Eur Biophys J (1995) 24: 107–115

European Biophysics Journal

Inward rectifier Potassium channels in plants differ from their animal counterparts in response to voltage and channel modulators

R. Hedrich[1], O. Moran[2], F. Conti[2], H. Busch[1], D. Becker[1], F. Gambale[2], I. Dreyer[1], A. Küch[3], K. Neuwinger[1], K. Palme[3]

[1] Institut für Biophysik, Universität Hannover, Herrenhäuserstrasse 2, D-30419 Hannover, Germany
[2] Istituto di Cibernetica e Biofisica, C.N.R., Via DeMarini 6, I-16149 Genova, Italy
[3] Max-Planck-Institut für Züchtungsforschung, Carl-von-Linne-Weg 10, D-50829 Köln, Germany

Received: 9 September 1994 / Accepted in revised form: 2 February 1995

Abstract. We have investigated the electrophysiological basis of potassium inward rectification of the *KAT1* gene product from *Arabidopsis thaliana* expressed in *Xenopus* oocytes and of functionally related K^+ channels in the plasma membrane of guard and root cells from *Vicia faba* and *Zea mays*. The whole-cell currents passed by these channels activate, following steps to membrane potentials more negative than –100 mV, with half activation times of tens of milliseconds. This voltage dependence was unaffected by the removal of cytoplasmic magnesium. Consequently, unlike inward rectifier channels of animals, inward rectification of plant potassium channels is an intrinsic property of the channel protein itself. We also found that the activation kinetics of *KAT1* were modulated by external pH. Decreasing the pH in the range 8.5 to 4.5 hastened activation and shifted the steady state activation curve by 19 mV per pH unit. This indicates that the activity of these K^+ channels and the activity of the plasma membrane H^+-ATPase may not only be coordinated by membrane potential but also by pH. The instantaneous current-voltage relationship, on the other hand, did not depend on pH, indicating that H^+ do not block the channel. In addition to sensitivity towards protons, the channels showed a high affinity voltage dependent block in the presence of cesium, but were less sensitive to barium. Recordings from membrane patches of *KAT1* injected oocytes in symmetric, Mg^{2+}-free, 100 mM-K^+, solutions allowed measurements of the current-voltage relation of single open *KAT1* channels with a unitary conductance of 5 pS. We conclude that the inward rectification of the currents mediated by the *KAT1* gene product, or the related endogenous channels of plant cells, results from voltage-modulated structural changes within the channel proteins. The voltage-sensing or the gating-structures appear to interact with a titratable acidic residue exposed to the extracellular medium.

Correspondence to: R. Hedrich

Key words: Potassium channel – *KAT1* – Voltage dependence – Cesium block – pH dependence – Kinetics

Introduction

Since the application of the patch-clamp technique to higher pants, K^+-selective channels have been identified in various species and cell types such as guard cells, mesophyll cells, aleuron cells, and root cells (Schroeder et al. 1984, 1987; Moran et al. 1988; Bush et al. 1988; Spalding et al. 1993; Colombo and Cerana 1991; Schachtmann et al. 1991; for review see Hedrich and Schroeder 1989). In the different cell types two major classes of voltage-dependent K^+ channels have been distinguished: inward rectifiers, that pass inward K^+ fluxes at negative membrane potentials, and outward rectifiers, activated by depolarisation. Both classes are characterised by their long activation and deactivation times (tens of ms), lack of inactivation, and pronounced selectivity for K^+ over the monovalent cations, with a permeability sequence $K^+ > Rb^+ > Na^+ >> Cs^+$ (Schroeder et al. 1987).

Two cDNAs encoding plant K^+ channels (*KAT1* and *AKT1*) have been isolated from *Arabidopsis thaliana* by complementation of K^+ transport deficient yeast mutants (Sentenac et al. 1992; Anderson et al. 1992). Following heterologous expression of *KAT1* in *Xenopus* oocytes and of *AKT1* in *Sf9* cells, it was demonstrated that both gene products mediate inward rectifying K^+ currents, as expected from the cloning strategy (Schachtmann et al. 1992). The predicted structure of the gene products comprise a membrane domain containing a putative pore forming region, which is highly homologous to the transmembrane segments S1–S6 and to the H5 segment common to all members of the voltage-gated K^+ channels of the *Shaker* gene family from animal cells (Pongs 1992), indicating a common ancestor (Jan and Jan 1992). Only very recently two members of a new family of inward rectifying K^+ channels from animal cells (*IRK1* and *ROMK1*) have been identified. Compared to the *Shaker*-type K^+ channels they

contain a much smaller membrane domain, which lacks the first four transmembrane segments, including the putative voltage sensor S4 (Kubo et al. 1993; Ho et al. 1993). Thus, plant K^+ channels present an apparent paradox of structure-function relation, having more functional similarities with the new K^+ channel family to which they are structurally more distantly related. A tentative explanation is that the membrane domain S1–S4, which seems to be a determinant of the activation of *Shaker* channels at more positive voltages, is simply not functional in *KAT1*. Consequently *KAT1* might share with *IRK1* and *ROMK1* the block by intracellular Mg^{2+}, a mechanism of voltage-dependence that obviates intrinsic voltage sensors (Matsuda et al. 1987; Vandenberg 1987; Matsuda 1988). Contrary to this hypothesis, it has already been shown that, unlike *IRK1* and *ROMK1*, the voltage range of activation of plant inward rectifiers is only weakly dependent on the extracellular K^+ concentration (Schroeder and Fang 1991; Schachtmann et al. 1992; Blatt 1992). The experiments reported here aim at further characterising the functional properties of these channels. We concentrated on the effects of potential channel-blockers: cytoplasmic Mg^{2+}, extracellular Cs^+ and Ba^{2+}, as well as extracellular protons.

Our results indicate that plant inward rectifier K^+ channels have voltage-sensing mechanisms profoundly distinct from those adopted by their functional relatives in the animal kingdom. This encourages speculation that apart from the voltage-dependence of voltage-gated channels, their property to either pass inward or outward K^+-flux, may be founded independently in the channel structure.

Material and methods

Molecular biology

KAT1 was recloned from an *Arabidopsis thaliana* cDNA library (Elledge et al. 1991) by PCR as described by Nitschke et al. (1992). The sequences of the oligonucleotides used were: 5 prime end: 5′TTC CTC GAG CTC CGT CAG GGA AAA GAT ATC GAT CTC TTG G 3′ 3 prime end: 5′TTC CTC GAG CTA CGT CAG GGG ATA ATC TAG ACT TTT C 3′. The resulting PCR product was subcloned in pBluescript. The DNA sequence analysis was performed using an automated DNA sequence analyzer (A.L.F., Pharmacia). The open reading frame was identical to the one reported by Anderson et al. (1992). However, it contained a mutation in the 3′ non-translated region. The PCR product was subcloned as an Xba I/BamH I fragment into the pGEMHE vector (Liman et al. 1992) (kind gift of E. Liman). Plasmid DNA was prepared using standard recombinant DNA techniques with minor variations according to Sambrook et al. (1989). In vitro transcription was performed using the T7 MegaScript Kit (Ambion).

