J. Membrane Biol. 137, 249–259 (1994)

The Journal of
Membrane
Biology

Selective Block by α-Dendrotoxin of the K^+ Inward Rectifier at the *Vicia* Guard Cell Plasma Membrane

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Received: 4 August 1993/Revised: 12 October 1993

Abstract. The efficacy and mechanism of α-dendrotoxin (DTX) block of K^+ channel currents in *Vicia* stomatal guard cells was examined. Currents carried by inward- and outward-rectifying K^+ channels were determined under voltage clamp in intact guard cells, and block was characterized as a function of DTX and external K^+ (K_o^+) concentrations. Added to the bath, 0.1–30 nM DTX blocked the inward-rectifying K^+ current ($I_{K,in}$), but was ineffective in blocking current through the outward-rectifying K^+ channels ($I_{K,out}$) even at concentrations of 30 nM. DTX block was independent of clamp voltage and had no significant effect on the voltage-dependent kinetics for $I_{K,in}$, neither altering its activation at voltages negative of −120 mV nor its deactivation at more positive voltages. No evidence was found for a use dependence to DTX action. Block of $I_{K,in}$ followed a simple titration function with an apparent $K_{1/2}$ for block of 2.2 nM in 3 mM K_o^+. However, DTX block was dependent on the external K^+ concentration. Raising K_o^+ from 3 to 30 mM slowed block and resulted in a 60–70% reduction in its efficacy (apparent K_i = 10 mM in 10 nM DTX). The effect of K^+ in protecting $I_{K,in}$ was competitive with DTX and specific for permeant cations. A joint analysis of $I_{K,in}$ block with DTX and K^+ concentration was consistent with a single class of binding sites with a K_d for DTX of 240 pM. A K_d of 410 μM for extracellular K^+ was also indicated. These results complement previous studies implicating a binding site requiring extracellular K^+ ($K_{1/2} \sim 1$ mM) for $I_{K,in}$ activation; they parallel features of K^+ channel block by DTX and related peptide toxins in many animal cells, demonstrating the sensitivity of plant plasma membrane K^+ channels to nanomolar toxin concentrations under physiological conditions; the data also highlight one main difference: in the guard cells, DTX action appears specific to the K^+ inward rectifier.

Key Words: *Vicia* — Stomatal guard cell — K^+ channel, inward rectifier — K^+ channel, outward rectifier — Pharmacology — Plasma membrane

Introduction

Combined with electrophysiological methods, the specificity of peptide toxins isolated from reptilian, arthropod, mollusc and insect venoms has provided some of the most powerful tools for identifying and characterizing ion channels in biological membranes. In the case of K^+ channels, for which a spectrum of channel types are often present in any one cell, peptide toxins have proven useful in dissecting the component K^+ currents to identify their physiological contribution to global cellular K^+ transport characteristics (Halliwell et al., 1986; Moczydlowski, Lucchesi & Ravindran, 1988; Jonas et al., 1989). Furthermore, the high affinity of toxin binding—often in the nanomolar to subnanomolar concentration range—has provided the means for biochemical characterizations of channel proteins (Talvenheimo, 1985; Catterall, 1988; Moczydlowski et al., 1988; Parcej & Dolly, 1989; Scott et al., 1990).

Remarkably, few studies have explored the utility of peptide toxins as potential tools in transport analyses of plants, especially of K^+ transport characteristics, despite growing evidence of the broad structural (Anderson et al., 1992; Jan & Jan, 1992; Sentenac et al., 1992) and biophysical (Tester, 1990; Blatt, 1991*a*; Blatt & Thiel, 1993) parallels between K^+ channels of animal and plant membranes. There are several prelimi-

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nary reports indicating that some of the toxins which target selected classes of K^+ channels in animals are effective in blocking K^+ channels of plant membranes. Ketchum and Poole (1990) described the block of a putative Ca^{2+}-dependent K^+ current in *Zea* protoplasts by the scorpion venom toxin charybdotoxin (ChTX), and a similar activity of ChTX was observed in *Chenopodium rubrum* vacuoles (Weiser & Bentrup, 1991). Both ChTX and α-dendrotoxin (DTX), a component of African Green Mamba venom, have also been reported to block K^+ channels of *Vicia* guard cells (Blatt, 1992).

It must be stressed that, to date, little information about the characteristics of channel block or toxin efficacy has been forthcoming. Indeed, in only one study have any quantitative characteristics of block been established: Weiser and Bentrup (1991) reported an apparent $K_{1/2}$ of 20 nM for ChTX block of the vacuolar SV-type channel from *Chenopodium*. Furthermore, almost no evidence is available which could bear on the selectivities of these toxins among plant K^+ channels. Indeed, channel block has more often been cited with reference a priori to the known toxin specificities among animal K^+ channels (Tester, 1988; Weiser & Bentrup, 1991), notably the affinity of ChTX for Ca^{2+}-activated K^+ channels (Miller et al., 1985; Moczydlowski et al., 1988; Lucchesi et al., 1989). There have been some reports of toxin insensitivity: Tester (1988) failed to identify an action of ChTX, DTX or the bee venom toxin apamin—effective in animal cells on a subclass of small conductance, Ca^{2+}-activated K^+ channels (Blatz & Magleby, 1986; Moczydlowski et al., 1988)—on K^+ channel currents in the giant alga *Chara*; and when incorporated in planar bilayers the activity of K^+ channels from *Secale* root plasma membrane has also proven to be unaffected by ChTX (White & Tester, 1992). Both studies are complicated, however, by questions of access to the extracellular mouth of the channel(s) (*cf.* Blatt, 1992) where toxin binding and block takes place (Moczydlowski et al., 1988; MacKinnon & Miller, 1989). Hence, the lack of effect in these instances cannot speak directly to the issue of specificity.

