Magnesium-independent activation of inward-rectifying K^+ channels in *Vicia faba* guard cells

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Abstract The activation of inward-rectifying K^+ channels in guard cells at membrane potentials negative of the K^+ equilibrium potential is important for their cellular function as proton pump-driven K^+ uptake pathways during stomatal opening. In animal cells the voltage-dependence of inward-rectifying K^+ channels is produced to a large extent by intracellular magnesium block. In guard cells, when cytosolic Mg^{2+} was either 3 mM or <1 μM, activation times, deactivation times and the steady-state voltage-dependence of K^+ channels remained unchanged. It is discussed that the activation mechanism of inward-rectifying K^+ channels in guard cells is independent of intracellular Mg^{2+} block.

Key words: Inward rectification; Magnesium block; Voltage-sensor; Stomata; K^+ transport; Polyamine

1. Introduction

Stomatal pores in the epidermis of leaves regulate the exchange of CO_2 and water vapor between leaves and the atmosphere. Each stomatal pore is surrounded by a pair of guard cells which controls opening and closing of the central aperture. Potassium uptake into guard cells produces turgor and volume increases of guard cells which in turn lead to opening of stomatal pores [1]. Inward-rectifying K^+ channels in guard cells have been shown to provide a major pathway for low-affinity K^+ uptake into guard cells required for stomatal opening [2–5]. The proton-extruding ATPase can provide sufficient driving force to create strong negative membrane potentials in plant cells [6] for inward-rectifying K^+ channel activation and for driving long-term low-affinity K^+ uptake into guard cells (for review see [7]).

Inward-rectifying K^+ (K_{in}^+) channels have been described in a number of higher plant cells, including barley aleurone layer cells, extensor cells in leaf pulvini, mesophyll cells and root hair cells [8–11] (for complete references see [7]). Similar to guard cells, K_{in}^+ channels in other plant cell types have been proposed to contribute to proton pump-driven K^+ uptake and to membrane potential control [8–11].

The molecular mechanisms remain unknown by which higher plant K_{in}^+ channels produce strong hyperpolarization-induced activation. Several distinct features of these voltage-dependent inward K^+ channels have been described. The activation potential of inward-rectifying K^+ channels in plant cells does not depend strongly on the extracellular K^+ concentration. Furthermore under most conditions, K_{in}^+ channels do not allow steady-state outward currents positive to the K^+ equilibrium potential, because of the steep hyperpolarization-induced activation [4].

Intracellular cations modulate K_{in}^+ channel properties in guard cells. Raising the cytosolic free calcium concentration in guard cells to micromolar levels inhibits K_{in}^+ channels [3,12]. Cytosolic Ca^{2+} inhibits K_{in}^+ currents by causing an apparent shift in the activation of these channels to more negative potentials [3].

In animal systems, the voltage-dependent activation of inward-rectifying K^+ channels (IRK) is strongly dependent on cytosolic Mg^{2+} and polyamines [13–15]. Cytosolic free Mg^{2+} has been shown to provide a major mechanism contributing to inward rectification in these animal K^+ channels: when the membrane potential is depolarized, cytosolic Mg^{2+} blocks inward-rectifying K^+ channels, thereby inhibiting K^+ currents [13,16,17]. Negative membrane potentials cause a dislodgement of cytosolic Mg^{2+} from the K^+ channel pore, leading to K^+ channel activation. Interestingly, inward rectification of IRKs in some animal systems does not depend on intracellular Mg^{2+} [18] or in other cases Mg^{2+} only partially modulates an instantaneous component of IRK currents [19] which may be explained by a recently revealed polyamine block [14,15].

Recent expression cloning studies have led to the isolation of the first K^+ channel clones from the higher plant *Arabidopsis thaliana*, which confer K^+ uptake into yeast [20,21]. *Xenopus* oocyte expression studies showed that one of these cDNAs, *KAT1*, confers the hallmark properties of plant K_{in}^+ channels [22]. This K_{in}^+ channel cDNA belongs to the superfamily of voltage-dependent K^+ channels which contain six putative membrane spanning domains, a fourth membrane spanning domain with positively charged amino acids [S4], and a pore region showing homology to a putative cation selectivity filter-forming domain (for review see [23]). Conversely, recent cloning of the first IRK channels from animal systems show that these channels differ in structure from the superfamily of voltage-dependent K^+ channels [24–26].