Injection of cRNA in Xenopus oocytes

Experiments were performed in Hannover and Genova. *Xenopus* frogs (Hannover) were purchased from Nasco (Wisconsin). Genova frogs were orginally purchased from Korlh (Hamburg) in 1991 and cultured in a small pond in the garden of Dr. R. Fioravanti in Camogli. Oocytes were isolated as described by Stühmer et al. (1987) and injected with *KAT1* cRNA (100 ng/μl) using a General Valve Picospritzer II microinjector (40 psi for 5–10 ms; Hannover) or Drumond "Nanoject" (46 nl, Genova). In order to compare *KAT1* expression in oocytes originating from the two different sources, preinjected oocytes and *KAT1* cRNA (Hannover) were transferred and analyzed in Genova.

Voltage-Clamp recordings

Whole cell K^+ currents were measured with a two microelectrode voltage-clamp (GeneClamp 500, Axon Instruments USA and Turbotec 01C, NPI Germany), using 0.2–0.4 MΩ (Genova) or 0.5–2 MΩ pipettes (Hannover) filled with 3 M KCl. Oocyte high potassium solution, KD98, contained (in mM): 98 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 TRIS-HCl, pH 7.4. The low potassium solution, KD10, was essentially the same as KD98, but contained only 10 mM KCl. All solutios were adjusted to 270 mOsm with sorbitol. Currents were filtered with a cut-off frequency of 3 kHz before acquisition.

Single channel recordings

Patch pipettes were made from aluminium silicate glass capillaries, silicone rubber coated, and fire polished to a final resistance of 0.6–1.2 MΩ. Pipettes were filled with 120 mM KCl, 20 mM TRIS-HCl pH 7.4, 5 mM EDTA and oocytes were bathed in the same solution. The membrane of the oocyte was partially broken to equilibrate the intracellular space with the bathing solution. Single channel properties of *KAT1* expressed in oocytes were studied in cell attached patches formed on the intact membrane. Currents were recorded with a standard patch-clamp amplifier (EPC-7, List, Germany), filtered with a low-pass four-pole Bessel filter at a cut-off frequency of 1–5 kHz. Single channel events were sampled at 5–20 kHz. Linear capacities and leak currents were digitally subtracted using appropriate scaled current traces without channel openings. Measurements were performed at 21.5 ± 1.5 °C.

Single channel events were analysed from >4 patches excised from different oocytes. Currents were studied during test pulses ranging from –60 to –200 mV from a holding potential of –40 mV. Average currents from single and multichannel patches to reconstruct macroscopic currents were performed on the basis of at least 30 consecutive voltage pulses.

Patch-clamp studies on guard cell and root protoplasts

Guard cell protoplasts were enzymatically isolated from 2–3 week old leaves of the broad bean, *Vicia faba* (Hedrich et al. 1990). Root protoplasts were enzymatically isolated from 3 day old *Zea mays* seedlings as described by Schachtmann et al. (1991). Ionic currents were studied

in the whole-cell configuration of the patch clamp technique (Hedrich and Schroeder 1989). Currents were elicited by voltage-steps from –80 to –200 mV and 300–600 ms duration, from a holding potential of –40 mV, and measured with either EPC-7, EPC-9 (HEKA, Germany) or Axoclamp 200A (Axon Instruments, USA) patch clamp amplifiers, and low-pass filtered with an eight-pole Bessel filter.

Guard cells were bathed in a solution containing (in mM): 30 Kgluconate, 0.1–1 $CaCl_2$, 2 $MgCl_2$, 10 MES/TRIS pH 5.6. The pipette (intracellular) solution contained (in mM): 146 Kgluconate, 4 KCl, 10 HEPES/TRIS pH 7.2 and 2 Na_2ATP, 0.1 EGTA. Root cell extracellular solution contained (in mM): 10 KCl, 88 Kgluconate, 1 $MgCl_2$, 10 MES/TRIS pH 5.6 in the bath and 10 KCl, 88 Kgluconate, 10 HEPES/TRIS pH 7.4 in the intracellular solution. The final osmolarity of the solutions of 450 mOsm was adjusted with sorbitol.

Results

General features of KAT1 currents

Members of the family of the plant inward rectifying K^+ channels were studied in vitro and in vivo. In vitro we analyzed the *KAT1* gene product following cRNA injection in *Xenopus* oocytes; in vivo we studied inward rectifying K^+ currents in the plasma membranes of *Vicia faba* guard and *Zea mays* root cells.

In voltage-clamped oocytes, inward currents resulting from *KAT1* expression were elicited by membrane potentials, V, more negative than –80 mV (Fig. 1 a). Stepping the membrane potential from a holding potential of –40 mV to various values between –80 and –200 mV activated voltage-dependent, slowly rising, K^+ currents, that did not reach a steady plateau even after 300 ms. Following long stimulation pulses of 3 s duration, the fraction of the steady-state current reached after 300 ms increased with more hyperpolarized potentials from 70% at –120 mV to 95% at –200 mV. The time course of the activation process at pH 7.4 was initially sigmoidal, but neither was of the Hodgkin-Huxley type (Hille 1992) nor could it be fitted by the sum of ≤2 exponentials (Fairley-Grenot and Assmann 1993). During a 300 ms step to –180 mV the current reached 50% of the plateau level after 37 ms, but at the end of the pulse still comprised only 92% of the steady state current reached after 3 s of activation. Since prolonged voltage stimulation tends to be deleterious to the cells, 300 ms pulses were routinely used in most of our experiments. Therefore, we characterised the voltage dependence of activation and kinetics by measurements of the late current, I_l, in response to 300 ms voltage pulses and the time, t_a, required to reach 50% of I_l. Both t_a and I_l are underestimates of the values that would characterise long stimulations, but their systematic use in comparative situations allowed us to extrapolate qualitatively our findings with respect to steady state conditions. Figure 1 c shows that t_a and the true half-activation time differed by up to 50%; however, they exhibited very similar voltage dependencies. The plot of I_l versus V in Fig. 1 b shows that activation became significant for $V \leq -100$ mV, but does not seem to saturate even for $V = -200$ mV, beyond which measurements were impaired by electrical breakdown phenomena. A better description of the voltage dependence of activation is obtained by converting the data of Fig. 1 b into estimates of activation probabilities, P(V), as shown in Fig. 1 d. This was per-

