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Identification and modulation of a voltage-dependent anion channel in the plasma membrane of guard cells by high-affinity ligands

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Guard cell anion channels (GCAC1) catalyze the release of anions across the plasma membrane during regulated volume decrease and also seem to be involved in the targeting of the plant growth hormones auxins. We have analyzed the modulation and inhibition of these voltage-dependent anion channels by different anion channel blockers. Ethacrynic acid, a structural correlate of an auxin, caused a shift in activation potential and simultaneously a transient increase in the peak current amplitude, whereas other blockers shifted and blocked the voltage-dependent activity of the channel. Comparison of dose–response curves for shift and block imposed by the inhibitor, indicate two different sites within the channel which interact with the ligand. The capability to inhibit GCAC1 increases in a dose-dependent manner in the sequence:

probenecid < A-9-C < ethacrynic acid < niflumic acid < IAA-94 < NPPB.

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, indicating an interaction with the open mouth of the channel. IAA-23, a structural analog of IAA-94, was used to enrich ligand-binding polypeptides from the plasma membrane of guard cells by IAA-23 affinity chromatography. From this protein fraction a 60 kDa polypeptide crossreacted specifically with polyclonal antibodies raised against anion channels isolated from kidney membranes. In contrast to guard cells, mesophyll plasma membranes were deficient in voltage-dependent anion channels and lacked crossreactivity with the antibody.

Key words: antibody/inhibitors/patch-clamp/plant anion channel/voltage-dependence

Introduction

Anion channels in the plasma membrane participate in a number of cellular functions, such as volume regulation, trans-epithelial transport, stabilization of the membrane potential or excitability. Abnormalities in anion channel properties or regulation underlie important diseases such as cystic fibrosis, cholera, secretory diarrhea and myotonia (Steinmeyer, 1991a,b).

In animal cells, anion channels comprise a diverse class of channel proteins divided into voltage-gated, agonist-gated and second messenger-gated subtypes. A strict separation is, however, often not possible e.g. when a channel of one class covers properties of another such as rectifying and cAMP-activated chloride channels in epithelia (Gögelein, 1988).

In contrast to animal cells, studies on single anion channels in plants are rather rare (Coleman, 1986; Schönknecht *et al.*, 1988; Schroeder and Hagiwara, 1989; Laver, 1991; Lew, 1991). Recently we analyzed the voltage-dependent activity, selectivity and regulation of the anion channel in the plasma membrane of guard cells (Keller *et al.*, 1989; Hedrich *et al.*, 1990; Marten *et al.*, 1991). This channel type is highly selective for anions, in particular nitrate, chloride and malate. Furthermore, these anion channels are characterized by a steep voltage-dependence. Chloride currents peak between −30 and −40 mV whereas a decline in channel activity results from potential shifts towards hyperpolarized values and towards the equilibrium potential of the anion. Prolonged stimulation by depolarizing voltages resulted in the inactivation of the channel. Comparative analysis of anion currents in whole cells and cell-free membrane patches has revealed that the macroscopic currents were carried by anion fluxes through single 32−40 pS channels (Keller *et al.*, 1989; Hedrich *et al.*, 1990). In previous studies it was demonstrated that the decrease in whole-cell current upon hyperpolarization resulted from an increase in the mean-closed time of the channel. In related investigations a rise in cytoplasmic Ca^{2+} in the presence of nucleotides was shown to evoke activation of the anion channels. Upon activation, these channels catalyze anion currents 10−20 times higher than in the inactivated state, thus shifting the resting potential of guard cells from a potassium-conducting state to an anion-conducting state (Hedrich *et al.*, 1990).

In further patch-clamp studies we have demonstrated that growth hormones directly affect the voltage-dependent activity of anion channels in a dose-dependent fashion. Auxin-dependent gating of the anion channel is side- and hormone-specific (Marten *et al.*, 1991). Furthermore, auxin action is channel-specific, since potassium channels coexisting in the same membrane were auxin-insensitive.

From the previous investigations it has been suggested that anion channels are involved in regulated volume decrease as well as targeting of auxins during hormone-induced cell swelling. Transient activation and inactivation of anion channels may also provide a potential mechanism for excitability in response to sudden changes in environmental conditions. In the following study we have therefore used different types of anion channel blockers to elucidate the structure requirement(s) for an effector to modify ligand-binding, voltage-dependence and peak current amplitude of the guard cell anion channel (GCAC1).