There is already substantial evidence in stomatal guard cells for two discrete populations of K^+ channels with different biophysical, pharmacological and functional characteristics (Blatt, 1991*a;* Blatt & Thiel, 1993). One class of channels generates a current which rectifies strongly outward ($I_{K,out}$) subject to the prevailing external K^+ concentration. This current shows a pronounced sensitivity to the K^+ channel blocker tetraethylammonium chloride (TEA) and intracellular pH (pH_i), but is largely insensitive to extracellular pH (pH_o) and cytoplasmic-free $[Ca^{2+}]$. The second class of K^+ channels generates a current which rectifies inward ($I_{K,in}$) but which, in its voltage dependence for gating, is insensitive to extracellular K^+ concentration. This current is roughly an order of magnitude less sensitive to TEA than $I_{K,out}$, but its gating is strongly dependent on pH_o and cytoplasmic-free $[Ca^{2+}]$. Thus, the recent observations of $I_{K,in}$ block by ChTX and DTX (Blatt, 1992) offered a unique opportunity to examine toxin efficacy and specificity between the two distinct classes of K^+ channels. Here, we report on studies with DTX carried out with intact *Vicia* stomatal guard cells. The results show that nanomolar concentrations of the toxin are effective in K^+ channel block and that block can be antagonized by raising $[K^+]_o$ prior to DTX additions. The results also demonstrate a pronounced selectivity of DTX for the K^+ inward rectifier.

Materials and Methods

Plants and Experimental Protocol

Plants of *Vicia faba* L. var. Bunyard's Exhibition were grown in vermiculite on modified Hoagland's medium (Blatt, 1987*a*) at 16–18°C and under controlled daylength (16 hr light: 8 hr dark). For experiments, only the youngest newly expanded leaves were chosen and harvested just prior to each experiment. Epidermal strips were taken from the abaxial surface and mounted on a glass chamber, precoated with an optically clear, pressure-sensitive silicon adhesive (Medical Adhesive 355, Dow Corning, Brussels). The chamber was then filled with standard buffer solution. All operations were carried out on a Zeiss IM inverted microscope (Zeiss, Oberkochen, FRG) fitted with Nomarski Differential Interference Contrast optics. Surface areas and volumes of impaled cells were calculated assuming a cylindrical geometry (Blatt, 1987*a*). The orthogonal dimensions (diameter, length) of the cells, and stomatal apertures, were measured with a calibrated eye-piece micrometer.

Experiments were carried out in solutions of 5 mM Ca^{2+}-MES buffer, pH 6.1 (5 mM 2-(N-morpholino)-propanesulfonic acid titrated to its pK_a with $Ca(OH)_2$, final $[Ca^{2+}] \sim 1$ mM). KCl, RbCl, NaCl, and α-dendrotoxin (DTX) were added as required. DTX was prepared as a 1 μM stock in H_2O, aliquoted and stored at −70°C prior to experiments. Solutions containing DTX were added directly to the chamber under stopped-flow conditions using a syringe. To ensure complete exchange, 10 chamber volumes were added (*ca.* 5 ml) in each trial. Control measurements were also carried out with buffer alone and showed no significant difference in membrane electrical characteristics with measurements carried out under the normal continuous flow conditions (*not shown*).

Electrical

Electrophysiological recordings were carried out using double-barrelled microelectrodes pulled using a modified Narashige horizontal puller (PD-5, Narashige Instruments, Tokyo). Current-passing and voltage-recording barrels were filled with 200 mM K^+-acetate, pH 7.5, to minimize salt leakage and salt-loading artifacts associated with the Cl^- anion (Blatt, 1987*a*). In all cases, connection to the amplifier headstage was via a 1 M KCl|Ag-AgCl halfcell, and a matching halfcell and 1 M KCl-agar bridge served as the reference (bath) electrode.

Mechanical, electrical and software design have been described in detail (Blatt, 1987*a,b,* 1991*b*). Current-voltage (*I-V*) relations were determined by the two-electrode method with the voltage clamp under microprocessor control using a WyeScience μP amplifier and

μLAB analog/digital interface and software (WyeScience, Wye, Kent). Steady-state *I-V* relations were recorded by clamping cells to a bipolar staircase of command voltages (Blatt, 1987*b*). Steps alternated positive and negative from the free-running membrane potential, V_m (typically 20 bipolar pulse-pairs) and were separated by equivalent periods when the membrane was clamped to V_m. The current signal was filtered by a 6-pole Butterworth filter at 0.3 or 1 kHz (−3 dB) before sampling, and currents and voltages were recorded during the final 10 msec of each pulse.

For time-dependent characteristics, current and voltage were sampled continuously at 0.5, 1, or 2 kHz while the clamped potential was driven through cycles of 1–4 programmable pulse steps. In most cases, the holding potential was set to V_m at the start of the clamp cycle. In some experiments, the time course for the development of DTX block was monitored with single voltage clamp steps of 4 sec duration, alternating positive and negative from V_m, and repeated every 10–30 sec. The sampling rate for most of the data shown was 0.5 kHz; however, recordings at 2 kHz (above the Nyquist limit) gave similar results. No attempt was made to compensate for the series resistance (R_s) to ground (Hodgkin, Huxley & Katz, 1952). Estimates for R_s indicated that it was unlikely to pose a serious problem in measurements of clamp potential (Blatt, 1988), despite the often high resistivity of the bathing media (=2.5 kΩ cm for 5 mM Ca^{2+}-HEPES with 0.1 mM KCl).

Numerical Analysis

Data analysis was carried out by nonlinear, least-squares (Marquardt, 1963) and, where appropriate, results are reported as the mean ± standard error (SE) of (n) observations.

Chemicals and Solutions

The pH buffer MES was from Sigma (St. Louis, MO) and α-dendrotoxin was from CalBiochem (San Diego, CA). Otherwise, all chemicals were Analytical Grade from BDH (Poole, Dorset, UK).

Results

Specificity of DTX for $I_{K,in}$

Previous studies have shown that the steady-state current-voltage (*I-V*) characteristics of intact guard cells are dominated by two distinct K^+ channel currents when bathed in 5 mM Ca^{2+}-MES, pH 6.1, with 3–30 mM KCl (Blatt, 1991*a*, 1992; Thiel, MacRobbie & Blatt, 1992; Blatt & Thiel, 1993). Comparable results were obtained in this study. In the presence of millimolar K_o^+, clamping the guard cell membrane to voltages negative of −120 mV uncovered the characteristic time- and voltage-dependent activation of current through inward-rectifying K^+ channels ($I_{K,in}$) which deactivated rapidly on a subsequent positive-going clamp step (*see* Fig. 1); in turn driving the membrane to voltages positive of the K^+ equilibrium potential ($E_K \sim -70$ mV in 10 mM K_o^+) yielded a complementary time- and voltage-dependent current (*not shown; see* Blatt, 1992; Blatt & Thiel, 1993) carried by a second class of outward-rectifying K^+ channels ($I_{K,out}$, *see* Fig. 2).

