Functional expression of the plant K^+ channel KAT1 in insect cells

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Abstract Following the biophysical analysis of plant K^+ channels in their natural environment, three members from the green branch of the evolutionary tree of life KAT1, AKT1 and KST1 have recently been identified on the molecular level. Among them, we focused on the expression and characterization of the *Arabidopsis thaliana* K^+ channel KAT1 in the insect cell line Sf9. The infection of Sf9 cells with KAT1-recombinant baculovirus resulted in functional expression of KAT1 channels, which was monitored by inward-rectifying, K^+-selective (impermeable to Na^+ and even NH_4^+) ionic conductance in whole-cell patch-clamp recordings. A voltage threshold as low as −60 to −80 mV for voltage activation compared to other plant inward rectifiers in vivo, and to in vitro expression of KAT1 in *Xenopus* oocytes or yeast, may be indicative for channel modulation by the expression system. A rise in cytoplasmic Ca^{2+} concentration (up to 1 mM), a regulator of the inward rectifier in *Vicia faba* guard cells, did not modify the voltage dependence of KAT1 in Sf9 cells. The access to channel function on one side and channel protein on the other make Sf9 cells a suitable heterologous system for studies on the biophysical properties, post-translational modification and assembly of a green inward rectifier.

Key words: Insect cell line (Sf9 cells); Functional expression; KAT1; Patch-clamp technique; Activation potential; Ammonium; Calcium

1. Introduction

Voltage-dependent potassium-selective ion channels in plants can be subdivided into outward and inward K^+ rectifiers [10]. On the molecular level the plant inward rectifiers are represented by transporters belonging to the family of KAT1- and AKT1-related channels [1,23]. So far, a functional analysis of an inwardly-rectifying K^+ channel in vivo as well as in vitro was only performed for a guard cell K^+ uptake channel identified in potato, and the related channel gene product KST1 [18]. For the comparative studies, KST1 was expressed in *Xenopus* oocytes, whereas isolated potato guard cells were used as the in vivo system. The functional features such as selectivity, kinetics, and voltage dependence of the channel-mediated inward K^+ currents were very similar in both experimental systems. KST1 activates negative to −80 mV, with a half-activation potential around −140 mV. This channel is highly permeable to K^+, less permeable to NH_4^+ and Rb^+, and impermeable to Na^+ and Li^+. Cs^+ which is phytotoxic for plant growth and development [24] blocks in a voltage-dependent manner [18, cf. 12,25]. The majority of these characteristics are related to the electrical properties described for the plasma membrane of higher plant cells [11] and KAT1 expressed in frog oocytes, yeast and insect cells [28].

In contrast to KST1, the in vivo features of the cloned *Arabidopsis* K^+ channels, KAT1 and AKT1, in the cellular tissue from which they originate, are still unknown. Both channel types (KAT1, AKT1) were cloned on the basis of their capability to complement K^+ uptake deficient yeast mutants [21,23]. In order to examine their electrical properties, expression systems were selected which tolerate transient membrane polarization in the range of −80 mV to −250 mV, characteristic for plant cells rather than for animal cells (−40 mV to −80 mV). So far, KAT1 could be functionally expressed in *Xenopus* oocytes and yeast [4,12,21,25,26], systems which allow the voltage-dependent stimulation of KAT1.

In the present work we used the insect cell line Sf9 derived from *Spodoptera frugiperda* for heterologous high-level expression of KAT1 to demonstrate (i) its functional expression in this cell line and (ii) the electrophysiological and biochemical characterization of KAT1. Sf9 cells have been reported as a suitable system for the functional expression and purification of animal ion channels [2,5,15,16]. But to our knowledge, insect cells were not yet used to express and to study plant ion channels. Since Sf9 cells are known to overproduce foreign channel protein, they represent a rich source for the plant ion channel protein for further analysis of the channel function and its molecular structure. Isolation and purification of sufficient amount of the channel protein may allow reconstitution and crystallization as well as search for interaction and assembly of subunits.