In our survey of compounds capable of interacting with GCAC1 we have included IAA-94 and IAA-23 in order to test their suitability as potential ligands for the future isola-

tion of the channel protein. We have selected these two compounds in particular since they have already been successfully employed in the isolation of anion channels from kidney and trachea membranes (Landry *et al.*, 1989; Redhead *et al.*, 1992).

Results

Modulation of voltage-dependence

In order to study inhibitor-induced alterations in plasma membrane anion fluxes we have applied the patch-clamp technique (Hamill *et al.*, 1981) to guard cell protoplasts (Schroeder *et al.*, 1984). Patch pipettes were sealed onto guard cell protoplasts isolated enzymatically from epidermal layers of *Vicia faba* leaves (Hedrich *et al.*, 1990). Patch-clamp recordings were performed in the whole-cell configuration and in outside-out membrane patches.

In the whole-cell configuration voltage-dependent anion channels are activated by nucleotides and calcium as described in our previous work (Hedrich *et al.*, 1990). In solutions containing 40 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM MES/Tris pH 5.6 in the bathing medium and 150 mM KCl, 2 mM $MgCl_2$, 10 mM MgATP and GTP, 0.1 mM EGTA, 10 mM HEPES/Tris pH 7.2 in the pipette (cytoplasm), outward and inward chloride fluxes were the predominant ionic conductances (Figure 1, trace I). Current–voltage curve analysis of voltage ramps or voltage pulses revealed that chloride efflux (inward current) is activated near −100 mV and peaks at −30 to −40 mV. Chloride efflux declines through the decrease in the electrochemical driving force when stepping the membrane potential to more depolarized values than −30 mV, reversing direction at the Nernst potential for chloride (E_{Cl^-} = +18 mV). At potentials 40–60 mV positive to E_{Cl^-} chloride influx (outward current) neither peaked nor became saturated but increased, often in a sigmoidal manner (Figure 1, trace I and Hedrich *et al.*, 1990).

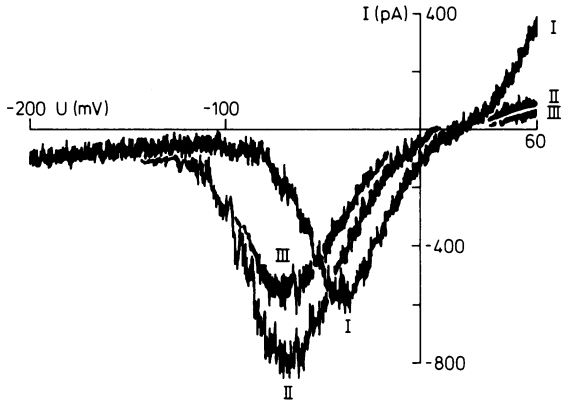


Fig. 1. Modulation of GCAC1 by ethacrynic acid (EA). Time course of EA-induced inhibition of outward current (chloride influx), modulation of the peak amplitude of inward current (chloride efflux) and shift in activation potential. Current–voltage relation of whole cell anion fluxes in the absence (I) and 15 and 75 s (II and III) after application of 100 μM EA resulting from 1 s voltage ramps from −200 to 60 mV.

Ethacrynic acid. When ethacrynic acid (EA) was applied to the bathing solution, both the activation potential and current amplitude were altered (Figure 1, traces I and II). Inhibition of outward current was observed before peak, and the activation potential of the inward current shifted to more hyperpolarized potentials. The shift in voltage-dependence and inhibition of inward current increased with incubation time (Figure 1, traces I–III). Steady-state conditions were reached within 1–2 min of effector treatment. The amplitude of the shifted inward current increased or remained unchanged when exposed to 100 μM EA (Figure 1). However, inhibition could also be observed during prolonged incubation with EA (compare action of auxins in Marten *et al.*, 1991). The modulation of GCAC1 was reversed when EA was removed from the extracellular medium. In order to determine whether GCAC1 is modulated through EA at the extracellular face of the channel or by cytoplasmic inhibitor levels, the cytoplasm was equilibrated for 15–25 min with EA through the patch pipette. Under these conditions anion currents were not affected, indicating that the EA binding-site is located at the external face of the channel. Equilibration half-times of the cytoplasm with EA and other inhibitors in the moleweight range 222–357 (see below) was monitored using 100 μM concentrations of the high quantum yield fluorochrome Lucifer Yellow (LY, mol. wt = 457) as a model. The transmission (Figure 2A) and fluorescence (Figure 2B) micrographs show an LY-loaded patch pipette attached to a guard cell protoplast. After the

