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Characterization of Ion Channels in the Plasma Membrane of Epidermal Cells of Expanding Pea (*Pisum sativum* arg) Leaves

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Abstract. Ion channels in isolated patches of the plasma membrane of pea (*Pisum sativum* arg) epidermal cells were studied with the patch-clamp technique. One anion and one cation channel were dominantly present in most trials. The anion channel conducts nitrate, halides and malate, with a conductance in symmetrical 100 mM Cl^- of 300 pS and can be blocked by SITS when applied to the cytoplasmic side of the membrane. The cation channel poorly discriminates between potassium, sodium and lithium, is not blocked by either TEA or Ba^{2+}, and has a conductance of 35 pS in symmetrical 100 mM K^+. The open probability of the cation channel increases with increase of the Ca^{2+} concentration on the cytoplasmic side of the membrane from 0.1 to 1 μM. The possible role of these two channels in the physiology of epidermal cells is discussed.

Key words: Patch clamp — Anion channel — Ca-dependent nonselective cation channel — *Pisum sativum* — Epidermal cell

Introduction

Plant cells grow by increasing cell volume, a process driven by turgor pressure exerted against a relatively stiff cell wall. Cellular regulation of growth rate relies on several physiological processes involving ion transport across the plasma membrane. First, for turgor maintenance, growing plant cells are faced with the task of adjusting osmotically, a major regulatory mechanism for leaf extension (Dale, 1988). Although synthesis or import of sugars may play a significant role in lowering the osmotic potential, potassium ions also contribute substantially to osmoregulation, as can anions such as malate, nitrate and chloride. Secondly, ion flux across the plasma membrane is likely to be involved in changing the membrane potential as part of signal transduction events (*references in* Schroeder & Keller, 1992) leading to, for instance, changes in growth rate. The third and possibly most important role for potassium in stimulating growth is to balance the charge of, and thereby facilitate the proton excretion induced by factors stimulating expansion growth such as light (Blum et al., 1992) or fusicoccin (Marré, 1979). Growth stimulation is specific for potassium or sodium (A.G. Van Elk and J.T.M. Elzenga, *unpublished results*).

The tissue layer exerting the most control over the growth rate is the epidermis. Attributing this role to the epidermis stems from theoretical considerations (Green, 1980), as well as studies on *Xanthium* leaves, that established that the epidermis cells start to elongate before the palisade cells (Maksymowych, 1973). Epidermal strips of expanding pea leaves, isolated from the underlying mesophyll, are capable of responding to light by increasing growth rate (A.G. Van Elk, J.T.M. Elzenga and E. Van Volkenburgh, *unpublished results*). Proton excretion is also stimulated by light, a stimulation that is enhanced by potassium in the external medium (M. Staal, J.T.M. Elzenga, A.G. Van Elk, H.B.A. Prins, E. Van Volkenburgh, *manuscript in preparation*). Regulation of the fluxes through ion channels and the proton pumping ATPase most likely are the underlying mechanisms of these light-induced processes.

The patch-clamp technique has been used to study other leaf cell types (guard cells [Schroeder, Hedrich & Fernandez, 1984], mesophyll cells [Moran et al., 1984] and cultured cells derived therefrom [Schauf & Wilson, 1987]), but to date little attention has been paid to epidermal cells. In this study, we set out to fill this gap and gain a better understanding of the mechanism of ion

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fluxes in epidermal cells of expanding leaves of *Pisum sativum.*

Materials and Methods

Plant Material

Seeds of *Pisum sativum* arg were obtained from the Plant Genetic Resources Unit USDA/ARS NYS Agr. Exp. Station, Geneva, NY. The seeds were germinated on wet filter paper and grown in soil in a Percival E54 growth cabinet under a light regime of 14 hr light: 10 hr dark at 20°C [fluence rate 180 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by Cool White fluorescent tubes (Philips)].

Isolation of Protoplasts

Protoplasts were isolated as described previously (Elzenga, Keller & Volkenburgh, 1991). In short, the epidermis of unfolding leaflets was stripped and floated on a buffered enzyme solution (1.7% w/v Cellulase RS [Yakult Honsha], 1.7% w/v Cellulysin [Calbiochem], 0.026% w/v Pectolyase Y-23 [Seishin], 0.2% w/v BSA [Sigma], 2.325% w/v Gamborg B5 [GIBCO], 2 mM $CaCl_2$ and 10 mM MES/KOH, pH 5.5 [Schroeder, 1988] and mannitol adjusting the osmolarity to 610 mOsm) for 5 min at 30°C on a rotary shaker at 50 rpm. The epidermal strips were transferred to a wash solution (identical to the enzyme solution except cellulase, cellulysin, pectolyase and BSA were omitted), transferred after 5 min to a second wash and after another 5 min to the bath solution. In this solution, which had an osmolarity of 240 mOsm, the protoplasts swelled and were released from the tissue. The protoplasts were allowed to settle on the glass bottom of the measuring cuvette.