The multiple effects of extracellular cations and of the cytosolic divalent cation Ca^{2+} on K_{in}^+ channels in guard cells as well as the unique structure of this class of inward-rectifying K^+ channels indicate complex mechanisms leading to hyperpolarization-induced activation. In the present study the effects of cytosolic Mg^{2+} on inward-rectifying K^+ channels in guard cells have been studied to gain further insight into the mechanism of K_{in}^+ channel activation. The presented data show that guard cell K_{in}^+ channels are not gated by cytosolic Mg^{2+}. Furthermore, it is discussed that hyperpolarization-induced activation in guard cells is largely due do to an intrinsic gating mechanism.

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2. Materials and methods

Vicia faba plants were grown, guard cell protoplasts isolated, patch clamp recordings performed and data analyzed as described previously [4]. The bath solution during recordings contained (in mM): 10 K^+-glutamate, 2 $MgCl_2$, 1 $CaCl_2$, 10 MES, KOH, pH 5.5, and 477 mannitol. The pipette solution contained (in mM) 100 K^+-glutamate, 10 HEPES, 1 K_2-EGTA, 3 KOH, pH 7.2, and 277 mannitol. In experiments with millimolar cytosolic Mg^{2+}, 4 mM MgATP and 3 mM $MgCl_2$ were added to the pipette solution. Free cytosolic Mg^{2+} was buffered to submicromolar levels by adding 4 mM Na_2ATP and 1 mM EDTA [28]. Liquid junction potentials were corrected as described [2].

3. Results

Whole-cell patch clamp studies were performed on guard cells from *Vicia faba* by imposing conditions which minimize anion channel currents while allowing characterization of K^+ channel currents [2]. When the membrane potential was stepped to voltages negative of approximately −80 mV, the slow activation of an inward-rectifying current was observed (Fig. 1A). Inward-rectifying currents have been shown to be carried by K^+ selective channels in the plasma membrane of guard cells [2,27]. These inward-rectifying K^+ channels allow large K^+ uptake currents into guard cells at hyperpolarized potentials. Inward-rectifying K^+ channel currents saturated at approximately −230 mV. The half activation potential of K^+_{in} channel currents under the imposed conditions was −170 ± 12 mV (± S.D., n = 5).

3.1. Effects of cytosolic Mg^{2+} on K^+_{in} channel activation

To determine whether intracellular Mg^{2+} block of K^+_{in} channels contributes to the strong voltage-dependence of K^+ channels, the cytosolic Mg^{2+} concentration was buffered to submicromolar concentrations. Furthermore, because cytosolic micromolar Ca^{2+} has been shown to inhibit K^+_{in} channels and shift the voltage-dependence of these K^+ channels [3,12], cytosolic Ca^{2+} was buffered to low concentrations using EGTA. Magnesium was buffered to low cytosolic concentrations using 4 mM

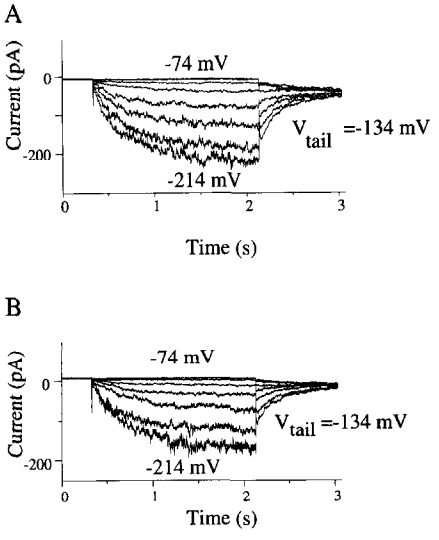

Fig. 1. K^+_{in} channels in guard cells show similar activation properties with 3 mM $MgCl_2$ (A) or 1 mM EDTA, 4 mM Na_2ATP and 0 Mg^{2+} (B) in the cytosol. Voltage clamped membrane potentials ranged from −74 to −214 mV in 20 mV increments.