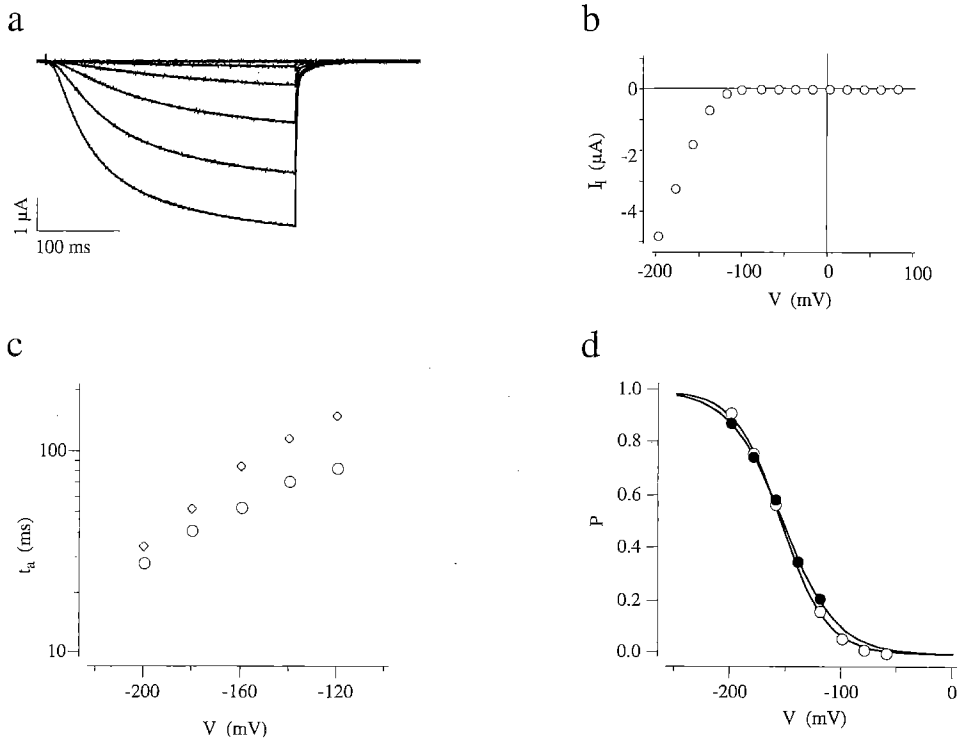

Fig. 1 a–d. Voltage-dependent properties of *KAT1* following expression in *Xenopus* oocytes. **a** Inward currents were elicited in response to voltage pulses from –80 to –200 mV of 300 ms duration, from a holding potential of –40 mV. **b** Late currents, measured at the end of the 300 ms voltage pulse, I_l, are plotted against the pulse potential, V. **c** The activation kinetics were evaluated as the time to reach the half of the amplitude at 300 ms (*circles*) and at 3 s (*diamonds*). **d** Activation curves corresponding to the current at the end of 300 ms voltage pulses (*open circles*) and at the end of 3 s pulses (*filled circles*). Data were fitted by a Boltzmann function (Eq. (1)), and normalized to the maximum value, yielding $V_o = -154$ mV and $V_a = 20.7$ mV for 300 ms pulses, and $V_o = -153$ mV and $V_a = 24.1$ mV for 3 s pulses

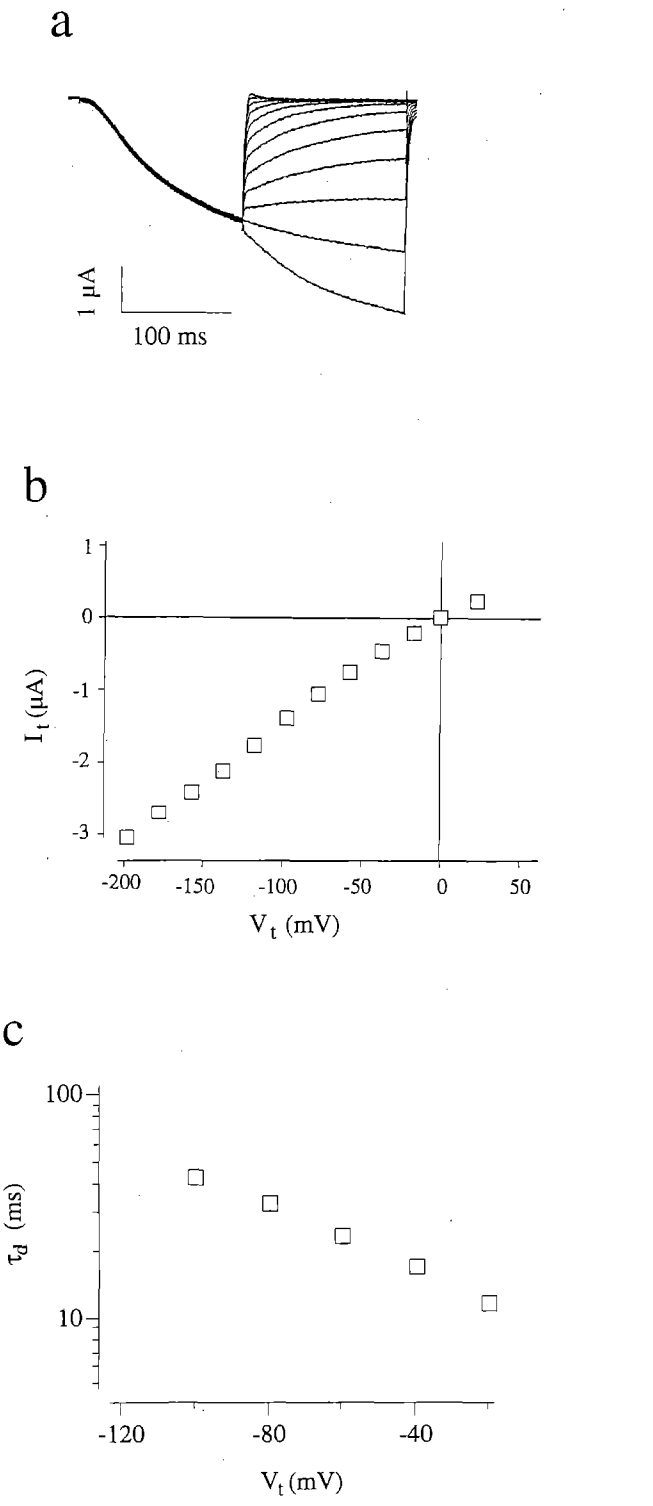

Fig. 2. **a** *KAT1* Currents elicited in oocytes by a tail pulse V_t from −200 to +20 mV (20 mV increments) following a conditioning prepulse to −180 mV for 150 ms, from a holding potential of −40 mV. Note further activation at tail potentials −180 and −200 mV indicating that activation was not yet completed during the prepuls. **b** Instantaneous current I_t is plotted against V_t. Notice the almost ohmic behaviour of the tails, which reverse direction at 2.5 mV in KD 98. **c** Voltage dependence of deactivation time constants τ_d, obtained by fitting the tail currents by a single exponential