Electrophysiology

Standard patch-clamp techniques were used (Hamill et al., 1981). Patch pipettes were pulled from 50 µl micropipettes (VWR, cat. no. 53432-783) on a Narishige PB-7 pipette puller. To reduce pipette capacitance, the tip and shank were dipped in Q-dope (polystyrene, GC Electronics, Rockford, IL) which was allowed to harden for at least 2 hr before use. The tips of the electrode were fire-polished immediately before use. Tip resistance was typically between 3 and 9 MΩ depending on solution composition and tip geometry. The bath solution contained 2 mM $MgCl_2$, 100 mM KCl (or as indicated in Results), 10 mM HEPES-BTP pH 7.0, mannitol to adjust the osmolarity to 240 mOsm. For pCa values between 7 and 5, 1 mM BAPTA was included. The $CaCl_2$ added was calculated using an algorithm with the protonation and Ca binding constants from Martell and Smith (1974) and adjusted for the ionic strength (Scharff, 1979, Eq. 13). The standard pipette solution was identical to the bath solution except that the osmolarity was adjusted to 290 mOsm. The Ag/AgCl reference electrode was connected to the bath via a salt bridge filled with the pipette solution. Electrode offsets were compensated electronically with the circuitry on the patch-clamp amplifier.

Seal formation was monitored by measuring the current induced by a 100 Hz 1 mV square pulse from a function generator (Topward type 8102). Typically $3 \cdot 10^{-3}$ Pascal suction was applied to the pipette to form the seal. Single channel currents were low-pass filtered at 3.5 kHz (3 dB corner frequency) with an 8-pole Bessel type filter (type 902, Frequency Devices, Haverhill, MA). The currents were monitored with a current voltage converter (List EPC-7), digitized by an A/D converter (custom made, based on a Labmaster ADC board [Scientific Solutions]) at a sample frequency of 33 kHz and stored on a PC hard drive. All experiments were performed in voltage-clamp mode. Command voltage protocols and A/D conversion were under software control (pCLAMP, Axon Instruments, Foster City, CA).

Fitting of the Data

Gaussian distributions were fitted to all-point histograms of the single channel recordings. The curves fitted to the *I-V* plots of the single channel currents are two- and four-state, class I kinetic models as described by Gradmann, Klieber and Hansen (1987) and Hansen et al. (1981). Fitting was done using simplex and/or maximum likelihood algorithms.

Sign Convention

The sign convention used throughout this report is the same as that proposed by Bertl et al. (1992). Voltages are referenced against the electrical potential of the apoplastic side of the membrane, regardless of the patch orientation (inside-out or outside-out). Negative membrane voltages mean cytoplasmic electrical potential negative to the apoplastic electrical potential. Positive currents mean positive charges moving from the cytoplasmic side to the apoplastic side.

Abbreviations

BAPTA: (1,2-Bis(2-aminophenoxy)ethane *N,N,N′,N′*-tetraacetic acid; BTP: Bis Tris Propane; MES: 4-morpholino-ethanesulfonic acid; HEPES: (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid); TEA: tetraethylammonium; SITS: 4-aceteamido-4-isothiocyanostilbene-2,2′-disulfonic acid.

Results and Discussion

Most patches of plasma membrane of epidermal cells used in this study (well over 100) contained active channels. One anion and one cation channel type were most abundant. These two channel types were observed at about the same frequency and often appeared simultaneously. In an estimated 10% of the patches, no channel openings could be observed.

Anion Channel

I-V Relation

Figure 1*A* shows single channel current traces at different membrane potentials (note the scale difference for currents at the three most negative potentials). At positive membrane potentials, the channel openings become burst-like, resulting in the more "noisy" traces when the channel is open. Channel openings at positive potentials could only be recorded immediately after keeping the membrane potential hyperpolarized, as the