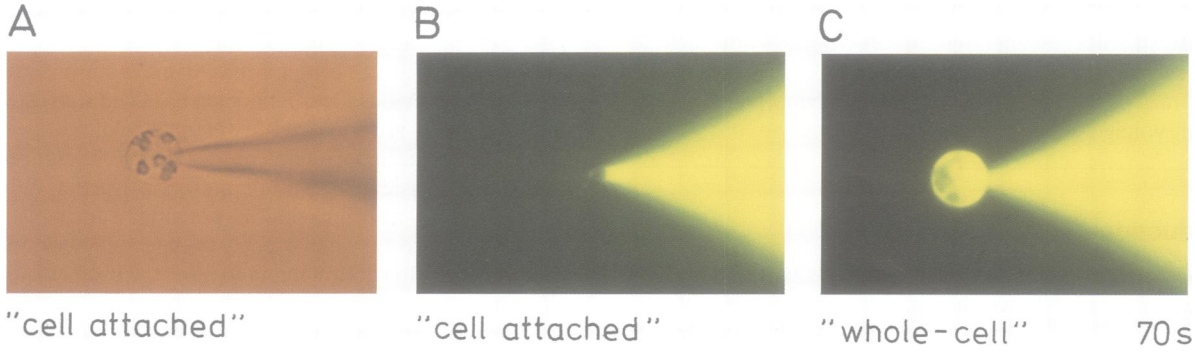


Fig. 2. Cytosolic loading of a guard cell protoplast by the fluorochrome LY through a patch pipette. Transmission (**A**) and fluorescence (**B**,**C**) micrographs of a protoplast in the cell attached configuration (A, B) and 70 s after establishment of the whole cell configuration (C). An equilibration time of ~10 min was calculated from experiments using 100 μM of the fluorochrome. In order to obtain higher resolution a 4 mM concentration of LY was used in these micrographs.

patch was ruptured and the whole-cell configuration established, the fluorochrome was equilibrated with the cytoplasm (Figure 2C). From similar experiments an equilibration time for LY of ~10 min was deduced.

Effect of other anion channel blockers

In order to compare the EA response with the effect of other anion channel blockers (abbreviations see Table I) we further tested probenecid, anthracene-9-carboxylic acid (A-9-C), indanyloxy-acetic acid (IAA-94), niflumic acid and nitrophenyl-propylamino-benzoic acid (NPPB) with respect to their modulation of GCAC1 (Table I).

All channel blockers caused a shift and block in the voltage-dependent activation of GCAC1 depending on their concentration (compare dose-dependent action of IAA-94). Figure 3 shows I−V curves for whole-cell anion channel activity in the absence of IAA-94 (closed symbols) and in the presence of 30 μM (open circles) and 100 μM IAA-94 (open triangles). The sensitivity of the channel to these inhibitors could be expressed by the sequence of inhibition constants or with respect to their action at 100 μM concentration as listed in Table I of

probenecid < A-9-C < EA < niflumic acid < IAA-94 < NPPB.

Within this group of blockers IAA-94 and NPPB with half-inhibition concentrations of 7 and 4 μM were most effective.

Modulation of single-channel characteristics

In our previous work single-channel analysis has already demonstrated that whole-cell anion currents are carried by 32−40 pS channels. Deactivation of GCAC1 at hyperpolarized potentials was shown to be the result of an increase in the closed times of the anion channel (Hedrich *et al.*, 1990; Marten *et al.*, 1991).

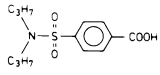
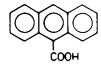
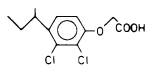
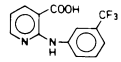
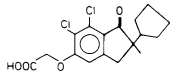
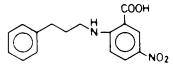
In order to determine the type of block imposed by the inhibitors, outside-out patches were exposed to inhibitor-containing media. A common observation with inhibitors effective in channel modulation was their ability to transform long open intervals of the channel into flickering bursts (Figure 4), reminiscent of the 'intermediate block' described for some animal anion channel inhibitors (Gögelein, 1988). Figure 4 shows fluctuations in open−closed transitions of up to four anion channels held at membrane potentials of −40 and −100 mV in the presence (left panel) or absence of 100 μM IAA-94 (right panel). Upon application of the effector, the peak activity shifted towards more hyperpolarized potentials as the macroscopic anion current in whole cells (for comparison see Figure 3, open triangles) from which patches were excised.

Different channel-sites for voltage-gating and block

We have compared dose−response curves for gate shifting and block of GCAC1 to examine whether the two superimposed inhibitor effects are closely related.