Figure 1 shows the response of the free-running membrane potential and of $I_{K,in}$ recorded from one guard cell before and after additions of DTX. Comparable results were obtained in all other 18 cells exposed to DTX in 3 mM KCl (*see also* Figs. 3 and 5). As shown, additions of DTX against a background of 3 mM K_o^+ resulted in progressive reductions in $I_{K,in}$ with DTX concentration. Toxin block resulted in a roughly scalar decline in $I_{K,in}$ and virtually complete block of the current was seen in 10 nM DTX. These characteristics are most evident in the steady-state *I-V* relations obtained after subtracting the background of instantaneous currents recorded at the start of each clamp step from the steady-state currents recorded at the end of each step (Fig. 1*C*, *see also* Fig. 4). DTX block was partially reversible, but the recovery was slow and variable, and was only observed following exposures to low toxin concentrations (≤2 nM); in no case was $I_{K,in}$ seen to recover after exposures to 10 or 30 nM DTX, even after subsequently washing with DTX-free medium for up to 34 min. For this reason, measurements were generally carried out with fresh tissue for each trial, although sequential challenge with increasing DTX concentrations gave comparable results (*see* Fig. 5), and analyses of data gathered in DTX were juxtaposed with similar measurements gathered prior to toxin exposures.

By contrast with its action on $I_{K,in}$, DTX was ineffective in blocking current through the K^+ outward rectifier ($I_{K,out}$), even at concentrations of 25 nM in 3 mM K_o^+. Figure 2 summarizes the steady-state characteristics for $I_{K,out}$ obtained concurrently with the data shown in Fig. 1. Recordings in this instance were obtained using a bipolar staircase protocol to minimize data acquisition time over the entire voltage spectrum, and background currents (dashed line, inset) were estimated by linear extrapolation from data points between −120 mV and E_K (Blatt, 1990, 1992). Qualitatively comparable results were obtained in all experiments and also using a two-step protocol with a conditioning voltage of −100 mV and test voltage steps between −80 and +50 mV (*not shown, see* Blatt, 1992; Blatt & Thiel, 1993). Indeed, in five of the nine cells challenged with 0.1–25 nM DTX in 3 mM KCl, $I_{K,out}$ *increased* in the steady-state by 4–12.3% at a clamp voltage of 0 mV.

$I_{K,IN}$ Activation and Deactivation under DTX Block

K^+ channel block by DTX and related peptide toxins is commonly associated with pronounced alterations in channel gating—marked in single channel recordings by the appearance of long-lived closed states associated with blocked configurations of the channels (Anderson et al., 1988; Stansfeld & Feltz, 1988; Rehm et al., 1989; Candia, Garcia & Latorre, 1992). Thus, we were interested to determine whether DTX block of $I_{K,in}$ might

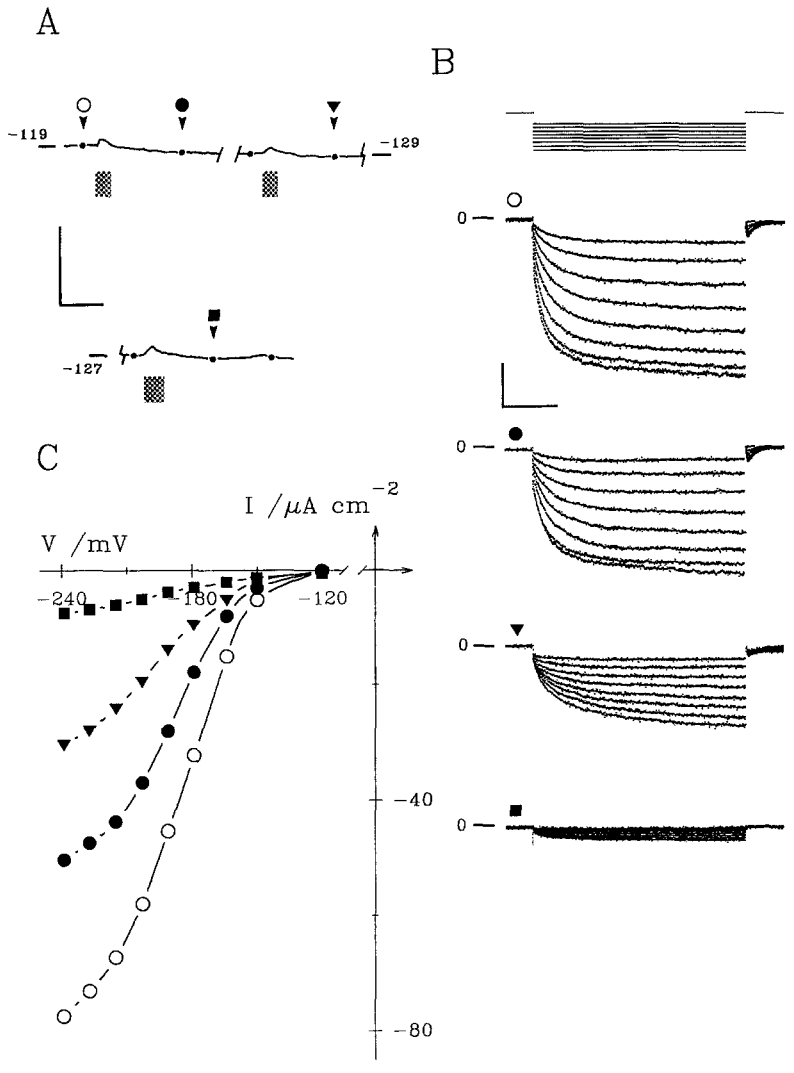

Fig. 1. K^+ inward-rectifier ($I_{K,in}$) block by α-dendrotoxin (DTX). Data from one *Vicia* guard cell bathed in 5 mM Ca^{2+}-MES, pH 6.1, with 3 mM KCl before (○) and after challenge with 1 (●), 5 (▼) and 10 nM (■) DTX. Cell parameters: surface area, 1.8×10^{-5} cm^2; volume 4.9 pl. (*A*) Free-running membrane potential trace (values in mV) with periods of DTX perfusion indicated by the bars below (clockwise, 1, 5 and 10 nM). Scale: vertical, 40 mV; horizontal, 3 min. Note the breaks in the trace. Total elapsed time of trace, 22 min. Periods of voltage-clamp scans (masked from trace) are indicated by dots, cross-referenced to *B* and *C* by symbol. (*B*) $I_{K,in}$ trajectories recorded before (○) and during exposures to 1 nM (●), 5 nM (▼) and 10 nM DTX (■). Current zero indicated on left. Clamp cycle voltages (above): conditioning, −120 mV; test (8), −150 to −240 mV; tailing, −120 mV. Scale: vertical, 100 mV or 20 μA cm^{-2}; horizontal, 500 msec. (*C*) Steady-state current-voltage relations for $I_{K,in}$ taken from currents recorded at the end of the test voltage pulses in *B* after subtracting the instantaneous background currents recorded <5 msec into pulses at each test voltage (*see* Blatt, 1992). Note the approx. voltage-independent block of the current. K^+ current block had little effect on the free-running potential in this cell, because the membrane voltage was already poised largely outside the voltage range in which the channels were active.