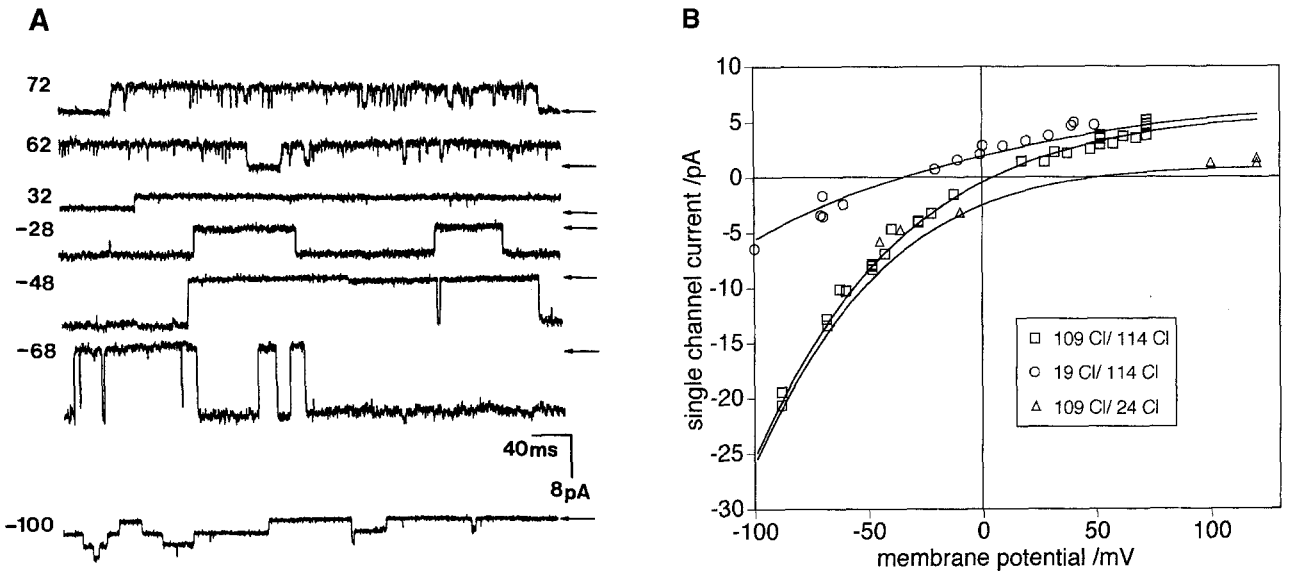

Fig. 1. Current-voltage relation of anion channel. (*A*) Single channel recording of inside-out patch. Composition of both the bath and pipette solution was (mM) 100 KCl, 5 $CaCl_2$, 2 $MgCl_2$ and 5 HEPES/BTP pH 7. The osmolarity was adjusted to 240 mOsm (bath) or 290 mOsm (pipette) by adding mannitol. Note the change in current scale for holding potentials of −100 mV and below. (*B*) Current-voltage relation of single channel currents in 100/100 (cytoplasmic/apoplastic concentration in mM), 10/100 and 100/10 KCl. The data were fitted simultaneously with an equation describing a four-state, class I model. (Fitted parameters are (10^6 sec^{-1}) k_{12} 12,000, k_{21} 315, k_{14} 11,600, k_{41} 12,100, k_{43} 444, k_{34} 2,740, k_{32} 3,230, k_{23} 19,200)

channel tends to inactivate at depolarized, positive, potentials. With voltage jumps during a channel opening, it was established that the open channel currents at positive and negative potentials were indeed carried by the same channel.

The *I-V* relation (Fig. 1*B*) shows that in symmetrical solutions the current through the open channel still shows a strong inward rectification (a slight inward rectification can even be seen in the 19/114 experiment, which sets up an inward Cl^- concentration gradient, favoring outward rectification). These curved *I-V* relations were fitted simultaneously with a four-state, class I kinetic model, with cytoplasmic and apoplastic Cl^- concentrations and the membrane potential as the three independent variables and the open channel current as the dependent variable. The fitted curves were used to determine the reversal potentials and the maximum conductance.

The reversal potential in asymmetric solutions is very close to the theoretical reversal potential for chloride (in 10/100 KCl solutions [contribution of $MgCl_2$ and $CaCl_2$ making the Cl^- concentration at the cytoplasmic side 19 mM and at the apoplastic side 109 mM], the reversal potential was −38 mV and in 100/10 KCl [Cl^- concentration at the cytoplasmic side 114 mM and at the apoplastic side 19 mM], it was +39 mV, the Nernst potentials for Cl^-, after correction for ionic activities, were −37 and +37 mV, respectively) indicating a high selectivity for Cl^- over K^+. The maximum conductance of the channel was about 300 pS, with 109 mM Cl^- at the cytoplasmic side and membrane potentials more negative than −50 mV.

Selectivity

By replacing Cl^- in the solution at one side of the membrane with other anions, we determined the relative permeability and relative conductance of these ions with respect to Cl^-. The relative permeability was calculated from the reversal potential using the Goldman-Hodgkin-Katz equation. The relative conductance was determined by comparing the slope of the *I-V* curve at negative potentials. The permeability sequence (Table 1) coincides for the halides with Eisenmann series number III and IV (Hille, 1984). The permeability sequence and the conductance sequence, however, do differ substantially from each other. Whereas, for instance, the reversal potential shifts to a value of about −50 mV when chloride is replaced by malate (Fig. 2), indicating

Table 1. Selectivity of anion channel

Anion	Relative permeability	Relative conductance
Cl^-	1	1
Br^-	1	0.96
I^-	0.42	1.21
F^-	0.11	2.6(?)
NO_3^-	1.34	
Malate	0.002	0.9
$NO_3^- > Cl^- = Br^- > I^- > F^- >> Malate^{--}$		

The relative permeability and relative conductance of other anions with respect to chloride were determined from plots of the current-voltage relation of single channel currents when the Cl^- at the cytoplasmic side of the membrane was replaced by other anion species (*see* Fig. 2). The relative conductance was determined by comparing the slope of the current-voltage relation at negative potentials when Cl^- had been replaced with the slope when the solutions on both sides of the channel contained Cl^-. The relative permeability was calculated from the reversal potential, using the Goldman-Hodgkin-Katz equation. The figure for the relative conductance of the channel for F^- is given tentatively, as the open frequency of the channel in the presence of F^- became very low, making measurements unreliable.