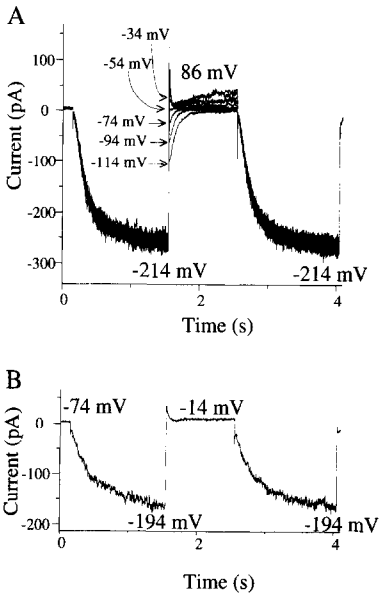

Fig. 2. The activation of K^+_{in} channels was largely independent of the prepulse potential with both 3 mM Mg^{2+} (A) and submicromolar Mg^{2+} (B) in the cytosol, indicating that strong depolarizations do not produce Mg^{2+} block of K^+_{in} channels.

Na_2ATP and 1 mM EDTA [28]. With the cytosolic Mg^{2+} concentration buffered to submicromolar levels, inward-rectifying K^+ channel currents were observed in guard cells (Fig. 1B).

When current–voltage curves were normalized with respect to K^+_{in} currents observed at −214 mV, it became apparent that the steady-state voltage-dependence of K^+_{in} channel currents was not significantly affected by cytosolic Mg^{2+} (not shown). In cardiac myocytes cytosolic Mg^{2+} has significant effects on the time-dependence of an instantaneous component of inward-rectifying K^+ channels [19]. To determine whether cytosolic Mg^{2+} has effects on the time-dependence of K^+_{in} channel currents in guard cells, half activation times of time-dependent currents and instantaneous currents were analyzed. Half activation times of the major time-dependent component of K^+_{in} channel currents under the imposed conditions with 3 mM Mg^{2+} in the cytosol were largely voltage-independent in the membrane potential range from −130 to −210 mV with an average value of $T_{1/2}$ = 340 ± 35 ms. When the cytosolic Mg^{2+} concentration was buffered to submicromolar concentrations, half activation times were also voltage-independent in the same voltage range with an average value of $T_{1/2}$ = 315 ± 40 ms which was similar to that with 3 mM Mg^{2+} in the cytosol. Instantaneous currents were not significantly different at 3 mM and submicromolar cytosolic Mg^{2+} and could be attributed to whole-cell background resistances of 5 to >20 GΩ [4]. In additional experiments 4 mM Na_2ATP without addition of EDTA was used to buffer the free Mg^{2+} concentration to submicromolar levels [28]. Use of Na_2ATP to chelate free Mg^{2+} also showed no effect on the voltage- and time-dependence of K^+_{in} channels (data not shown).

Experiments as illustrated in Fig. 1 were performed with voltage pulses departing from a holding potential of −74 mV. It is possible that at −74 mV, cytosolic Mg^{2+} is already dislodged from K^+_{in} channels such that Mg^{2+} would show no strong

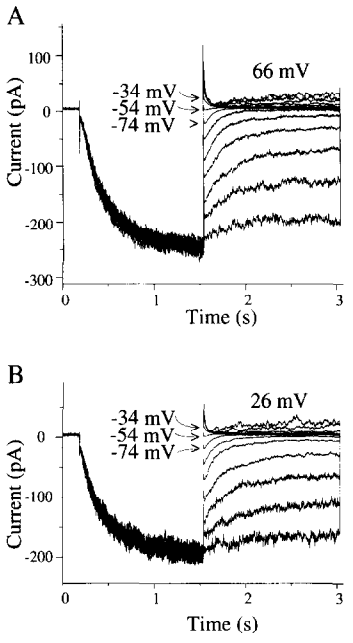

Fig. 3. Deactivation of K_{in}^+ channel currents with 3 mM cytosolic Mg^{2+} (A) or submicromolar Mg^{2+} (B). Membrane potentials close to the reversal potential are indicated to the left of deactivating currents (voltage increments: 20 mV).