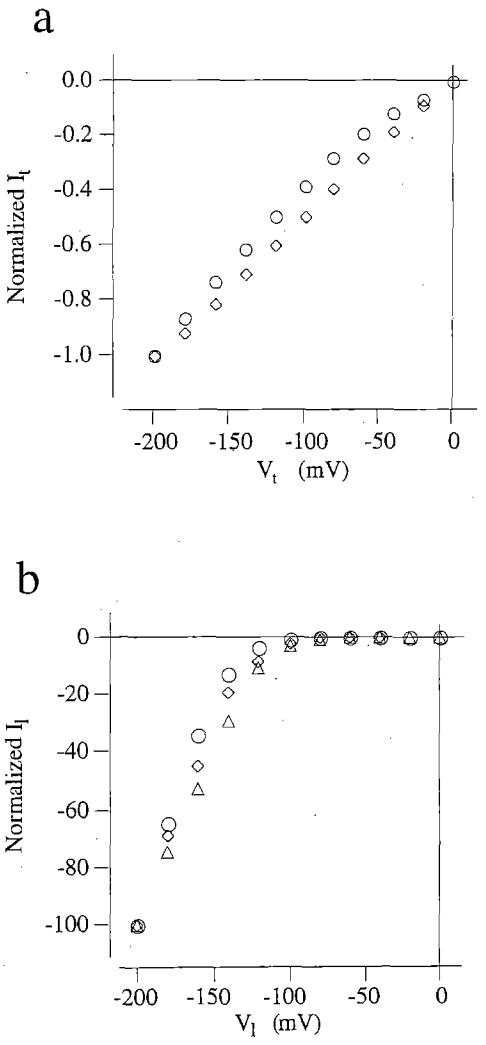

Fig. 3 a, b. Effect of intracellular Mg^{2+} on plant K^+ channels. **a** Instantaneous current-voltage relationships evaluated from a control *KAT1* injected oocytes (*circles*) and from an oocyte with zero intracellular Mg^{2+}, obtained by injecting EDTA, to an intracellular concentration of ~10 mM (diamonds). Values of I_t were normalized to the instantaneous current obtained at −200 mV. Note that the slight rectification in the control experiment almost diappeared after injection with EDTA. **b** Voltage-dependent inward K^+ currents in the plasma membrane of *KAT1* injected oocytes (*circles*), guard cell (diamonds), and root cell protoplasts (*triangles*) in the absence of cytoplasmic Mg^{2+}. I_l were normalized to the I_l obtained at −200 mV

formed by dividing $I_l(V)$ by the instantaneous tail current, $I_t(V)$, measured as described below, and normalizing the results to the asymptotic value (A) at infinite negative voltage, estimated from a Boltzmann fit of the data:

$$\frac{I_l}{I_t} = A \cdot P(V) = \frac{A}{1 + e^{\frac{(V - V_0)}{V_a}}}. \quad (1)$$

For the experiment illustrated in Fig. 1 the latter fit yields an half-activation voltage, $V_o = -154$ mV, and a voltage sensitivity, $V_a = 21$ mV. In Fig. 1 d we also plotted the P(V) estimates that would have been obtained from true steady-state currents measured for 3 s stimuli. A fit of the latter with Eq. (1) yields estimates of V_o and V_a, of −153 mV

and 24 mV, respectively, justifying the use of I_1 for our routine analyses.

Instantaneous current-voltage relationships and the effect of intracellular Mg^{2+}

The kinetics of the deactivation following the return from an hyperpolarizing prepulse to less negative voltages, were studied by tail current analysis (Fig. 2 a). Following an activating prepulse of 150 ms at V = −180 mV, steps to tail potentials, V_t, between −160 and +20 mV, elicited tail currents, I_t, that decayed exponentially with time constants decreasing with V_t from 50 ms to 10 ms (Fig. 2 c). The tail current-voltage relationship, between the early I_t and V_t, showed some non-linearity upon approaching the zero-current potential, near the estimated Nernst potential for K^+ (−3 ± 5 mV in KD98, Fig. 2 b; −60 mV in KD10). However, this rectification is insignificant in comparison with that of the late currents, shown in Fig. 1 b. Protoplasts from *Vicia faba* guard cells and *Zea mays* root cells as well exhibited slowly activating and voltage dependent inward rectifier K+ channels (data not shown), indicating the existence of *KAT1* related currents in these two preparations.

In order to determine whether the strong inward rectification of the late currents results from a voltage-dependent block by cytoplasmic Mg^{2+} ions, as suggested for animal inward rectifiers (Matsuda et al. 1987; Vandenberg 1987; Matsuda 1988), we performed experiments at low intracellular Mg^{2+} concentrations. Injection of oocytes with 40 nl of 200 mM EDTA solution was expected to result in ~10 mM final concentration of EDTA inside the cell, buffering intracellular Mg^{2+} to below 100 nM. Voltage-clamp measurements on EDTA treated oocytes were performed 5 min to 2 h after EDTA injection. In all 10 oocytes tested, K^+ currents with the same properties as described before were observed (Fig. 3 a, circles). Likewise, in the two plant cell types in the absence of Mg^{2+} and addition of 2 mM cytoplasmic EDTA in whole-cell recordings, we found the voltage-dependence of the K^+ currents not to depend on the presence of intracellular Mg^{2+} (Fig. 3 b). The only difference found in Mg^{2+}-free conditions was the absence of significant non-linearities in the instantaneous I_t–V_t relation (Fig. 3 a). This indicates that cytoplasmic Mg^{2+} may act as a fast voltage-dependent blocker of inward currents, as in most other cation selective channels (Matsuda 1988; Johnson and Ascher 1990; Horie et al. 1987; Lin et al. 1991; Pusch 1990), but does not play an important role in the voltage dependence of the slow activation of plant inward rectifier K^+ channels.

5 pS channels underlie KAT1 currents

Single-channel properties were analysed from patch-clamp recordings of *KAT1*-cRNA injected oocytes. Using 0.8–1 MΩ pipettes filled with KD98 solution, attached-patch seal resistances larger than 10 GΩ were obtained. This allowed the analysis of single channel events, with recording from 5–40 channels. In patches, lacking stretch activated channels, we never detected any channel activity at a steady holding potential of −40 mV, whereas 300 ms pulses from −120 to −200 mV caused channel openings, whose frequency increases with pulse duration and the amplitude of the voltage step (Fig. 4 a). This activity was clearly attributable to *KAT1* channels because the average of many (25–40) responses to the same voltage step reproduced the behaviour of the macroscopic current recorded in whole oocytes in response to the same type of stimulation (Fig. 4 b). Owing to the low steady state probability of opening for V = −120 or −140 mV, a large number of distinct single-channel events could be resolved at these potentials, even in patches showing up to 40 active channels at −200 mV (see upper trace of Fig. 4 a, c). Unitary events were occasionally observed also at the beginning of pulses to V = −160 mV, when the open channel probability was still small. However, the presence of multiple channel openings did not allow a reliable statistical analysis of single-channel kinetics.