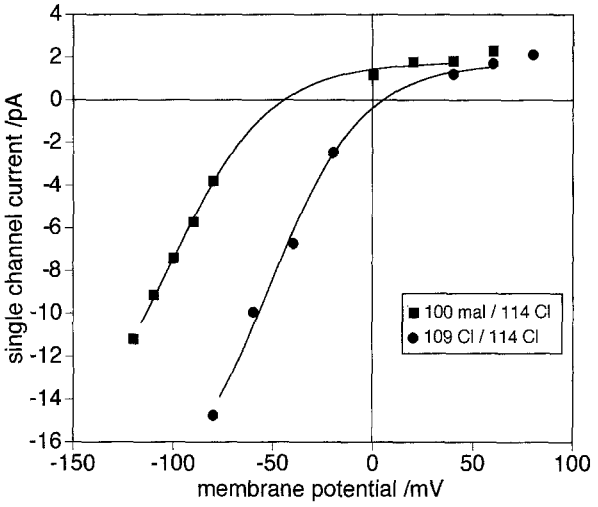

Fig. 2. Effect of changing the anion species on the current-voltage relation of anion channel. Current-voltage relation of single open channel currents in 109 Cl/114 Cl and 100 Malate/114 Cl (convention and further composition of bath and pipette solution as in Fig. 1). This and similar plots were used to compile the data summarized in Tables 1 and 2.

a very low relative permeability of malate, the slope of the *I-V* curve is hardly affected, indicating similar conductances for malate and chloride. This observed difference between sequences points to interaction between permeating ions and the channel protein (Hille, 1984). The relative conductance of fluoride is given tentatively, as the open frequency of the channel became very low in the presence of F^-, making measurements unreliable.

Effect of Channel Blockers

Two reported anion channel blockers, Zn^{2+} and SITS, were tested for their effect on the anion channel. Of these, Zn^{2+} at 10 μM did not have an effect on either side of the membrane. When applied at the apoplastic side, SITS had no effect either, but when added at a concentration of 0.1 or 0.5 mM at the cytoplasmic side, it blocked the channel in a reversible manner (Fig. 3). In the absence of SITS, all openings were at a conductance level of 300 pS, whereas channel openings at both 300 and 150 pS were seen when 0.1 or 0.5 mM SITS was present. In other membranes, this phenomenon has been observed and taken as indicative of a "double-barrelled" conductive pathway in which gating of both pathways is normally strictly coupled, but where each of the pathways can be blocked by SITS independently (Miller & White, 1984).

Comparison with Other Channels

The anion channel in pea epidermal plasma membrane has characteristics that set this channel apart from all the other anion channels described from plant plasma membranes. The anion channel in *Vicia* guard cell plasma membrane of which single channel data are available, has a much lower conductance, is inhibited by Zn^{2+} (Keller, Hedrich & Raschke, 1990) and is activated by Ca^{2+} and nucleotides (Hedrich, Busch & Raschke, 1990). The chloride channel described from *Chara* internode cells has a much lower conductance and seems inactivated by $[Ca^{2+}]$ at the cytoplasmic side higher than 10^{-6} M (Okihara et al., 1991), whereas the pea chloride channel does not seem to be inactivated by changing pCa from 6 to 3 (*data not shown*). The chloride channel from *Asclepias* suspension cells has also a high conductance (100 pS in 100 mM Cl^-) but could be inhibited by both ethacrynic acid and Zn^{2+} (Schauf & Wilson, 1987). The anion channel described from *Amaranthus* cotyledons (Terry, Tyerman & Findlay, 1991), although it has a conductance of about half that of the pea channel, could be a similar channel, except that the channel in pea seems to lack the smaller substates.