effects on K_{in}^+ current activation. To test whether more positive membrane potentials can result in voltage-dependent blocking of K_{in}^+ channel currents by cytosolic Mg^{2+}, further experiments were conducted. A pulse protocol was designed to compare activation properties of K_{in}^+ channel currents from holding potentials in the range from −114 mV to +86 mV (Fig. 2A). The initial pulse to −214 mV in Fig. 2A illustrates that when the membrane potential was hyperpolarized from −74 mV to −214 mV, typical activation of K_{in}^+ channel currents was observed. Following this pulse the membrane potential was stepped to potentials ranging from −114 mV to +86 mV. Subsequently, the membrane potential was stepped again to −214 mV from various depolarized holding potentials. When 3 mM Mg^{2+} were added to the cytosolic solution (Fig. 2A), activation properties of K_{in}^+ channel currents were unaffected by holding potentials in the range from −114 to +86 mV (Fig. 2A). When the cytosolic Mg^{2+} concentration was buffered to submicromolar concentrations, thereby removing any possible Mg^{2+} block, the activation of K_{in}^+ channel currents was similar for prepulse potentials of −74 mV, −14 mV (Fig. 2B) or more positive potentials (not shown). Small changes in instantaneous currents could be accounted for by the linear membrane resistance of guard cells. These data together indicate that cytosolic Mg^{2+} did not produce a measureable voltage-dependent block and Mg^{2+} has no significant effects on the activation of K_{in}^+ channel currents.

3.2. Mg^{2+} effects on K_{in}^+ channel deactivation

Further experiments were performed to determine whether cytosolic Mg^{2+} has effects on the voltage-dependent closing (deactivation) of K_{in}^+ channel currents. The voltage- and time-dependence of K_{in}^+ channel deactivation was studied at millimolar and submicromolar cytosolic Mg^{2+} concentrations. Typical deactivating tail currents of K_{in}^+ channels were observed when the cytosol of guard cells was perfused with a solution containing 3 mM Mg^{2+} (Fig. 3A). The deactivation of K_{in}^+ currents showed a strong voltage-dependence (Fig. 3A) [2,29,30]. As illustrated in Fig. 3A, the reversal potential of deactivating K_{in}^+ channel currents was approximately −50 mV, which lies very close to the equilibrium potential for K^+ (= −52 mV after correction for ionic activities). This confirms previous results showing reversal of K_{in}^+ channel currents within 5 mV of the K^+ equilibrium potential [2], while contrasting results suggest reversal potentials of K_{in}^+ channels at approximately −25 to −33 mV [31]. The large outward tail currents at strong positive potentials indicated that Mg^{2+} does not instantaneously block K_{in}^+ channels upon depolarization (Fig. 3A) as is the case for animal inward-rectifying K^+ channels [19].

To determine whether lowering cytosolic Mg^{2+} can influence the strong voltage-dependence of K_{in}^+ channel deactivation, the cytosolic Mg^{2+} concentration was buffered to submicromolar levels. As shown in Fig. 3B, typical deactivation currents were also observed with <1 μM free cytosolic Mg^{2+}. To further determine whether cytosolic Mg^{2+} has a weak effect on the strongly voltage-dependent K_{in}^+ channel deactivation, half deactivation times were analyzed as a function of the membrane potential (Fig. 4). In the presence of 3 mM Mg^{2+} in the cytosol, tail currents of K_{in}^+ channels showed a strong dependence of half-deactivation times on membrane potential. When the cytosolic Mg^{2+} concentration was buffered to submicromolar levels, similar half deactivation times and a similar voltage-dependence of K_{in}^+ channel deactivation was observed (Fig. 4), indicating that the depolarization-induced closing of K_{in}^+ channels does not depend on the cytosolic Mg^{2+} concentration at physiological membrane potentials.

4. Discussion

The presented data show that the voltage- and time-dependences of inward-rectifying K^+ channels in guard cells are not modulated by changes in the cytosolic Mg^{2+} concentration. Cytosolic Mg^{2+} did not significantly affect the steady-state voltage-dependence, K_{in}^+ channel current magnitudes, half activation times or deactivation times of guard cell K_{in}^+ channel currents. These data show that guard cell K_{in}^+ channels differ in