2. Materials and methods

2.1. Construction and isolation of recombinant baculovirus

All recombinant DNA manipulations were carried out by standard procedures [3]. KAT1 cDNA was introduced in pGmAc 34T transfer vector [6]. KAT1 cDNA was provided in the yeast/*E. coli* shuttle vector pFL61 between two *Not*I sites [17, 23]. In pGmAc 34T plasmid, the initiator ATG of polyhedrin was removed by changing G to T, and a linker containing a *Not*I site was introduced at the *Sma*I cloning site present at a deletion between nucleotides +44 and +462. KAT1 cDNA consisted of 186 base pairs of 5′- and 101 base pairs of 3′-untranslated nucleotides. In the resulting plasmid, the initiator methionine is preceded by the 5′ untranslated region of the polyhedrin gene along with an additional 245 bp (Gaymard et al., unpublished). Sf21 cells were transfected with the resulting plasmid and wild type viral DNA as described in Davrinche et al. [6]. Recombinant virus clones were identified visually as occlusion negative plaques. Three independant plaques were purified by three rounds of infection of Sf21 cells. Pure recombinant viruses were amplified to 108 pfu/ml, and stored at 4°C.

2.2. Insect cells culture

Spodoptera frugiperda Sf9 and Sf21 cell lines were grown as mono-

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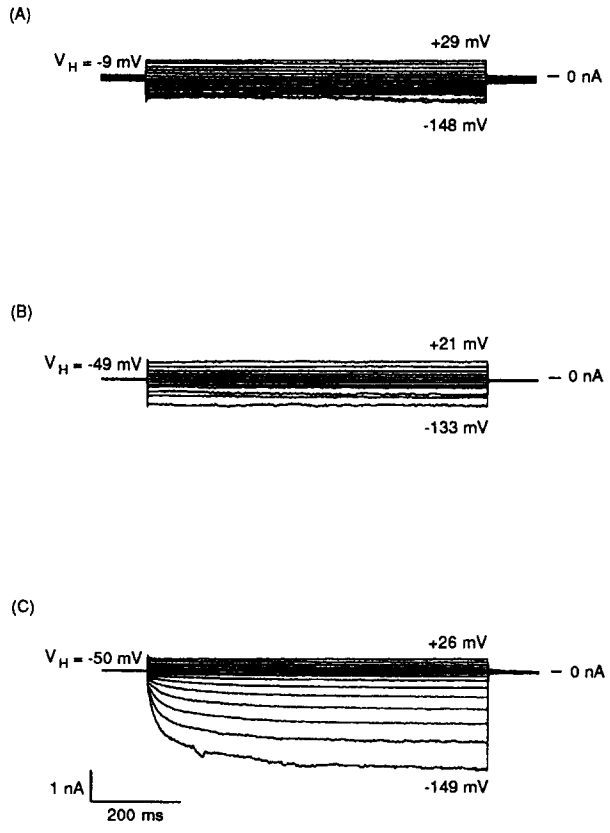


Fig. 1. Electrical properties of the plasma membrane of non-infected (A) and baculovirus-infected insect cells (B,C). Infection with KAT1-recombinant virus (C) resulted in slowly activating inward-rectifying currents through voltage stimulation to hyperpolarized potentials. The experiments shown in Fig. 1B and C were performed in the presence of 120 mM Kglutamate, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.2/TRIS in the pipette and 10 mM KCl, 120 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose, 10 mM MES, pH 6.3/TRIS in the extracellular medium.

layer cultures at 28°C in TC-100 medium (Gibco-BRL) supplemented with 10% (v/v) fetal calf serum (growth medium). Cells were split every 4 days, and kept at a density of about 7×10^5 cells/ml. Log phase cells were layered in 3 cm diameter cell culture dishes at a density of 5×10^5 cells/ml and infected by wild-type or KAT1 recombinant virus (multiplicity of infection, M.O.I. = 10). After 24 to 48 h of incubation at 28°C, Sf9 cells were electrophysiologically assayed using the patch-clamp technique [9].