also be detected in the gating kinetics of the macroscopic current and its voltage dependence. Figures 3, 4, and the Table, summarize the analyses of $I_{K,in}$ activation and deactivation kinetics in 3 mM KCl and their dependence of DTX concentration as well as the steady-state voltage dependence of block. Both activation (Fig. 3*A*) and deactivation (Table) halftimes ($t_{1/2}$) were determined by exponential fittings of the current trajectories after first subtracting the instantaneous current component. In neither case was a significant effect of DTX on macroscopic current kinetics evident. Much the same conclusion was drawn from analyses carried out on a cell-by-cell basis (Fig. 3*B*), and no indication of a voltage dependence to DTX block could be deduced from the steady-state currents (Fig. 4).

Measurements were also carried out to determine whether K^+ channel activation promoted DTX block (use dependence). In these experiments, guard cells (n = 5) were clamped from holding potentials positive of −100 mV to membrane voltages of −200 or −220 mV for periods of up to 4 sec and the clamp cycles repeated 8–16 times at 10–30 sec intervals once an apparent steady-state of block was achieved. However, in no case was there an appreciable time-dependent "inactivation" or rundown of the clamp current, nor was DTX block further enhanced by the steady-state activation of $I_{K,in}$ (*not shown*).

$I_{K,IN}$ Block and its Dependence on DTX and Extracellular K^+

The data outlined above demonstrated a potency for $I_{K,in}$ block by DTX in the nanomolar concentration range and comparable to that for K^+ channel block of neuronal tissues in vivo (Halliwell et al., 1986; Stansfeld & Feltz, 1988; Schweitz et al., 1989; Bräu et al., 1990) and of expressed RCK clones (Stühmer et al.,

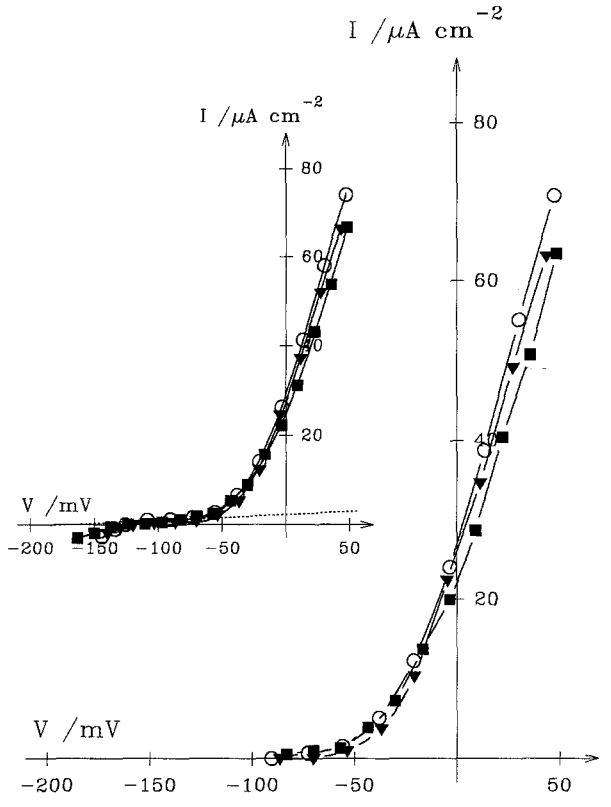

Fig. 2. K^+ outward-rectifier ($I_{K,out}$) insensitivity to DTX. Data from the same guard cell as in Fig. 1, cross-referenced to DTX treatment by symbol (○, control; ▼, 5 nM DTX; ■, 10 nM DTX). Steady-state currents were recorded using a bipolar staircase protocol to record whole membrane current (inset). $I_{K,out}$, shown in the main frame, was determined by subtracting the background current from points positive of E_K. Background current estimated by linear extrapolation from data points between E_K and −120 mV (inset, dotted line).