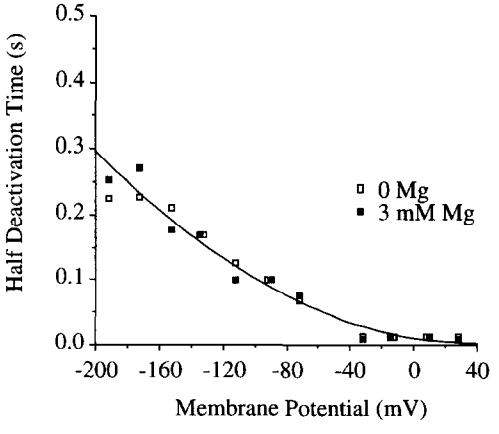

Fig. 4. Deactivation half times with 3 mM cytosolic $MgCl_2$ and submicromolar Mg^{2+}. When half times were similar at 0 Mg^{2+} and 3 mM Mg^{2+}, data points were slightly shifted horizontally for visibility of data.

their response to intracellular free Mg^{2+} from IRK channels in animal cells.

In animal cells, reduction of cytosolic Mg^{2+} to submicromolar levels leads to loss of a significant portion of the voltage-dependence of IRK channels [13,16,17]. However, even after buffering cytosolic Mg^{2+} to submicromolar levels a second component of IRK channels retains voltage- and time-dependence [18,19]. Recent cloning of IRK channels show that these channels do not include a highly amphipathic positively charged S4 domain [24–26], indicating that the Mg^{2+}-independent voltage-dependence [18,19] is most likely due to a novel type of channel activation. Recent studies support this model showing that channel block by diffusible polyamines provides a mechanism for the Mg^{2+}-independent voltage-dependent component of IRK channels [14,15].

Data from previous studies indicate an independence of guard cell K_{in}^+ channel activation on cytosolic diffusible polyamines. Outside-patch recordings of single K_{in}^+ channels show that the time and voltage dependence of these channels is maintained over periods lasting >30 min after membrane patch excision [2,3] while diffusible polyamines alter the voltage-dependence of IRK channels within 30 s of patch excision [14,15]. Furthermore, inside-out macropatch recordings from KAT1-expressing oocytes show that the time and voltage-dependence of K_{in}^+ channel currents is unchanged with micromolar free Mg^{2+} in the cytosolic solution showing that diffusible cytosolic polyamines are not responsible for inward rectification in K_{in}^+ channels (W. Gassmann and J.I. Schroeder, unpublished).

The primary structures of plant K_{in}^+ channels [20,21] differ significantly from those of animal IRK channels. Animal IRK channels have 2 predicted membrane spanning domains with a central loop proposed to function in determining cation selectivity [24–26]. The *Arabidopsis* cDNA KAT1 was shown to encode an inward-rectifying K^+ channel with similar selectivity, pharmacology and voltage and time dependences to K_{in}^+ channels in guard cells and other higher plant cells [22]. All plant K_{in}^+ channels cloned to date show a structural similarity to depolarization-activated K^+ channels and include a positively charged amphipathic S4 domain, a P-domain (H5) with the proposed K^+ selectivity signature sequence (23) and a predicted membrane topology with 6 putative membrane-spanning domains [20,21]. Thus plant K_{in}^+ channels belong to the superfamily of voltage-dependent cation (K^+, Na^+ and Ca^{2+}) channels, which are all activated by depolarization. The hyperpolarization-induced activation of plant K_{in}^+ channels is unique for this superfamily of cation channels. The Mg^{2+} and polyamine insensitivity, the strong voltage-dependence of K_{in}^+ channel deactivation (Figs. 3 and 4), the time dependence of K_{in}^+ channel activation (Fig. 1) as well as the structure of these channels indicate that the voltage-dependence of K_{in}^+ channels may be regulated by an intrinsic gating mechanism similar to that of depolarization-activated K^+ channels [32]. Further research will be required to determine whether the S4 domain in plant K_{in}^+ channels functions as a voltage sensor which contributes to channel activation.

In conclusion, these data show that guard cell K_{in}^+ channels differ in their mechanism of voltage-dependent regulation from animal IRK channels. Furthermore, the possibility that the voltage sensor of plant K_{in}^+ channels may be related to that of animal depolarization-activated K^+ channels indicates that plant K_{in}^+ channels provide an interesting hyperpolarization-activated model system for studying intrinsic activation mechanisms of voltage-dependent K^+ channels.

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