Single-channel currents were estimated from raw data amplitude histograms, as shown in Fig. 4 d for measurements at V = −160 mV. Several peaks were observed, corresponding to current amplitudes fairly equally spaced at an interval of 0.8 pA, the unitary current flowing through a single *KAT1* channel at that potential. Similar data could also be obtained for membrane potentials positive to −100 mV by analysis of the tail currents at various voltages following hyperpolarizing prepulses. The single-channel current-voltage relation shown in Fig. 4 e was obtained from 5 different membrane patches (4 oocytes). In agreement with the measurements of macroscopic currents described above, this current-voltage relation is linear in the range of −200 mV to +60 mV and intersects the voltage axis near the origin, as expected for Mg^{2+}-free symmetric K^+ conditions. Under these conditions a voltage independent conductance, γ, of *KAT1* channels of the order of 5 pS was calculated from the single channel current-voltage relation in Fig. 4 e. Occasionally, we saw discrete current events corresponding to much higher γ values (34 pS, Fig. 4 f), close to those reported by Schachtmann et al. (1992). However, the correlation of these events with *KAT1* channels is very doubtful, because in these membrane patches the average of many records failed to reproduce the time course and the voltage dependence of macroscopic *KAT1* currents.

High-affinity Cs^+ block

A characteristic feature of K^+ inward rectifiers is their sensitivity to blockage by extracellular Cs^+ and Ba^{2+} (Hagiwara et al. 1976; Standen and Stanfield 1978; Kubo et al. 1993; for review see Hille 1992). We also expected *KAT1* channels to be blocked by Cs^+ because this cation was recently found to suppress K^+ uptake and growth in *Arabidopsis* at concentrations between 50 μM and 2 mM (Sheahan et al. 1993). Indeed, when Cs^+ was applied to the bath solution in this concentration range, *KAT1* currents were strongly affected: the current amplitude was reduced and the activation kinetics were slowed. In KD98 the current at the end of a 300 ms voltage pulse to −160 mV was halved in the presence of 1 mM Cs^+, and the reduction was larger at more negative voltages and at higher Cs^+ concentrations

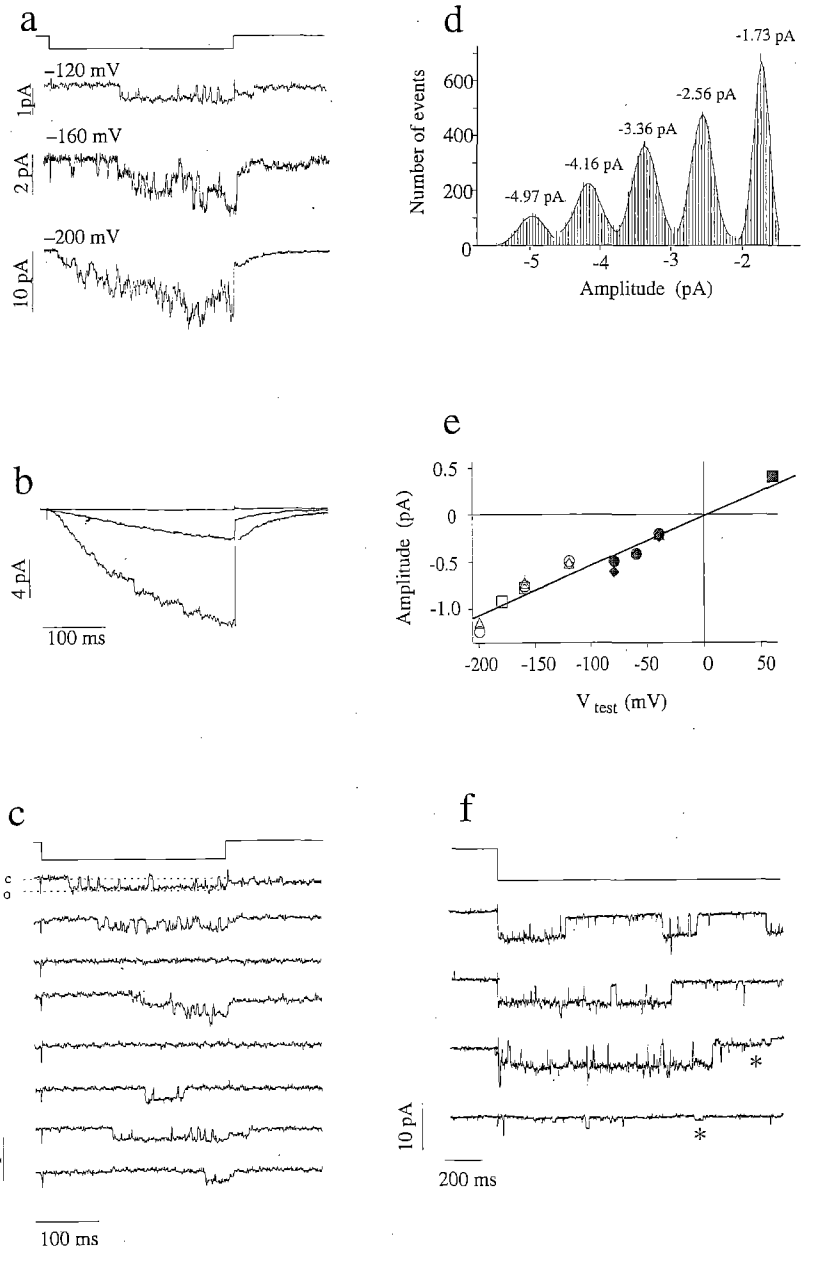

Fig. 4 a–f. Unitary events of *KAT1* channels expressed in oocytes measured from attached membrane patches. **a** Currents elicited by voltage pulses to −120, −160 and −200 mV, from a holding potential of −40 mV. The linear capacity and leakage was digitally subtracted by appropriate scaled responses to positive potentials. The patch contained at least 32 active channels. **b** Averages of 25–40 current traces as those shown in **a** reconstruct the macroscopic inward currents (compare with Fig. 1 a, time scale as in Fig. 1 a). **c** Single channel events in a membrane patch with few channels (5), stimulated by voltage pulses to −120 mV. Closed (c) and open (o) states are indicated. **d** Raw data histogram of single *KAT1* channels in a patch was constructed from 40 consecutive pulses to V = −160 mV. Each peak was well fitted by a Gaussian distribution. The first peak at −1.73 pA corresponds to the closed state. **e** Current-voltage relation of single *KAT1* channels obtained from four different experiments (different symbols). Channel amplitudes marked with filled symbols were measured during channel closure (tails) following a double-voltage pulse experiment (in analogy to Fig. 2 a). **f** In some patches we observed 34 pS channels (c.f. Schachtman et al. 1992) superimposed to 5 pS *KAT1* channels (*asterisks*)

(Fig. 5 a). A tenfold reduction of the K^+ concentration in the control solution (KD10) increased the Cs^+ sensitivity, e.g. the blocking effect of 2 mM Cs^+ in KD98 was comparable to that of 200 μM Cs^+ in KD10 (not shown). This suggests a competition between K^+ and Cs^+ for the same site.