The shift and block of the anion channel saturate as a function of the effector concentration. However, the shape of the curve and thus the kind of interaction with a putative 'binding-site' differed strongly (Figure 5A and B). Besides differences in shift and block resulting from the effector in question, we also observed effector-specific variations (compare NPPB and IAA-94 in Figure 5A and B). Whereas the channel block can be explained by an interaction of the ligand with the open mouth of the channel, the 'gate-shifting'

Table I. Action of various anion channel blockers on the modulation of the voltage-dependent activity of GCAC1 protoplasts

Inhibitor	Structure	Shift of I_{Cl-} peak (mV)	Inhibition of I_{Cl-} (%)	K_i (μM)	*n*
Benzoic acid		n.d.[a]	n.d.	−	3
Probenecid[c]		−13.7 ± 3.0	34.8 ± 17.1	>100	4
A-9-C[d]		−20.8 ± 3.4	51.8 ± 10.8	100	13
Ethacrynic acid[e]		−37.12 ± 8.9	65.7 ± 13.8	m.e.[(2)]	23
Niflumic acid[f]		−76.3 ± 11.0	98.3 ± 2.4	20	19
IAA-94[g]		−58.4 ± 12.2	90.5 ± 7.4	7	36
NPPB[h]		−108 ± 11.8	100	4	39

Inhibition is expressed with respect to the peak current potential of the control cells. Values for shift and block were obtained in the presence of inhibitors at 100 μM. K_i represents effector concentrations causing a 50% inhibition of the anion current.
Abbreviations: [a]n.d., not detected; [b]m.e., moderate effect (see text); [c]*p*-(dipropylaminosulfonyl) benzoic acid; [d]anthracene-9-carboxylic acid; [e][2,3-dichloro-4-(2-methylenebutyryl)-phenoxy] acetic acid; [f]2-(α,α,α-trifluoro-*m*-toluidino)-pyridine-3-carboxylic acid; [g][(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)oxy] acetic acid; [h]5-nitro-2-(3-phenylpropylamino) benzoic acid.

action may result from adsorption of the ligand(s) at sites which directly or indirectly alter the intramembraneous electrical field (see Armstrong and Cota, 1991).

Channel specificity

To demonstrate the channel specificity of IAA-94, the behavior of plasma membrane potassium channels was monitored during inhibitor action on GCAC1 (Figure 6). In the presence of external 40 mM $CaCl_2$ and 150 mM KCl in the pipette, inward currents were carried by chloride only, as characterized by the inward hump between the Nernst potential of chloride and −100 mV and a high resistance range negative to −100 mV (Figure 1, trace I). Upon replacement of 40 mM $CaCl_2$ by 30 mM KCl, 2 mM $CaCl_2$ inward potassium currents were elicited at voltages negative to −100 mV (Figure 6). Voltage ramps from −250 to 60 mV from a holding potential of −100 mV allowed us to record the activity of K^+ and Cl^- currents simultaneously. Upon the application of IAA-94 anion currents were altered as predicted (Figure 6, I and II), whereas potassium currents were not inhibited significantly (Figure 6, I−III). This indicates that K^+ channels of guard cells do not possess binding sites for IAA-94.

On the other hand Ba^{2+} and TEA^+, known potassium channel blockers in this system (Schroeder *et al.*, 1987; Marten *et al.*, 1991) did not inhibit the anion channel. Ba^{2+} was even able to replace Ca^{2+} for stimulation (not shown).

IAA-23 affinity chromatography with plasma membrane proteins and crossreactivity with antibodies raised against anion channels

In order to determine whether the abundance and properties of the anion channel are unique for guard cells (GC in Figure 7A, open circles in 7B) or whether they are expressed in cell types that do not undergo pronounced volume changes, we also analyzed the current−voltage relationship and IAA-94 response of mesophyll cells from the same tissue (MC in Figure 7A, closed circles in 7B). Under the identical experimental conditions as optimized for guard cells (Hedrich *et al.*, 1990) anion currents were not observed for mesophyll cells. Whether these differences in both cell types resulted from a higher abundance of the anion channel in guard cells or from its regulation was further analyzed at the protein level. For this reason we selected the derivative IAA-23 which could be coupled to a CNBr−Sepharose column and thereby exposes a structure very similar to IAA-94. Using this affinity column, an IAA-23-binding protein fraction was enriched (Figure 8) through elution by IAA-94 as described before (Landry *et al.*, 1989). In order to reduce non-specific binding, all buffers contained 100 μM benzoic acid since this arylcarboxylate does not alter the channel properties (Table I). In the final step we attempted to identify the channel-specific protein immunologically in analogy to recent approaches in various animal cell types (Redhead *et al.*, 1992). Plasma membrane proteins or an IAA-94 eluate of guard cells and mesophyll cells were separated by SDS−PAGE. Membrane proteins were transferred onto nitrocellulose filters and incubated with a polyclonal antibody raised against kidney anion channels (Figure 8). The antibody crossreacted with a polypeptide of ~60 kDa in guard cell plasma membranes (Figure 8, GC lanes), whereas only a minor fraction in mesophyll membranes crossreacted with the antibody (Figure 8, MC lanes). Incubation of guard cell protoplasts with the antibody for 0.5−2 h, on the other hand, did not inhibit the anion channel activity. This indicates that either the antigen is not located on the extracellular surface of the plasma membrane or it cannot be accessed by the antibody.