Cation Channel

I-V Relation, Selectivity and Effect of Blockers

Figure 4*a* shows current traces of a cation channel in symmetrical 100/100 KCl solutions. As the reversal potential in the 10/100 KCl asymmetric solutions resembles the Nernst potential for K^+ (measured reversal potential 41 mV, *vs.* Nernst potential for K^+ of 49 mV, based on ionic activities), this channel has a high se-

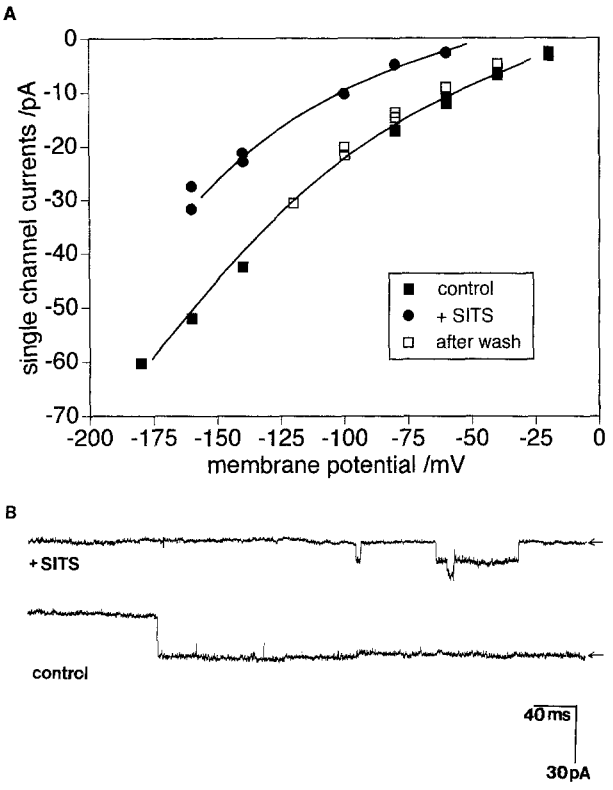

Fig. 3. Effect of SITS on the single channel conductance of the anion channel (*A*) Current-voltage plot of single open channel currents in one inside-out patch in control solution (*see* Fig. 1), after changing the solution in bath with a solution containing 0.5 mM SITS, and after replacing the solution containing SITS with the control solution. (Other patches occasionally displayed currents at both conductance states in the presence of SITS.) (*B*) Single channel recordings of inside-out patch at a holding potential of −100 mV in the presence of 0.5 mM SITS at the cytoplasmic side of the membrane (+SITS trace) and after replacing the solution with a solution without SITS (control trace).

lectivity of cations over anions. The channel shows some outward rectification even in symmetrical solutions (Fig. 4*B*). The fitted curve to the *I-V* plot in 100/100 KCl is based on a two-state class I kinetic model. The maximum conductance of this channel in 100/100 KCl is about 35 pS. Both 1 mM TEA and 1 mM Ba^{2+}, applied to the cytoplasmic side of the membrane, failed to block the channel. The relative permeability and relative conductance of this channel, determined in a similar manner as for the anion channel, are given in Table 2. As both Na^+ and Li^+ are more permeable than K^+, this channel is a nonselective cation channel.

Effect of $[Ca^{2+}]_{cyt}$ on the Open Probability

Figure 5*A* shows two current traces of an inside-out patch with pCa 3 and 7 in the bath, respectively. The patch, which contained at least five channels, exhibited multiple openings at pCa 3 and is virtually silent in pCa 7. The channel open probability is dependent on the $[Ca^{2+}]_{cyt}$ (Fig. 5*B*). There is an increase in the open probability from almost zero at pCa 7 to about 0.1 at pCa 6, which is in the physiological $[Ca^{2+}]_{cyt}$ range and might have regulatory significance. There is, however, a further increase at unphysiologically high calcium concentrations. A similar activating effect of millimolar Ca^{2+} was observed in yeast vacuolar cation channels (Bertl & Slayman, 1990) after channels were "run down." When this "rundown" was prevented by including DTT in the bath solution, micromolar Ca^{2+} proved already sufficient for channel activation. In pea, applying millimolar DTT had no effect on channel activity.

Comparison with Other Channels

The poor discrimination between cations and the lack of blocking by Ba^{2+} and TEA clearly distinguishes the cation channel from most of the cation channels described from other cell types. Cation channels with similar conductances (20 to 40 pS) mostly have a high pK^+/pNa^+, (root cells [Vogelzang & Prins, 1992], oat mesophyll cells [Kourie & Goldsmith, 1992], inward and outward K^+ current-carrying channels in *Vicia* guard cells [Schroeder, Raschke & Neher, 1987], *Amaranthus* cotyledon cells [Terry et al., 1991], *Asclepias* suspension cells [Schauf & Wilson, 1987] and PKC1 and PKC2 in *Arabidopsis* mesophyll cells [Spalding et al., 1992]) and/or are blocked by Ba^{2+} (*Vicia* guard cells) or TEA (root cells).