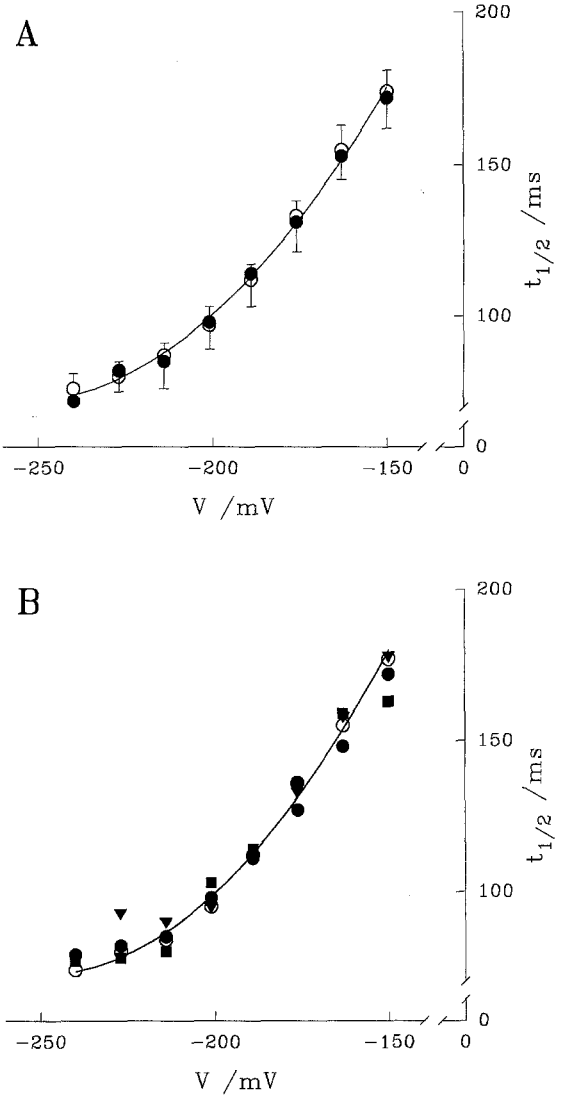

Fig. 3. Insensitivity to DTX of the activation kinetics for the K^+ inward rectifier. Halftimes ($t_{1/2}$) for activation of $I_{K,in}$ determined as a function of clamp voltage following conditioning clamp steps at −120 mV after subtracting the instantaneous current recorded at each voltage. (*A*) Mean halftimes (±SE) pooled for exposures to 2–10 nM DTX (n = 9 *Vicia* guard cells). Open circles (○) are measurements from the same cells prior to DTX additions. (*B*) Half-times for the data shown in Fig. 1, cross-referenced by symbol (○, control; ●, 1 nM DTX; ▼, 5 nM DTX; ■, 10 nM DTX).

1989). They also highlighted a striking disparity between animal and plant K^+ channels targeted by the toxin, the principal channels affected in the guard cells being a class of *inward* rectifiers. This difference raises a question about the process(es) of DTX action in the guard cells. There are good reasons to anticipate that a common mechanism of block depends on toxin entry into the mouth of the channel pore and subsequent interactions between the (basic) toxin and acidic residues in channel mouth (Moczydlowski et al., 1988; MacKinnon & Miller, 1989; Stühmer et al., 1989). DTX block in nerve (Bräu et al., 1990), like that of ChTX (Anderson et al., 1988) and iberiotoxin (IbTX) (Candia et al., 1992) block of skeletal Ca^{2+}-activated K^+ channels, is subject to the ionic strength of the medium, a behavior which reflects a competition between toxin and monovalent cation within the mouth of the channel. Thus, an immediate issue was whether $I_{K,in}$ block by DTX exhibited a comparable dependence on monovalent (permeant) cation concentration.

To further detail toxin interaction with the K^+ channels, measurements of $I_{K,in}$ were carried out over a range of DTX and external K^+ concentrations. Figure 5 summarizes the results from the 18 cells challenged with DTX concentrations ranging from 0.1 to 25 nM against a background of 3 mM KCl. DTX block was adequately fitted by a simple bimolecular binding function according to the scheme

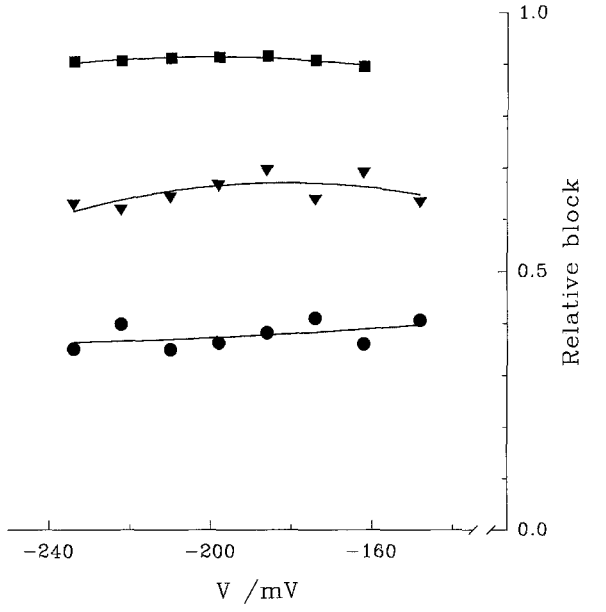

Fig. 4. Voltage dependence of $I_{K,in}$ block by DTX. Block calculated for the data in Fig. 1 relative to the steady-state current in the absence of DTX ($I_{K,in}{}^o$) as 1-($I_{K,in}/I_{K,in}{}^o$) at each clamp voltage. Symbols cross-reference to Fig. 1 (●, 1 nM DTX; ▼, 5 nM DTX; ■, 10 nM DTX). Comparable results were obtained in all guard cells examined.

Table. DTX insensitivity of $I_{K,in}$ deactivation.[a]

[DTX]/nM	$t_{1/2}$/msec	n
	47 ± 4	6
1	54 ± 5	3
2	44 ± 6	3
10	51 ± 6	3

[a] Halftimes for deactivation determined from exponential fittings to currents recorded at −120 mV following conditioning clamp steps to −220 mV (*see* Blatt, 1992). Data from n cells bathed in 5 mM Ca^{2+}-MES, pH 6.1, with 3 mM KCl with data from each cell pooled from ≥4 separate recordings.

$$\text{active channel} \underset{k_{-1}}{\overset{k_1[\text{DTX}]}{\rightleftharpoons}} \text{blocked channel} \qquad [S1]$$

which predicts a dependence on DTX concentration obeying the relationship for relative block

$$1 - I_{K,in}/I_{K,in}{}^o = \frac{B_{max}}{1 + K_d/[\text{DTX}]} \qquad (1)$$

The analysis yielded an apparent K_d ($=k_{-1}/k_1$) of 2.2 ± 0.6 nM and B_{max} (maximum fractional block) of 0.98 ± 0.04, consistent with a single DTX-binding site and 100% block at saturating DTX concentrations.

Experiments were also carried out at external K^+ concentrations ranging from 3 to 30 mM while challenging guard cells with 10 nM DTX. Within this concentration range, raising K_o^+ did not uncover a steady-state voltage dependence to DTX block, nor again was an effect of DTX evident in the voltage-dependent kinetics for $I_{K,in}$ activation and deactivation (*not shown*). However, elevating K_o^+ prior to DTX additions was observed to protect the current from block by the toxin. In K_o^+ concentrations above 3 mM, the progress of block was markedly slowed such that in 30 mM KCl a steady-state level of block was reached only after 6–8 min exposure. Figure 6 shows the time courses for block with 10 nM DTX from three guard cells bathed in standard buffer with 3, 7 and 30 mM KCl. The measurements shown were carried out by monitoring $I_{K,in}$ with single voltage-clamp steps of 4 sec duration repeated at 10–30 sec intervals. Comparable results were obtained in trials with the other cells at each K_o^+ concentration, albeit from measurements carried out over broader intervals.