When membrane fractions other than plasma membranes were analyzed immunologically, crossreactions with other polypeptides that were not IAA-binding proteins were also observed. Whether these bands represent other anion-permeable channels in vacuolar, photosynthetic, or mitochondrial membranes (Hedrich and Neher, 1987; Hedrich and Kurkgjian, 1987; Schönknecht *et al.*, 1988; Sorgato *et al.*, 1987) needs further elucidation.

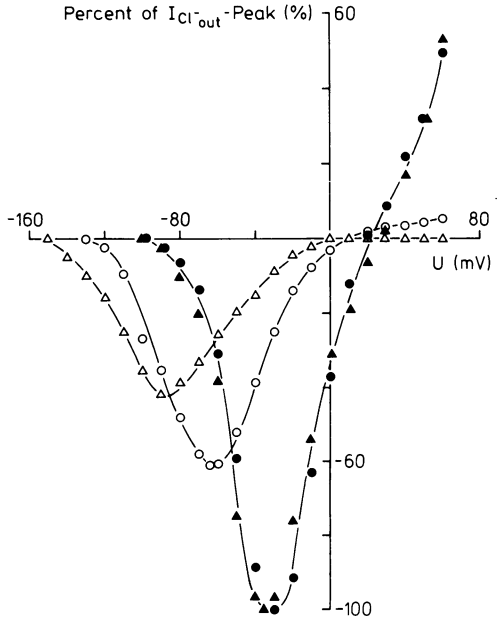


Fig. 3. Dose-dependent modulation of GCAC1 by IAA-94. Alteration of peak amplitude and shift in activation potential of anion efflux. Current−voltage relation of whole cell anion fluxes in the absence (closed symbols) and 105 s after application of 30 (open circles) and 100 μM (open triangles) IAA-94 (see legend to Figure 1).

Discussion

Stomatal movement requires guard cells to respond to sudden changes in environmental conditions by changes in cell volume. In contrast to guard cells, photosynthetically active cells do not undergo pronounced volume changes. Based on these fundamental differences in cell function one would expect that the two cell types would differ in their composition and density of ion channels and ion pumps. In a previous study based on plasma membrane isolation, purification and Western blot analysis we were able to demonstrate that the difference in H^+ pump activities in both cell types resulted from a higher abundance of the H^+-ATPase in guard cell plasma membranes (Becker *et al.*, 1992; Lohse and Hedrich, 1992). Our finding that mesophyll cells lack anion channel activity (Figure 7B) and exhibit a largely reduced crossreactivity of the antibody with the supposed channel protein (Figure 8) provide further support for the physiological demands of the two cell types. This gives rise to the idea that cell- or tissue- and probably plant-specific subtypes, densities or regulation do exist.