The Ca^{2+} dependence of the cation channel is also found for the outward current carried by K^+ in corn suspension cells (Ketchum & Poole, 1991). The cation channel in pea epidermal cells resembles most closely the nonselective cation channels described from endosperm cells in *Heamanthus* and *Clivia* fruits (Stoeckel & Takeda, 1989) and *Arabidopsis* mesophyll cells (PCC1) (Spalding et al., 1992). These channels are highly selective for cations over anions, but discriminate poorly between monovalent cations. The resemblance of the cation channel in pea with the cation channel in endosperm cells goes even further as they both have about the same conductance, are not blocked by Ba^{2+} and are both activated by micromolar Ca^{2+}. However, the channel from endosperm cells was reported to pass outward current only, whereas we also found inward current and a slight outward rectification.

Channels with Lower Abundance

Apart from the cation and anion channel described above, several other channel types were occasionally

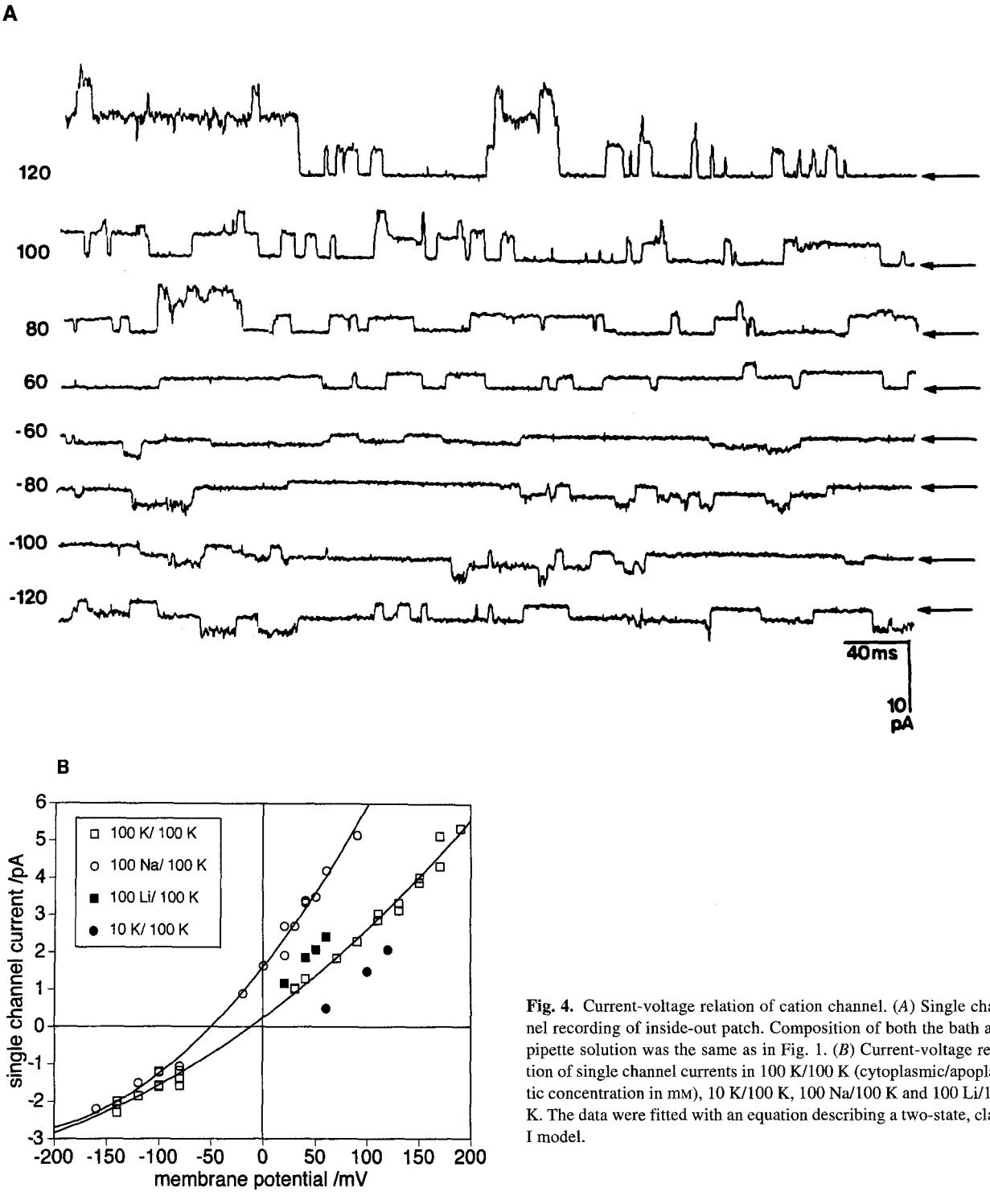

Fig. 4. Current-voltage relation of cation channel. (*A*) Single channel recording of inside-out patch. Composition of both the bath and pipette solution was the same as in Fig. 1. (*B*) Current-voltage relation of single channel currents in 100 K/100 K (cytoplasmic/apoplastic concentration in mM), 10 K/100 K, 100 Na/100 K and 100 Li/100 K. The data were fitted with an equation describing a two-state, class I model.

seen. One of these was seen five times (Fig. 6*A* and *B*). This channel conducts anions (very similar channel openings were observed in solutions containing either Cl^- or glutamate [*see* Fig. 6]) as inferred from the reversal potential (extrapolated reversal potential -30 ± 6 mV [the channel did not open at voltages that were positive enough to encompass the reversal potential], *vs.* Nernst potential for glutamate of -22 mV). This channel has a distinct voltage dependence, opening only at very hyperpolarized membrane potentials (Fig. 7).