2.3. Electrophysiological analysis

Current measurements were made in the whole-cell configuration with an Axopatch 200 A amplifier (Axon Instruments, Inc. Foster City, CA, USA). Whole-cell currents were filtered at 1 or 5 kHz, digitized and stored for analysis on hard disc. Software of Axon Instruments was used for voltage stimulation and analysis on a personal computer. Holding potentials V_H were applied for 10 s between successive voltage pulses. The voltages were corrected for series resistance (R_{series}) and liquid junction potential [19]. Patch pipettes were prepared from Kimax-51 34500 glass (Kimble products, Vineland, NY, USA). The reference electrode was filled with 3 M KCl and a 3 M KCl/2% agarose-plug. The experiments were performed at room temperature (20–22°C).

2.4. Patch-clamp solutions

The standard pipette solution (cytoplasm) was composed of 120 mM KCl, 5 mM $MgCl_2$, 5 mM glucose, 10 mM EGTA, 10 mM MgATP, 10 mM HEPES, pH 7.2/TRIS (Figs. 1A, 3B and 2A circles). The extracellular solution (bath) contained 55 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM MES, pH 6.3/TRIS (Fig. 1B). Modifications of the solutions in the individual experiments were mentioned in the figure legends. The final osmolality was adjusted to 300 mosmol kg^{-1} with sorbitol.