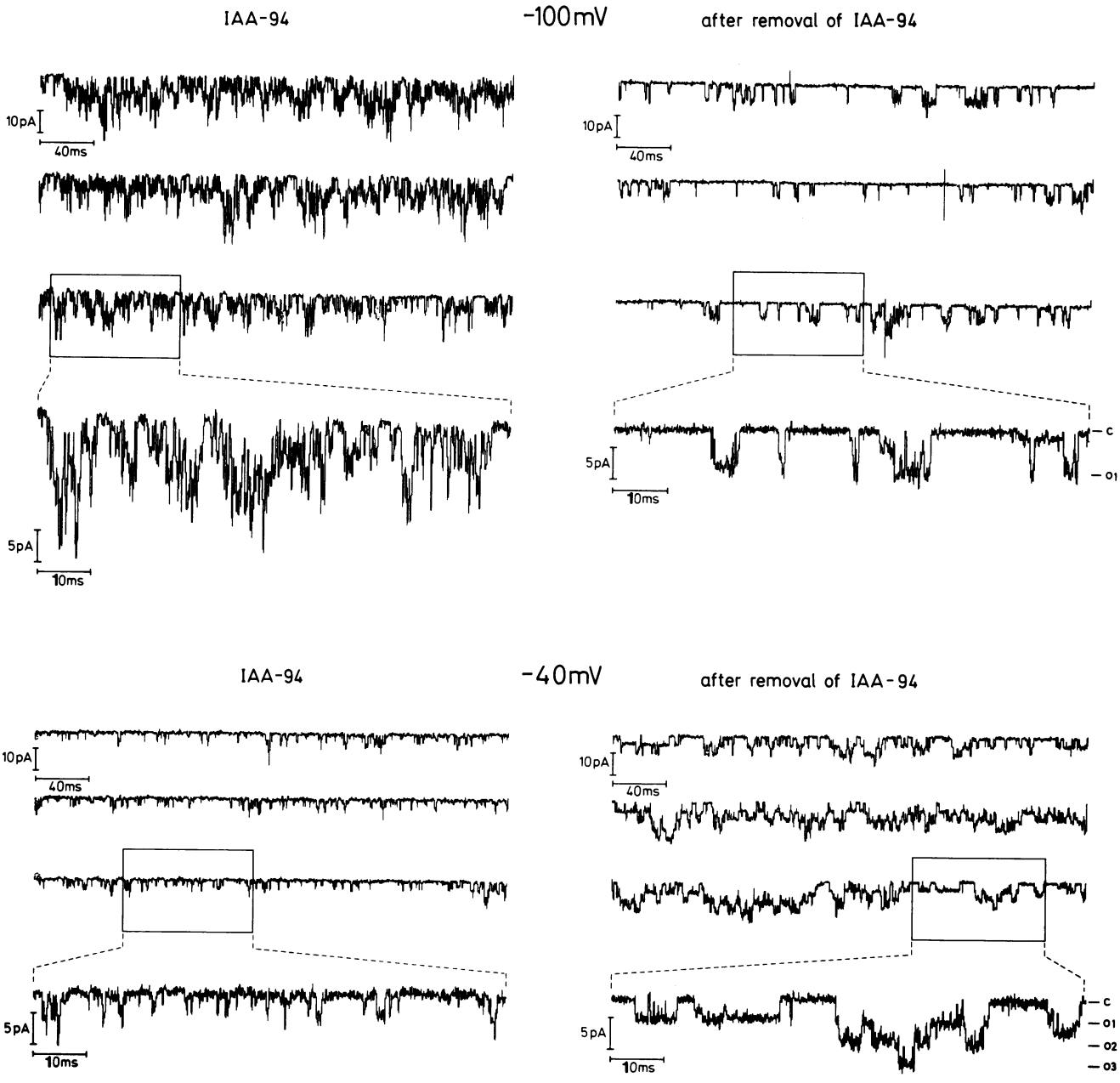


Fig. 4. Modulation of anion channel properties by IAA-94. Single-channel activity recorded from an outside-out membrane patch excised from whole cells as shown in Figure 3 in the presence (left panel) and absence of 100 μM IAA-94 (right panel) with the membrane potential clamped as indicated. C, closed channel; O_1-O_3, one to three open channels.

In this investigation we have demonstrated that a broad spectrum of anion channel blockers was capable of modulating and blocking the anion channel of guard cells (Table I). The effect of EA is reminiscent of the action of growth hormones (Marten *et al.*, 1991). EA, a structural correlate of indolyl-3-acetate, the naturally occurring auxin, could therefore at least partially mimic the hormone effect and thus serve as a tool to study hormone targeting to GCAC1 and the transduction pathway linked to it (Palme, 1992).

Structurally non-related anion channel blockers (Wangemann *et al.*, 1986) altered the voltage-dependence of the channel in a similar fashion. From the dose–response characteristics (Figure 5A and B) it could be deduced that sites responsible for gate-shifting and block are different from each other.

It is too early to define the structural requirement of a compound to either block or shift the voltage-dependent activity of GCAC1. However, it is very likely that compounds used in our studies possess sites of interaction with the channel such as negatively charged carboxylate, chloro, and nitro groups, positively charged amines and polar interactions with cycloalkyl or aryl residues. Since benzoate (Table I), glutamate, gluconate and lysine (not shown) were not found to affect the anion channel, the presence of a carboxylate, amine or aryl group alone does not suffice to modulate GCAC1.