The data in Fig. 6 also mark a decline in the efficacy of steady-state DTX block with increasing K_o^+. The effect is clearly seen in Fig. 7 in which the fractional current relative to the control before DTX addition, $I_{K,in}/I_{K,in}{}^o$, is plotted as a function of monovalent cation concentration. The ability for K_o^+ to protect against DTX block could be overcome by raising the

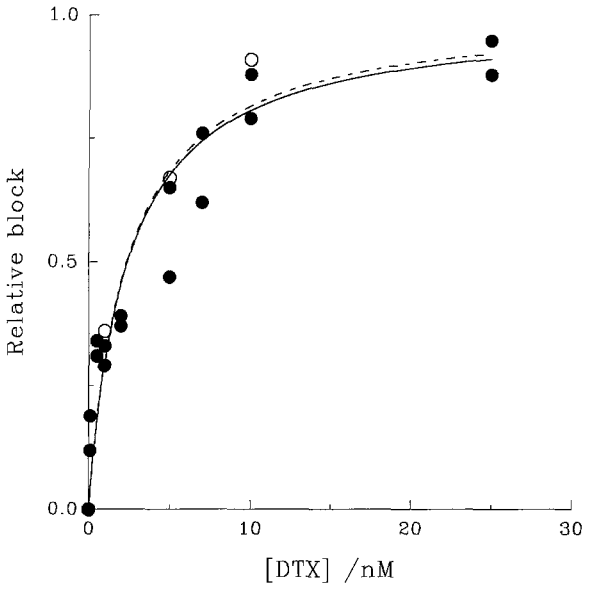

Fig. 5. Concentration dependence for DTX block of $I_{K,in}$. Data pooled from 18 *Vicia* guard cells bathed in 5 mM Ca^{2+}-MES, pH 6.1, with 3 mM KCl. Data from the cell in Fig. 1 indicated by the open symbols. Relative block calculated as in Fig. 4 for currents recorded at −200 mV. The unbroken curve is the result of fitting to Eq. (1), the dashed curve is the result of fitting to Eq. (2) jointly with the data in Fig. 7. Unbroken curve parameters: apparent K_d, 2.2 ± 0.6 nM; B_{max}, 0.98 ± 0.04. Dashed curve parameters: K_d, 240 ± 20 pM; K_i ($=K_d$ for K_o^+), 410 ± 30 μM; B_{max}, 0.97 ± 0.05.

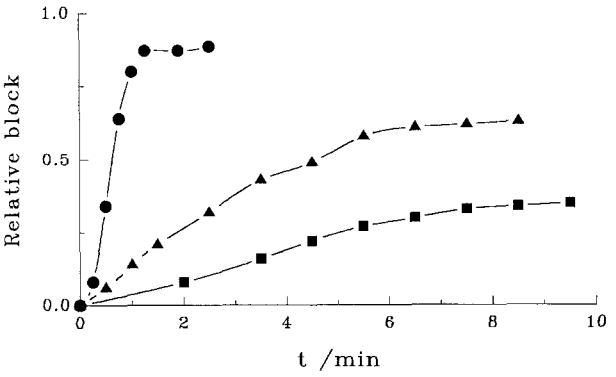

Fig. 6. Time course for DTX block of the K^+ inward rectifier. Data from three *Vicia* guard cells challenged with 10 nM DTX. The cells were bathed in 5 mM Ca^{2+}-MES, pH 6.1, with 3 mM (●), 7 mM (▲) and 30 mM (■) KCl. Measurements shown were carried out by monitoring $I_{K,in}$ with single voltage-clamp steps to −220 mV of 4 sec duration repeated at 10–60 sec intervals. Note the slowing and protection from block afforded by raising the KCl concentration. Steady-state block was achieved in 1–2 min with 3 mM KCl in all cases, while in all three trials against a background of 30 mM KCl, steady-state block was realized only after 6–8 min in DTX.

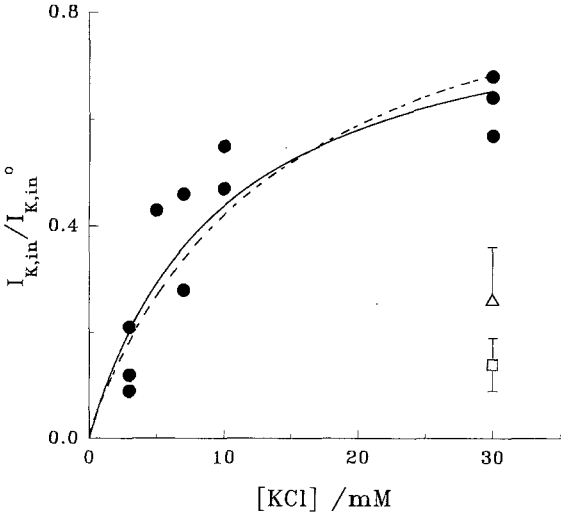

Fig. 7. Protection of $I_{K,in}$ from DTX block by monovalent cations outside. Data from 17 *Vicia* guard cells challenged with 10 nM DTX. Cells bathed in 5 mM Ca^{2+}-MES, pH 6.1, with the monovalent cation concentrations indicated on the abscissa. Data are plotted as the steady-state current at −200 mV relative to the controls ($I_{K,in}/I_{K,in}^o$). The unbroken curve is the result of fitting measurements in KCl alone to the equation $I_{K,in}/I_{K,in}^o = B_{min}/(1 + Ki/[K^+]_o)$. Fitted parameters: K_i, 10 ± 3 mM; B_{min}, 0.89 ± 0.09. The dashed curve is the result of the joint fitting to Eq. (2) with the data in Fig. 5 (*see* Fig. 5 for details). Also shown are results of additional measurements (mean ± SE) with RbCl (Δ, n = 4 cells) and NaCl (□, n = 5 cells), including 3 mM KCl as the primary current-carrying ion [(Blatt, 1992); total monovalent cation concentration, 30 mM] and calculated as the current fraction over the mean in 3 mM KCl.

DTX concentration (*not shown*) and, to an extent, external Rb^+ could substitute for K_o^+; however, Na^+ substituted for K_o^+ only poorly (Fig. 7, open symbols). Protection from block can be treated following a scheme analogous to that for DTX block. In this case, analysis of the data gathered in K_o^+ yielded an apparent K_i of 10 ± 3 mM and a B_{min} (=1 − B_{max}) of 0.11 ± 0.09 for the cation in 10 nM DTX (Fig. 7, unbroken line). The results, thus, were consistent with a simple competition between permeant cation and the toxin.