The voltage-dependence of the Cs^+ block is more clearly seen in the instantaneous tail currents following an activating prepulse to −180 mV (Fig. 5 b). In 2 mM Cs^+, for $V_t = -200$ mV the early tail current is smaller, and for V_t between −160 and −60 mV larger than the current at the end of the prepulse. This indicates a further block at −200 mV and a partial unblock of the channels between −160 and −60 mV that was faster than our time resolution. Thus, the instantaneous current-voltage relation exhibits a region of negative resistance. The absolute value of the current reaches a maximum at a voltage that is about −120 mV in 1 mM Cs^+ and increases with increasing Cs^+ concentration (Fig. 5 c).

In contrast to Cs^+, extracellular Ba^{2+} did not affect the *KAT1* currents at concentrations up to 5 mM (c.f. 30 μM in *IRK1*, Kubo et al. 1993).

pH-dependence

We studied the effect of extracellular pH on the properties of *KAT1* channels because K^+ uptake in plants and fungi is often accompanied by changes in the extracellular proton concentrations. We explored whether titratable amino acids in the *KAT1* polypeptide are susceptible to extracellular pH changes. In particular, low pHs might modify Asp and His residues located at or close to the putative pore region H5. Furthermore, protons are known blockers of some

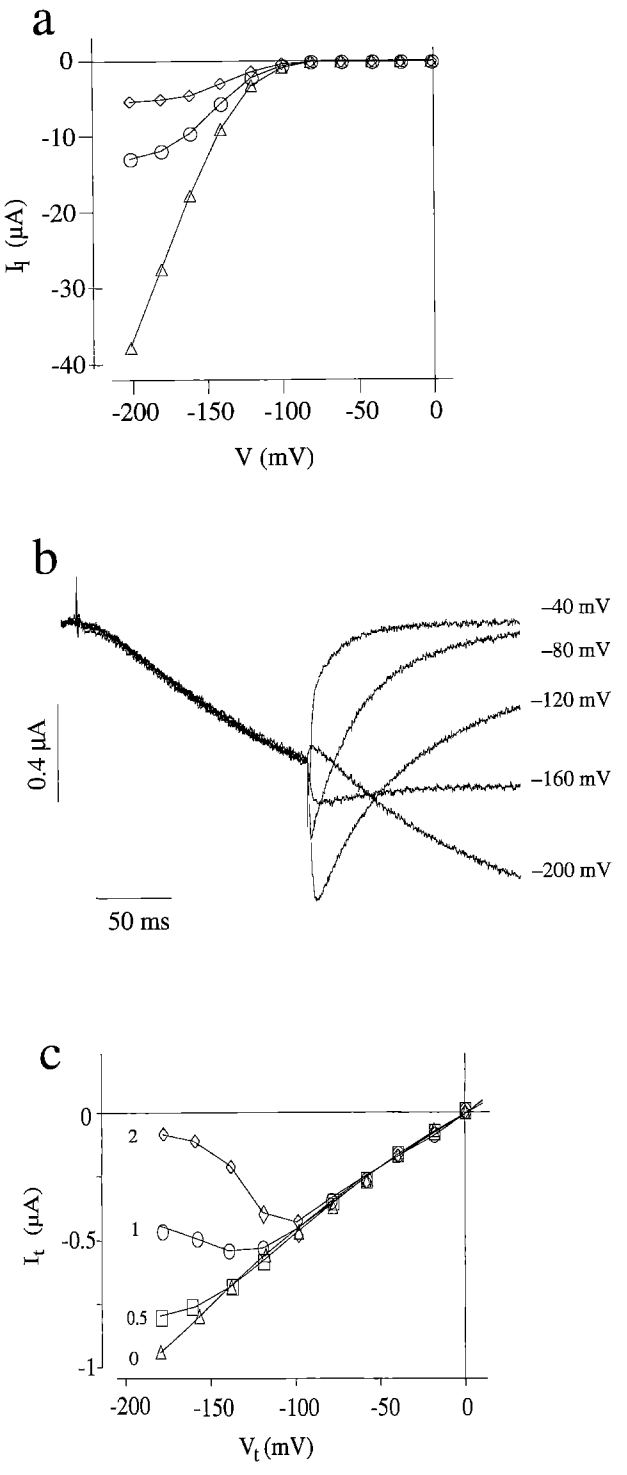

Fig. 5 a–c. Block of *KAT1* currents in oocytes by Cs^+. **a** I_1-V relation measured in control conditions and in the presence of 1 mM (*circles*) and 2 mM extracellular Cs^+ (diamonds). **b** Tail currents measured in the presence of 2 mM extracellular Cs^+. Currents were elicited by a prepulse to –180 mV for 150 ms, followed by a tail pulse to –200 mV to –40 mV. The value of V_t is indicated near to each trace. **c** Instantaneous currents determined by extrapolation of single exponential fits (t=0) at different extracellular Cs^+ concentrations were plotted against V_t. The Cs^+ concentration (mM) is given next to each curve

cation-selective channels, notably the voltage-gated Na^+ and K^+ channels (Hille 1992), as well as the SV-type channel in plant vacuoles (Schulz-Lessdorf and Hedrich unpublished).

By raising the H^+ concentration from pH 7.4 to 6.5, 5.6 as well as pH 4.5, which is closer to the physiological pH range in the extracellular space of plant cells, the currents at the end of 300 ms pulses were increased, but the effect became progressively smaller at more negative voltages (Fig. 6a). This observation might be explained by a pH-induced positive shift of the activation curve rather than modification of the open-channel conductance. This interpretation was further supported by measurements of instantaneous current-voltage characteristics, which were independent of pH, apart from a scaling factor related to the differences in the extent of activation at the end of the prepulse (data not shown). Figure 6b shows activation curves at pH 7.4 and pH 4.5 on the same oocyte (obtained as described for Fig. 1d). These data demonstrate that the increase in pH by one unit produces a positive shift of approximately 19 mV in the voltage-dependence of activation of *KAT1* channels (Fig. 6b inset). Consistent with this observation we found a pronounced pH-dependence of the gating kinetics of *KAT1* channels. As shown in Fig. 6c, d the activation times decreased and the deactivation times increased upon the decrease in extracellular pH.

Discussion

Cloned members of plant homologues of the *Shaker* potassium channel family showed striking structural similarities, but significant differences in their physiological function from their animal counterparts (shown here and in Anderson et al. 1992; Sentenac et al. 1992; Schachtmann et al. 1992). Unlike animal *Shaker*-related channels these plant K^+ channels are inwardly rectifying. The inwardly rectifying K^+ channels from animals do belong to a different, distantly related family (Aldrich 1993) and rectification rather than being an intrinsic property appears to be dependent on the presence of intracellular magnesium (Ho et al. 1993; Kubo et al. 1993; Matsuda 1988). The results reported here now show that inward rectification of *KAT1* and functionally-related channels in guard and root cells does not require the presence of cytoplasmic Mg^{2+}. In retrospect, this result is not surprising, since the overall structures of the animal inward rectifiers cloned so far, *IRK1*, *ROMK1* and *RACTK1* (Suzuki et al. 1994), are not related to *KAT1*. Thus, like their outwardly rectifying relatives in animals, the voltage dependence of *KAT1* is genuine and may be attributed to an intrinsic voltage sensor.