Further analysis of inhibitor structure and channel function

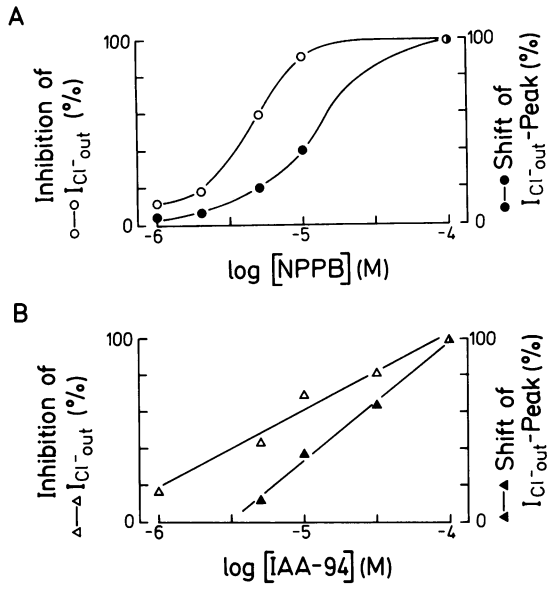


Fig. 5. Concentration-dependence of NPPB (**A**) and IAA-94-induced (**B**) block of outward anion channels at the peak current potential and shift in the activation potential. Data were normalized with respect to the peaks in inhibition and gate-shifting at 100 μM NPPB and IAA-94.

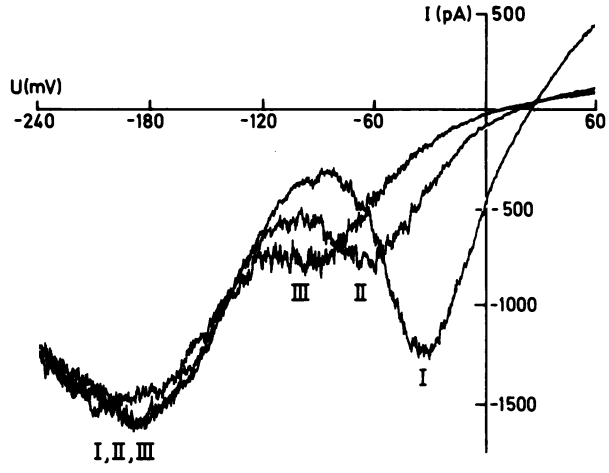


Fig. 6. Simultaneous recording of voltage-dependent potassium and anion channels in the absence (I) and presence 5 s (II) and 15 s (III) after application of 100 μM IAA-94. Note that potassium currents in contrast to anion channels were not significantly affected by IAA-94. Current–voltage relationships of potassium and anion channels resulting from 1.2 s voltage ramps from −250 mV to 60 mM.

may give clues to the nature and location of channel sites responsible for gate-shifting and block.

Materials and methods

Protoplast isolation and patch-clamp recording

Guard cell and mesophyll protoplasts were enzymatically isolated from 2–3 week old leaves of the broad bean, *Vicia faba* (Hedrich *et al.*, 1990; Lohse and Hedrich, 1992). Patch pipettes were sealed against the plasma membrane to study ion fluxes in the whole cell configuration and in outside-out patches as previously described (Hedrich and Schroeder, 1989). Current measurements were made with an EPC-7 and EPC-9 patch-clamp amplifier (List Electronic, Darmstadt and HEKA, Lambrecht, Germany), low-pass filtered at 1 or 10 kHz with an eight-pole Bessel filter. Data were digitized (VR-10, Instrutech. Corp., USA), stored on video tape and analyzed using patch-clamp software (Instrutech. Corp.) on an Atari Mega ST4.

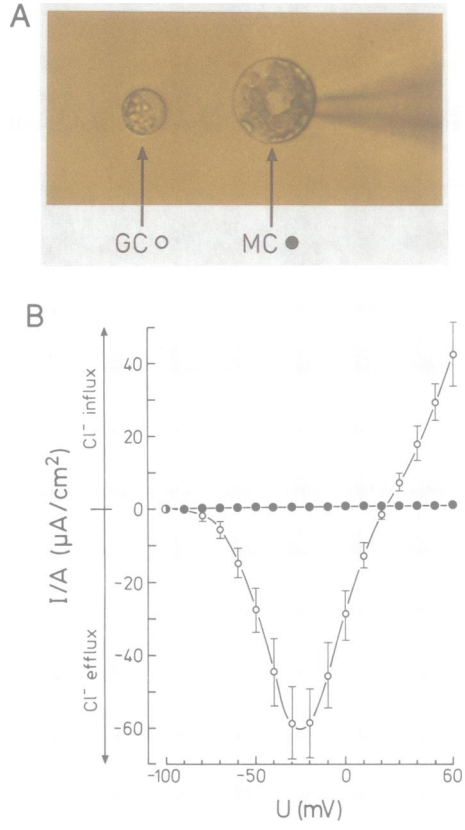


Fig. 7. Contribution of voltage-dependent anion channels to the electrical properties of guard cells and mesophyll cells. (**A**) Light micrograph of a mesophyll protoplast with a patch pipette attached to it close to a guard cell protoplast. (**B**) Average current–voltage relationship of guard cell (open circles, n = 7) and mesophyll plasma membrane (closed circles, n = 6). Solutions were identical to those used in Figures 1–5.