Although an economical interpretation favors the simple bimolecular binding and competition model, it will be seen that the analysis is complicated on one account: while extracellular K^+ appeared to compete with DTX for binding, it was also nominally the charge-carrying ion and a prerequisite for the activity of $I_{K,in}$ (Blatt, 1992). So, a significant degree of interaction between DTX and K_o^+ is inherent to these measurements. To accommodate DTX block and its antagonism by K_o^+ Scheme S1 may be expanded to incorporate both components as

$$\text{Unbound channel} \underset{k_{-1}}{\overset{k_1[\text{DTX}]}{\rightleftharpoons}} \text{Blocked channel} \qquad [S2]$$

$$k_2[K^+] \Big\| k_{-2}$$

K^+-bound channel
(active)

This purely competitive scheme predicts a joint dependence on DTX and K_o^+ concentrations which obeys the relationship for relative block

$$1 - I_{K,in}/I_{K,in}^o = \frac{B_{max}}{1 + K_d(1 + [K^+]/K_i)/[DTX]} \qquad (2)$$

where the dissociation constant for DTX, $K_d = k_{-1}/k_1$, and the constant for K_o^+, $K_i = k_{-2}/k_2$. Joint fitting of the data in Figs. 5 and 7 was carried out according to Eq. (1). The analysis yielded visually satisfactory results with best fittings giving a value for K_d of 240 ± 20 pM, for K_i of 410 ± 30 μM and for B_{max} of 0.97 ± 0.05. These results are shown in each figure by the dashed lines.

Discussion

The results above clearly establish the K^+ inward rectifier as a target for DTX action at the plasma membrane of a higher plant cell type, the stomatal guard cell. That plant ion channels might be subject to experimental attack with neurotoxic peptides, including DTX, had been mooted in several recent studies (Ketchum & Poole,

1990; Weiser & Bentrup, 1991; Blatt, 1992). However, apart from these few preliminary investigations, essentially no information has been available until now which could speak to the efficacy, specificities or mechanisms of plant K^+ channel block by these toxins. Our data offer primary evidence for a high-affinity interaction in vivo of the guard cell K^+ channels with DTX. Block of the K^+ channel current—with an apparent $K_{1/2}$ of 2.2 nM in 3 mM KCl and a predicted K_d of 0.24 nM—highlights an efficacy comparable to that reported for a number of voltage-dependent K^+ channels in animal tissues (Moczydlowski et al., 1988; Stansfeld & Feltz, 1988; Jonas et al., 1989; Ruppersberg et al., 1990; Garcia et al., 1991). The data support recent findings of an (^{125}I)DTX-binding component in leaf plasma membrane fractions with an apparent K_d near 3 nM under similar K_o^+ conditions (G. Obermeyer, A. Butt, S. Jarvis and M. Blatt, *unpublished observations*). Furthermore, the electrophysiological analyses demonstrate an insensitivity to DTX of the other dominant class of (outward-rectifying) K^+ channels at the guard cell plasma membrane. Indeed, even in 25–30 nM DTX—concentrations roughly 10-fold above the $K_{1/2}$ for $I_{K,in}$—we found no evidence for toxin block of the K^+ outward rectifier. This specificity for the K^+ inward rectifier, thus, carries immediate implications for the utility of DTX in further biochemical and physiological work.

Comparative Pharmacology

Indeed, the most striking feature of DTX action in the guard cell is its selectivity for the K^+ inward rectifier, despite the otherwise broad parallels to channel block in animal cell preparations. In animal cells, DTX appears limited in action to a subfamily of outward-rectifying K^+ channels which includes neuromuscular delayed rectifiers, A-type and Ca^{2+}-dependent K^+ channels (Halliwell et al., 1986; Moczydlowski et al., 1988; Jonas et al., 1989; Rehm et al., 1989; Garcia et al., 1991). Block in each of these cases is evoked with nanomolar to subnanomolar toxin concentrations. In the guard cells, by contrast, both DTX and ChTX actions are evident in $I_{K,in}$ (*see also* Blatt, 1992) and, at least for DTX (*see* Figs. 1 and 2), this activity is clearly targeted specifically to this current.

Several biophysical characteristics of the guard cell K^+ channels do ally them with their "opposite" numbers found in the plasma membranes of many animals cells (Blatt & Thiel, 1993): (i) K_o^+ does not alter the voltage dependence of $I_{K,in}$ (Blatt, 1992), by contrast with the classic anomalous rectifiers of neuromuscular membranes and egg cells for which channel gating follows E_K (Hagiwara, Miyasaki & Rosenthal, 1976; Hagiwara & Yoshii, 1979; Balser, Roden & Bennett, 1991; Kubo et al., 1993), instead this dependence is evident in the gating of $I_{K,out}$ (Blatt, 1991*a*; Blatt & Thiel, 1993); (ii) gating of $I_{K,in}$ is strongly dependent on cytoplasmic-free [Ca^{2+}] and [H^+] (Schroeder & Hagiwara, 1989; Blatt, 1992; Blatt & Armstrong, 1993) in a manner analogous to the outward-rectifying epithelial and neuromuscular maxi-K^+ channels (Christensen & Zeuthen, 1987; Blatz & Magleby, 1987; Laurido et al., 1991); while (iii) gating of $I_{K,out}$ in the guard cells is independent of cytoplasmic-free [Ca^{2+}] (Schroeder & Hagiwara, 1989; Blatt, Thiel & Armstrong, 1990; Blatt & Armstrong, 1993).

These parallels also find support now in molecular characterizations of plant K^+ channels. Two distinct K^+ inward rectifiers have been identified from *Arabidopsis* (Anderson et al., 1992; Schachtman et al., 1992; Sentenac et al., 1992) which share fundamental structural features with the superfamily of K^+ outward rectifiers originally identified with the *Shaker* locus of *Drosophila* (Jan & Jan, 1992). This same parallel, thus, may go some way in explaining the selectivity of DTX for $I_{K,in}$, assuming that the guard cell channels share structural similarities with these proteins. [A complementary question relates to the nature of the guard cell K^+ outward rectifier and its insensitivity to DTX. We note that recent studies have uncovered the primary structures for two nucleotide-sensitive K^+ inward rectifiers from kidney and a macrophage cell line (Ho et al., 1993; Kubo et al., 1993). These appear to form a new structural class of K^+ channels. It will indeed be remarkable if their counterparts in plants behave as outward rectifiers!]