Table 2. Selectivity of cation channel

Cation	Relative permeability	Relative conductance
K^+	1	1
Na^+	5.12	1.7
Li^+	2.03	1.2
$K^+ > Na^+ > Li^+$		

The relative permeability and relative conductance of other cations with respect to potassium were determined from plots of the current-voltage relation of single channel currents when the K^+ at the cytoplasmic side of the membrane was replaced by other cation species (*see* Fig. 4). The relative conductance was calculated from the slopes of the current-voltage relation at positive membrane potentials. The relative permeability was calculated from the reversal potential, using the Goldman-Hodgkin-Katz equation.

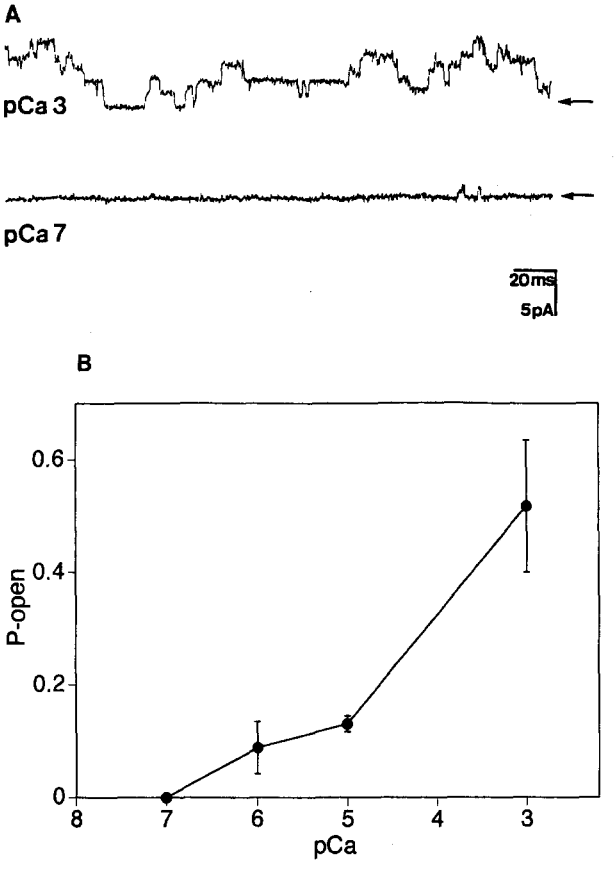

Fig. 5. Effect of $[Ca^{2+}]_{cyt}$ on the open probability of the cation channel. (*A*) Single channel recordings of one inside-out patch at a holding potential of +60 mV. The bath and pipette solution had the same composition as in Fig. 1, except that the $[Ca^{2+}]$ in the bath initially was 1 mM (pCa 3) and was later replaced by a solution buffered with 1 mM BAPTA at a $[Ca^{2+}]$ of 0.1 μM (pCa 7). (*B*) Dependence of the open probability of the cation channel on the $[Ca^{2+}]_{cyt}$. Mean and standard deviation of three independent experiments are given. The open probability is calculated according to Sokabe, Sachs and Jing (1991).