Voltage sensitivity of the animal *Shaker* channels appears to lie in the S4 transmembrane domain. This region contains repeating basic amino acids at every third or fourth residue, whose presence appears to be important for the sensitivity of the channel to voltage (Papazian et al. 1991). Such repeating basic residues are also evident in the S4 region of *KAT1*. However, what is lacking in *KAT1* are leucine heptat repeats, another structural motif found in the S4 and S5 region of *Shaker* related channels. These

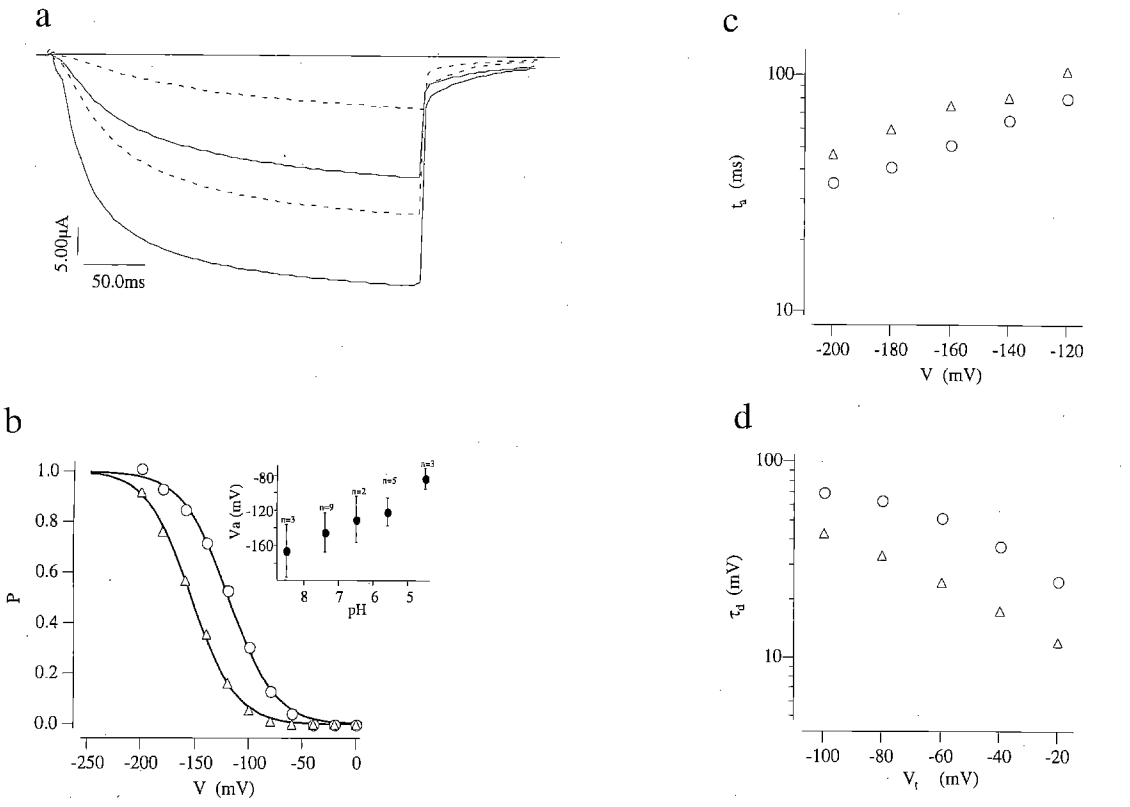

Fig. 6a–d. Effect of protons on *KAT1* currents in oocytes. **a** Currents elicited by a voltage pulse to –140 and –160 mV, with an extracellular proton concentration of pH=7.4 (*broken lines*) and pH=4.5 (*continuous lines*), from a holding potential of –40 mV. Inset: pH-dependent shift in the half-activation voltage, V_a **b**. The corresponding activation curves reveal a shift of the half activation potential, from V_o=–154 mV at pH=7.4 (*triangles*) to V_o=–119 mV at pH=4.5 (*circles*), without a significant change on V_a=20 mV. Voltage dependence of the activation time t_a **a** and deactivation time constants τ_d **d**, evaluated at pH=7.4 (*triangles*) and pH=4.5 (*circles*)

heptad repeats are also supposed to be involved in voltage sensing possibly through interactions with other helices (McCormack et al. 1991). Consequently, the absence of these heptads in the *KAT1* sequence may suggest that the interaction of the S4 helices with other parts of the channel molecule may be altered, leading to opposed rectification.

The only highly conserved motif in *Shaker*, *IRK1*, *ROMK1*, and *KAT1* is the pore region, including the selectivity filter. In order to prove whether the plant and animal inward rectifiers share other properties related to the pore, besides K^+ selectivity, we compared their susceptibility to inhibition by Cs^+ and Ba^{2+}. A high-affinity voltage-dependent block by Cs^+ is a characteristic feature of most K^+ selective channels that is also apparent in *KAT1*. However, we found that Ba^{2+}, the other common blocker of animal voltage-dependent K^+ channels, had no effect on *KAT1*, even at millimolar concentrations. An observation in contrast to Schachtmann et al. (1992).

What is possibly even more significant is the sensitivity of these plant channels to pH. For most potassium and sodium channels protons act as a blocker (Hille 1992; Suzuki et al. 1994). In contrast, *KAT1* has an increased probability of opening in response to decreasing pH, showing an increased current amplitude resulting from a shift in the voltage-threshold for activation. This sensitivity to pH is particularly interesting given the role of the H^+-ATPase in generating the membrane potential negative to –100 mV. Thus H^+ pumping and potassium channel activity may be coordinated in a voltage and pH dependent manner as has been discussed for the inwardly rectifying potassium channel in guard cells (Blatt 1992). Compared to their animal counterparts, acid-activation, a consequence of H^+ extrusion (Shimazaki and Zeiger 1986), as shown for *KAT1* constitutes a plant K^+ channel specific property.

In conclusion, we think that the present work has provided evidence that *KAT1* differs from animal counterparts in response to voltage and channel modulators. With respect to the susceptibility of *KAT1* towards inhibition by low Cs^+ concentrations, our studies together with those on the cesium toxicity (Sheahan et al. 1993) may underline the vital function of K^+ channels for plant growth and development.

Acknowledgements. We gratefully acknowledge E. Liman for providing us with pGEMHE vector. We thank C. Redhead for helpful comments on the manuscript. This work was funded by DFG grants to RH, CNR target project Genetic Engeneering to FC, special project RAISA, subproject N.2.1 paper 1142 to FG and RH and HSFPO to KP.

