and presence (*squares*) of 100 μM DIDS. Replacement of extracellular Ca^{2+} by K^+ causes a decrease in the anion current (*circles*) and an increase in the inward K^+ current (*triangles*). Similar observations were made in seven other experiments. Current-voltage relations of K^+ and anion channels resulted from voltage-ramps as described in the legend to Fig. 1

Stilbenes as blockers specific for GCAC1

To demonstrate the channel-specifity of anion channel blockers, the most effective blocker of guard cell anion channels, DIDS, was selected to investigate the behaviour of plasma membrane K^+ channels during inhibitor action. In the presence of external 40 mM $CaCl_2$ and 150 mM KCl in the pipette inward currents were carried by chloride efflux only, as characterized by the inward hump between E_{Cl^-} and −100 mV and a high resistance range negative to −100 mV. Upon replacement of 40 mM $CaCl_2$ by 30 mM KCl, in the presence of 1 mM $CaCl_2$ inward K^+ currents were elicited at voltages negative to −100 mV (Fig. 5). Voltage-ramps from −200 to +60 mV from a holding potential of −100 mV allowed us to record the activity of K^+_{in} and Cl^- channels simultaneously (see also Marten et al. 1991 a). Upon the application of DIDS, anion currents were blocked (Fig. 5), whereas potassium currents were not affected. This indicates that K^+_{in} channels of guard cells are not inhibited by DIDS.

Discussion

Ion channel inhibitors such as NPPB, IAA-94, A-9-C, or niflumic acid (Marten et al. 1992) have in common that they all block GCAC1 and shift its voltage gate. Since these blockers are chemically non-related we were not able to conclude the structural requirements for a compound to either block or modulate the voltage sensor of GCAC1.

Here we selected stilbene derivatives, a class of closely related and chemically rather simple compounds to elucidate their effects on the two sites of GCAC1. Stilbenes have already been used to generate a pharmacological fingerprint to compare various anion transporters (Greger 1992, Cabantchik and Greger 1992). The efficiency (K_d) of DIDS within anion transporters from animal origin ranges from ineffective to high-affinity block (for review see Greger 1992).

In agreement with studies on the anion exchanger of red blood cells (Passow 1992) the efficiency (K_d in μM) of stilbene action followed the sequence DIDS (0.2) > DNDS (0.5) > SITS (4), indicating the presence of a high-affinity ligand binding site in GCAC1. A detailed analysis of the high affinity stilbene block was provided on band 3, where the primary structure is known. Site-directed mutagenesis and functional expression of anion transport in frog oocytes give rise to the conclusion that at least two lysine residues represent stilbene binding sites. DIDS interaction (two isothiocyanate groups, NCS) with both sites was supposed to result in an intramolecular cross-link and in turn block of the transporter. Whereas DIDS block of GCAC1 is not in conflict with this hypothesis, the reversibility of DIDS block and the fact that shift and block also result from structurally non-related anion channel inhibitors (Marten et al. 1992) is not in line with Lys-stilbene-Lys interaction. In the absence of sequence information of the plant anion channel it is thus too early to ask for a conserved "stilbene pocket" on GCAC1.

Whereas DIDS only affected the ion permeation, SITS and DNDS action was characterized by the superposition of the block by a shift in the voltage gate. We could, however, not exclude the possibility that DIDS shifts the voltage sensor out of the potential range tolerated by guard cell protoplasts. In addition to block and shift, DNDS caused a voltage-(depolarization) induced decrease in decay times of the anion current (Fig. 3). Tail currents resulting from voltage-pulses (hyperpolarization to −200 mV following depolarizing voltage steps) were not reduced to the same extent as the steady state currents. This observation as well as 'unblock' during hyperpolarization indicates a voltage-dependent action of SITS and DNDS on GCAC1 rather than a change in inactivation (data not shown).

In conclusion, it seems that analysis of additional stilbene derivatives is required to correlate the structure of an effector to its function as a blocker or gating modifier on GCAC1.

Extracellular growth hormones have already been shown to alter the voltage sensor (Marten et al. 1991 a). Up to now all gating modifiers studied were anionic under our experimental conditions. It is thus tempting to speculate that the nature of the ligand occupying an 'anion binding site' at the external face of GCAC1 controls the location of the voltage gate. On the other hand, residues such as aryl-, alkyl- or sulfonyl groups may determine whether and how (fast-slow block) the compound occludes the channel pore.

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Note added in proof. Zn^{2+} was shown to block background anion channels in a previous study (Keller et al. 1989), in suspension cultured cells (Schauf and Wilson), or SV-type channel of the vacuolar membrane (Hedrich and Kurkdjian 1988). However, in a recent investigation, 100 μM Zn^{2+} ($n = 10$ and 6 for extracellular and intracellular application) neither blocked nor shifted the activation potential of the Ca^{2+}- and nucleotide-activated anion channel.

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Appendix

Statistics to Fig. 2 B, C: The number (n) of experiments, the inhibition of GCAC1 (I) ± standard deviation (SSD) in %, and the shift (S) of peak current ±SSD in mV for the various stilbene concentrations are as follows:

Fig. 2 B

Concentration (μM)	n	I±SSD (%)
100	17	100
10	4	97.4±5.8
1	6	80.5±9.4
0.1	4	34.5±10.6

Fig. 2 C

Concentration (μM)	n	I±SSD (%)	S±SSD (mV)
100	3	98.4±2.8	−74.6±2.2
10	6	92.6±4.5	−52.1±6.6
5	6	85.0±8.7	−40.7±5.7
2	4	75.4±8.2	−25.5±6.7
1	7	66.6±12.3	−23.2±7.2
0.5	9	48.0±10.3	−10.6±3.6
0.2	8	31.4±12.1	−4.0±2.3
0.1	3	0	0