Mechanism of $I_{K,IN}$ Block by DTX

In animal cells, K^+ channel block by DTX, ChTX and related peptide toxins (Moczydlowski et al., 1988; Schweitz et al., 1989; Garcia et al., 1991) is thought to arise from electrostatic interactions with the channel and an occlusion of the channel mouth at the extracellular face of the membrane, rather than interference with the channel gate or with access for permeant ions from inside. Several lines of evidence support this interpretation. Among these, ChTX and IbTX block of Ca^{2+}-activated K^+ channels depend on toxin additions to the extracellular face of the membrane and follow simple bimolecular kinetics (Anderson et al., 1988; Candia et al., 1992); block is largely independent of channel state, open or closed [(Bräu et al., 1990; Candia et al., 1992) *but see* (Anderson et al., 1988)]; and at the single channel level block gives rise to long-lived, nonconducting states, but gating during unblock appears unaltered (Anderson et al., 1988; MacKinnon & Miller, 1988; Stansfeld & Feltz, 1988; Rehm et al., 1989; Candia et al., 1992). In addition, ChTX block is relieved by K^+ entering the channel pore from the inside and destabilizing the channel-ChTX complex ("knock-on" effect), a feature which accounts for the

voltage dependence of ChTX block (MacKinnon & Miller, 1988). Finally, DTX block, as well as that of ChTX and IbTX, is sensitive to the ionic strength of the medium: toxin block in each case is relieved competitively on raising the extracellular cation concentration, and both ChTX and IbTX block are antagonized by TEA which lodges in the channel mouth (Anderson et al., 1988; Bräu et al., 1990; Candia et al., 1992).

Block of the guard cell K^+ channels by DTX is broadly consistent with this picture on several accounts relating to block kinetics and the interaction with permeant cations, although there are some notable differences in detail. First, $I_{K,in}$ block, its dependence on DTX concentration and its antagonism by K_o^+ can be accommodated within the framework of a simple, bimolecular interaction between the toxin (and/or permeant cation) and the channel. Likewise, that recovery from block was slow and often incomplete within the timescale of these experiments (20–40 min) accords with the very slow off times anticipated for toxin block [(Anderson et al., 1988; Candia et al., 1992); (*see also* Weller et al., 1985; Benoit & Dubois, 1986; Penner et al., 1986)]. [The proviso to this conclusion holds that $I_{K,in}$ is predominated by one K^+ channel characteristic. In fact, all available evidence indicates that the assumption is reasonable (Blatt & Thiel, 1993), and the concentration dependencies for steady-state block (Figs. 5 and 7) were consistent with this interpretation. Thus, while measurements from the intact cells necessarily reflect the ensemble of active channels, an embedded characteristic of more complex block kinetics from several component currents is unlikely.]

Second, antagonism of DTX block was achieved on raising the extracellular cation concentration with K^+, to a lesser extent with Rb^+, and only minimally with Na^+. This cation dependence contrasts with the nonspecific effect of ionic strength reported for ChTX block of neuromuscular Ca^{2+}-activated K^+ channels (Candia et al., 1992). However, it accords with the relative equilibrium selectivities of $I_{K,in}$ between these monovalent cations (Blatt, 1992) and, thus, with the ability of the cations to enter and bind within the channel pore. So, the characteristics of cation protection argue for DTX interaction at a specific site associated with the K^+ channel, rather than for nonspecific charge screening. The data also concur with a single site, capable of binding either DTX or K^+ (but not both together), since DTX block and its interaction with K^+ could be accommodated by a simple, competitive reaction scheme.

Third, DTX was found to be equally effective regardless of whether the prevailing membrane voltage favored steady-state current activation or deactivation. Block was unaffected when the guard cell membrane was deliberately clamped to voltages sufficient to achieve maximal activation of $I_{K,in}$. Likewise, both the activation and deactivation kinetics as well as the voltage dependence of $I_{K,in}$ were insensitive to the presence of the toxin (Figs. 3 and 4, Table). Thus, no appreciable indication could be found for block dependent on channel activity or membrane voltage.

Taken together, these observations are consistent with an occlusion model for DTX action. According to this scheme, block is effected when one toxin molecule binds externally at the mouth of the guard cell K^+ channel to obstruct cation permeation. The voltage insensitivity of block argues for a site outside the membrane electric field, and its competition with K_o^+ indicates that DTX may displace a K^+ ion from the same site. The argument finds support also in observations that, like DTX, external K^+ concentration does not alter the voltage dependence for $I_{K,in}$ activation nor its relative conductance (Blatt, 1992).

A Distal Site for DTX and K^+ Modulating $I_{K,IN}$?

Finally, one observation raises a question about the site of DTX binding and suggests an alternative model in which DTX modulates channel activity by binding to an external (distal?) regulatory site normally occupied by a K^+ ion. There is some evidence indicating that K^+ outside the guard cell is required for $I_{K,in}$ activity, independent of its role as the charge-carrying species (Blatt, 1992; Blatt & Thiel, 1993). The voltage dependence for gating of the K^+ inward rectifier is fixed to the voltage range negative of approximately -120 mV and—with E_K situated positive of this voltage in millimolar K_o^+—effectively restricts the net K^+ flux to uptake (Thiel et al., 1992; Blatt & Thiel, 1993). The same voltage dependence, however, anticipates a substantial *outward-directed* current through the channels when K_o^+ falls below approximately 1 mM ($E_K < -120$ mV).

In fact, this current does not materialize, suggesting that cation binding outside is an integral part of the gating mechanism and that the binding site(s) must fill as K_o^+ rises above approx. 1 mM (Blatt, 1992). Juxtaposed with this observation, the DTX/K^+ competition analysis above indicates a K_d for K_o^+ of approx. 0.4 mM, remarkably close to the value anticipated for such a regulatory site. Thus, it can be argued that DTX may effect channel block by displacing K^+ from this regulatory site, rather than by a direct occlusion of the channel mouth. Work directed to distinguishing between these two alternatives will now benefit from recordings at the single channel level.

We thank J.O. Dolly (Imperial, London) and S.M. Jarvis (University of Kent, Canterbury) for several helpful discussions. This work was supported by SERC grant GR/H07696 and was aided by equipment grants from the Gatsby Foundation, the Royal Society and the University of London Central Research Fund. G.O. was supported by an Ausbildungsstipendium (OB 85/1-1) from the Deutsche Forschungsgemeinschaft. F.A. holds a Sainsbury Studentship.

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