References

Anderson JA, Huprikar SS, Kochian LV, Lucas WJ, Gaber RF (1992) Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. Proc Natl Acad Sci 89:3736–3740

Aldrich R (1993) Advent of a new family. Nature 362:107–108

Blatt MR (1992) K^+ channels of stomatal guard cells – Characteristics of the inward rectifier and its control by pH. J Gen Physiol 99:615–644

Bush DS, Hedrich R, Schroeder JI, Jones RL (1988) Channel-mediated K^+ flux in barley aleurone protoplasts. Planta 176:368–377

Colombo R, Cerana R (1991) Inward rectifying K^+ channels in the plasma membrane of *Arabidopsis thaliana*. Plant Physiol 9:1130–1135

Elledge SE, Mulligan JT, Ramer SW, Spottswood M, Davis (1991) λYES: A multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc Natl Acad Sci, USA 88:1731–1735

Fairley-Grenot KA, Assmann SM (1993) Comparison of K^+-channel activation and deactivation in guard cells from a dicotyledon (*Vicia faba*) and an graminaceous monocotyledon (*Zea mays*). Planta 189:410–419

Hagiwara S, Miyazaki S, Rothenthal NP (1976) Potassium current and the effect of cesium on this current during anomalous rectification of the egg cell membrane of a starfish. J Gen Physiol 67:621–638

Hedrich R, Schroeder JI (1989) The physiology of ion channels and electrogenic pumps in higher plants. Ann Rev Plant Physiol 40:539–569

Hedrich R, Busch H, Raschke K (1990) Ca^{2+} and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. EMBO J 9:3889–3892

Hille B (1992) Ionic Channels of Excitable Membranes. Sinauer, Sunderland, MA, USA

Ho K, Nichols CG, Lederer WJ, Lytton J, Vassilev PM, Kanazirska MV, Herbert SC (1993) Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. Nature 362:31–38

Horie M, Irisawa H, Noma A (1987) Voltage-dependent magnesium block of adenosine-triphosphate-sensitive potassium channel in guinea-pig ventricular cells. J Physiol (London) 387:251–271

Jan LY, Jan YN (1992) Tracing the roots of ion channels. Cell 69:715–718

Johnson JW, Ascher P (1990) Voltage-dependent block by intracellular Mg^{2+} of *N*-methyl-D-aspartate-activated channels. Biophys J 57:1085–1090

Kubo Y, Baldwin TJ, Jan YN, Jan LY (1993) Primary structure and functional expression of a mouse inward rectifier potassium channel. Nature 362:127–133

Liman ER, Tytgat J, Hess P (1992) Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. Neuron 9:861–871

Lin F, Conti F, Moran O (1991) Competitive blockage of the sodium channel by intracellular magnesium ions in central mammalian neurones. Eur Biophys J 19:109–118

Matsuda H (1988) Open-state substructure of inwardly rectifying potassium channels revealed by magnesium block in guinea-pig heart cells. J Physiol (London) 397:237–258

Matsuda H, Saigusa A, Irisawa H (1987) Ohmic conductance through the inwardly rectifying K channel and blocking by internal magnesium. Nature 325:156–159

McCormack K, Tanouye MA, Iverson LE, Lin J-W, Ramaswami M, McCormack T, Capanelli JT, Mathew MK, Rudy B (1991) A role for hydrophobic residues in the voltage-dependent gating of shaker K^+ channels. Proc Natl Acad Sci USA 88:2931–2935

Moran N, Ehrenstein G, Iwasa K, Mischke C, Bare C, Sutter RI (1988) Potassium channels in motor cells of *Samanea saman* – A patch-clamp study. Plant Physiol 88:643–648

Nitschke K, Fleig U, Schell J, Palme K (1992) Complementation of the cs *dis2* – 11 cell cycle mutant of *Schizosaccharomyces pombe* by a protein phosphatase from *Arabidopsis thaliana*. EMBO J 11:1327–1333

Papazian DMT, Timpe LC, Jan YN, Jan LY (1991) Alteration of voltage-dependence of *Shaker* potassium channels by mutations in the S4 sequence. Nature 349:305–310

Pongs O (1992) Voltage-gated K^+ channels. In: Molecular aspects of transport proteins. Amsterdam, Elsevier

Pusch M (1990) Open-channel block of Na^+ channels by intracellular Mg^{2+}. Eur Biophys J 18:317–326

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual 2nd. Cold Spring Habour Laboratory Press, Cold Spring Habour N.Y.

Schachtmann DP, Tyerman SD, Terry BR (1991) The K^+/Na^+ selectivity of a cation channel in the plasma membrane of root cells does not differ in salt-tolerant and salt-sensitive wheat species. Plant Physiol 97:598–605

Schachtmann DP, Schroeder JI, Lucas WJ, Anderson JA, Gaber RF (1992) Expression of an inward-rectifying potassium channel by the *Arabidopsis KAT1* cDNA. Science 258:1654–1658

Schroeder JI, Fang MM (1991) Inward-rectifying K^+ channels in guard cells provide a mechanism for low affinity K^+ uptake. Proc Natl Acad Sci 88:11 583–11 587

Schroeder JI, Hedrich R, Fernandez JM (1984) Potassium-selective single channels in guard cell protoplasts of *Vicia faba*. Nature 312:361–362

Schroeder JI, Raschke K, Neher E (1987) Voltage dependence of K^+ channels in guard-cell protoplasts. Proc Natl Acad Sci 84:4108–4112

Sentenac H, Bonneaud N, Minet M, Lacroute F, Salmon J-M, Gaymard F, Grignon C (1992) Cloning and expression in Yeast of a plant potassium ion transport system. Science 256:663–665

Sheahan JJ, Ribeiro-Neto L, Sussman MR (1993) Cesium insensitive mutants of *Arabidopsis thaliana*. Plant J 3:647–656

Shimazaki K, Iino N, Zeiger E (1986) Blue-light-dependent proton extrusion by guard cell protoplasts. Nature 319:324–326

Spalding EP, Goldsmith MH (1993) Activation of K^+ channels in the plasma membrane of *Arabidopsis* by ATP produced photosynthetically. Plant Cell 5:477–484

Standen NB, Stanfield PR (1978) A potential- and time-dependent blockade of inward rectification in frog skeletal muscle fibres. J Physiol 280:169–191

Stühmer W, Methfessel C, Sakmann B, Noda M, Numa S (1987) Patch clamp characterization of sodium channels expressed from rat brain cDNA. Eur Biophys J 14:131–138

Suzuki M, Takahashi K, Ikeba M, Hayakawa H, Ogawa A, Kawaguchi Y, Sakai O (1994) Cloning of a pH-sensitive K^+ channel possessing two transmembrane segments. Nature 367:642–645

Vandenberg CA (1987) Inward rectification of a potassium channel in cardiac ventricular cells depends on internal magnesium ions. Proc Natl Acad Sci 84:2560–2564