Solutions

Solutions in Figures 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 2 $MgCl_2$, 10 mM MgATP and GTP, 0.1 mM EGTA, 10 mM HEPES/Tris pH 7.2 in the pipette (cytoplasm). In the experiments shown in Figure 5, guard cell protoplasts were exposed to 150 mM KCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 10 mM MgATP and 10 mM GTP, 10 mM HEPES/Tris pH 7.2 in the pipette, and 30 mM KCl (or 40 mM $CaCl_2$ to pre-activate anion channels), 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 MES/Tris pH 5.6 in the bath.

Channel blockers

Anion channel blockers were dissolved in ethanol. Final ethanol concentration in the experiments was 0.1 or 1%. Ethanol at 1% (v/v) itself did not affect the anion channel.

Isolation of guard cell and mesophyll cell plasma membranes and analysis of membrane proteins

Plasma membranes from guard and mesophyll cells were isolated from 2–3 week old *Vicia faba* leaves according to Becker *et al.* (1992). Membrane proteins were solubilized in 10% SDS, 0.8 M NaOH and precipitated with methanol at −80°C. Pellets were used for protein determination according to Bradford (1976) and SDS–PAGE. SDS–PAGE was performed according to Laemmli (1970). Proteins were separated on 8–15% acrylamide gels. Membrane proteins were electroblotted from polyacrylamide gels onto nitrocellulose membranes using a semi-dry apparatus (Biometra) at 5 mA/cm^2 for 45 min. Western blotting was performed according to Towbin *et al.* (1984). Immunodetection of blotted or dotted proteins with polyclonal antibodies ('alpha 64') raised against anion channels from kidney were performed as described by Redhead *et al.* (1992). A second antibody coupled to alkaline phosphatase was used as described by Blake *et al.* (1984).

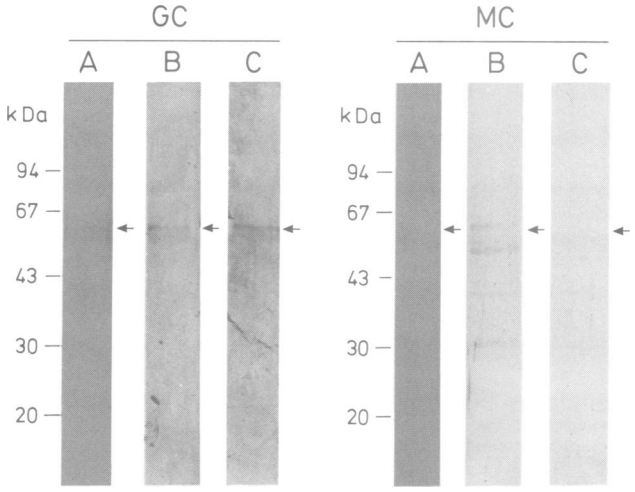


Fig. 8. Western blot analysis of the anion channel protein of plasma membrane-derived proteins from guard cells (GC) and mesophyll cells (MC). Mesophyll and guard cell plasma membranes were solubilized in 60 mM Mega 9, 100 μM benzoic acid, 10 mM HEPES/KOH pH 7.2 and protease inhibitors (100 μM *o*-phenanthroline, 1 μg/ml leupeptin). Supernatants (15 min, 150 000 *g*) were diluted to 20 mM Mega 9 and incubated with an IAA-23 matrix for 2 h. The matrix was washed with buffer to remove unbound proteins. Proteins eluted from the IAA-23 column by 200 μM IAA-94 were subjected to SDS–PAGE and Western blot analysis. 10 μg (A), 10 μg (B) and 2 μg (C) for the guard cell fraction and 15 μg (A), 10 μg (B), and 5 μg (C) for mesophyll fractions were subjected to SDS–PAGE on an 8–15% gel, transferred to nitrocellulose filters and challenged with antibodies raised against a 64 kDa anion channel of kidney membranes. Lane A, total plasma membrane; lane B, membrane fraction not bound to the column; lane C, fraction eluted by IAA-94.

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