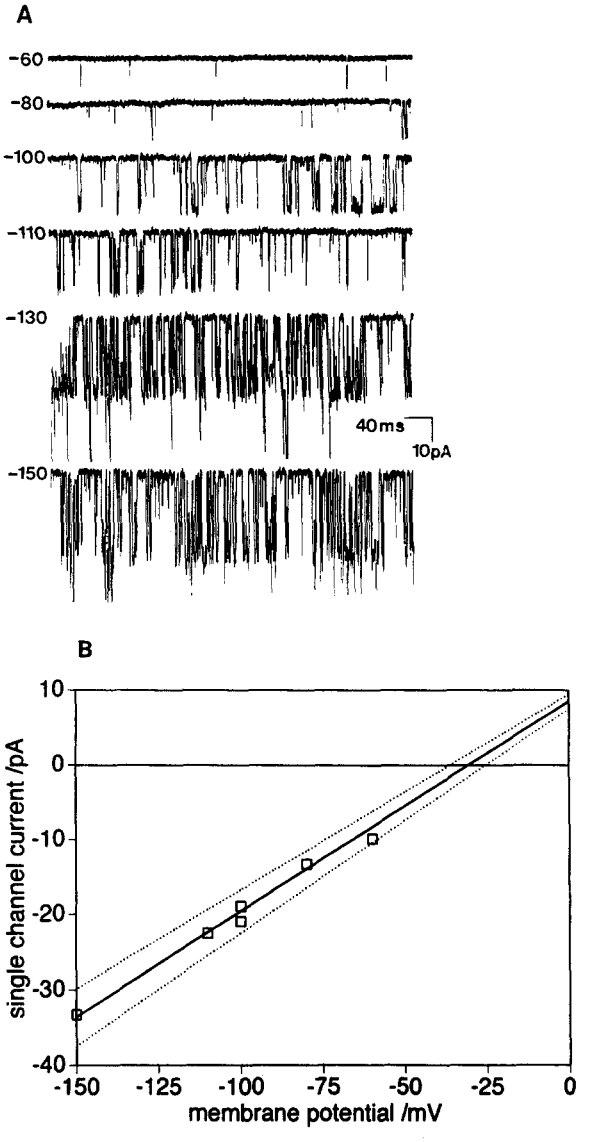

Fig. 6. Current-voltage relation of second anion channel. (*A*) Single channel recording of inside-out patch. The bath solution contained 100 mM KGlutamate and the pipette solution 20 mM KGlutamate. Both contained 5 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM HEPES/BTP pH 7. (*B*) Current-voltage relation of single open channel currents shown in *A*. A straight line was fitted to the data (additional lines given are plus and minus one standard deviation for slope and intercept).

Physiological Implications

Cation Channel

Stoeckel and Takeda (1989) discuss the role of the non-selective cation channel in endosperm cells, pointing out the role of analogous channels in animal cells in modulating the membrane potential. By analogy with animal cells, external stimuli might act through increase of $[Ca^{2+}]_{cyt}$ as a second messenger. Epidermal strips of young pea leaves respond to light by extracellular acid-

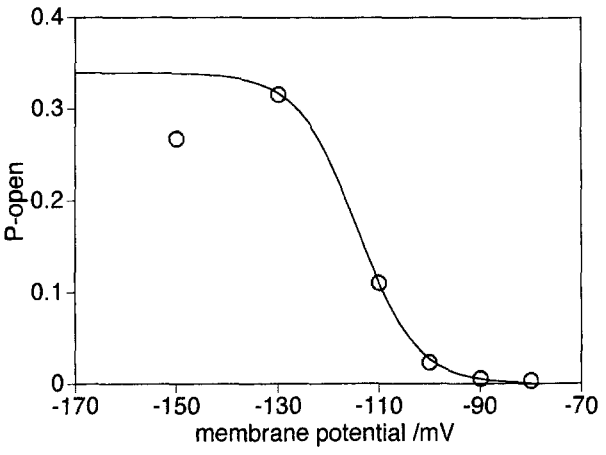

Fig. 7. Voltage dependence of the open probability of the second anion channel. Open probabilities of the anion channel are plotted against the membrane potential. A Boltzmann distribution curve has been fitted to the data (Hille, 1984) (half-maximal activation potential -113 mV; valency of gating charge 4.2). The open probability was calculated as in Fig. 5.

ification, mediated by the proton pumping plasma membrane ATPase. The acidification is strongly stimulated by extracellular K^+ (M. Staal, J.T.M. Elzenga, A.G. Van Elk, H.B. A. Prins, E. Van Volkenburgh, *manuscript in preparation*). Light-induced membrane potential changes in epidermal strips indicate that light also induces influx of Ca^{2+} across the plasma membrane (J.T.M. Elzenga et al. *manuscript in preparation*). Influx of calcium could activate the Ca^{2+}-dependent nonselective cation channel, mediating K^+ influx. Influx of K^+ will, by charge-balancing the H^+ efflux through the ATPase, allow sustained acidification of the extracellular space, and solute accumulation for the maintenance of turgor.

Anion Channel

Anion channels have been implicated in turgor regulation of *Characeae* (Okazaki & Tazawa, 1990) and tobacco culture cells (Falke et al., 1988). Anion channels are well suited to function as "osmotic valves" in plant cells since Cl^- is the only major ionic species for which the electrochemical gradient practically always will lead to an outward flux of the ion. In young, growing epidermal cells the 300 pS anion channel (Fig. 1) could be responsible for balancing the osmotic pressure of the cytoplasm and cell wall yielding. This is supported by the observation (*data not shown*) that, occasionally, in the cell-attached patch configuration, increased suction activated a channel with kinetics similar to the 300 pS anion channel. In isolated patches, increased suction never led to increased activity of the anion channel. It is presumed that a channel can be activated by the tension in the cytoskeletal elements connected to the channel that is set up by deformation of the cell (Christensen & Hoffmann, 1992). The absence of "stretch activation" in isolated patches could be the result of disruption of the cytoskeleton during the isolation of the patches.

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