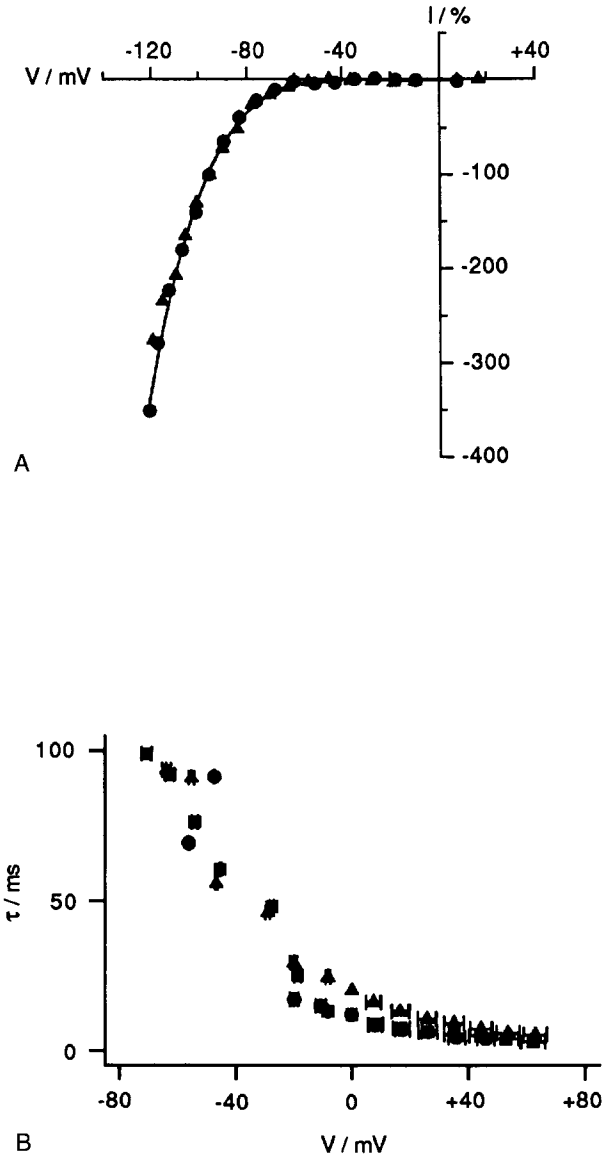


Fig. 2. Voltage dependence and deactivation kinetics of KAT1 expressed in Sf9 cells. (A) A change in the cytoplasmic Ca^{2+} level (circles: 10 mM EGTA, and triangles: 1 mM $CaCl_2$ instead of EGTA) did not alter the voltage dependence of KAT1 currents in Sf9 cells. The steady-state currents evoked by hyper- and depolarizing 756 ms voltage steps from a holding potential of −30 mV (circles) or −40 mV (triangles) were normalized with respect to the currents at −96 mV and expressed as a function of voltage after linear subtraction of instantaneous currents. The bath solution was composed of 120 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM MES, pH 6.3/TRIS. (B) Deactivation time constants of the inward rectifier KAT1 versus voltage. After stimulating KAT1 with a hyperpolarizing prepulse, the decay of the K^+ currents was induced by voltage steps towards more positive potentials (c.f. Fig. 3A). Symbols represent individual experiments and membrane potentials mean values from R_{series}-corrected voltages relative to the onset and termination of the second voltage pulse. Experiments were performed in the presence of 25 mM KCl in the bath.

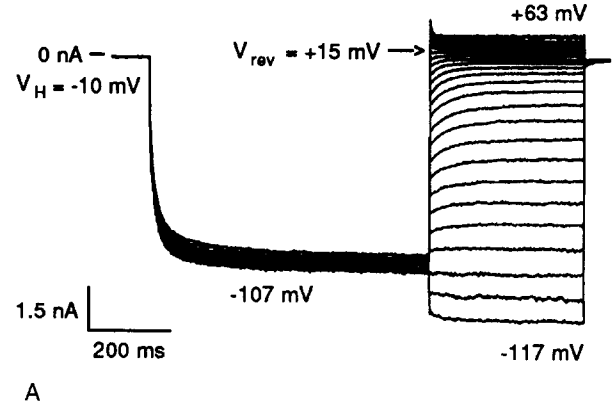
3. Results and discussion

One to two days after infection of the Sf9 cells, we studied the expression of the foreign, plant specific K^+ uptake channel KAT1 from *Arabidopsis thaliana* by patch-clamp analysis. Upon the attachment of 2–4 MΩ patch pipettes onto the plasma membrane of Sf9 cells and application of light suction, seals of >0.1 GΩ were obtained. After rupturing the membrane spanning with the pipette tip in the whole-cell configuration, the membrane potential was clamped to about −10 or −50 mV. Hyper- and depolarizing voltage pulses between −150 and +30 mV were applied (Fig. 1). KAT1-infected Sf9 cells[1] exhibited the characteristic features of inward-rectifying K^+ currents during voltage steps more negative than −60 mV (Figs. 1C and 2A). Besides the instantaneous currents of about 1 nA in magnitude found in non-infected (Fig. 1A), wild-type (Fig. 1B) or KAT1-recombinant virus-infected cells (Fig. 1C), the appearance of time- and voltage-dependent K^+ currents accompanied KAT1 expression only. The instantaneous currents might be at least partially related to the activity of endogenous, voltage-independent ion transporters of the Sf9 expression system [8]. Indeed, in excised outside-out membrane patches, single ion fluctuations were found at potentials (not shown) where time-dependent currents were not observed. For this reason, the analysis of the KAT1-related channel activity on the level of single ion channels was not possible.

Application of single voltage pulses of at least 764 ms duration towards hyperpolarized potentials was well suited to separate slowly activating inward-directed currents from instantaneous currents (Figs. 1C and 3A). In the presence of 25 mM K^+ in the extracellular medium, currents elicited at −94 mV required about 334 ms to half-saturate and upon repolarization to −70 mV declined with a time constant of about 100 ms (Fig. 2B). Activation as well as deactivation kinetics were voltage-dependent. While the activation time constants decreased with increasing hyperpolarization, the decay times decreased with depolarization (Figs. 1C and 2B). A more elaborate characterization of the rise times, however, was hindered by the magnitude of the whole cell currents (1–10 nA) in relation to the access resistance (2–10 MΩ).

In contrast to previous studies of Schroeder and Hagiwara [22] elevated intracellular Ca^{2+} did not affect K^+ uptake currents. The presence of 1 mM intracellular Ca^{2+} on the cytoplasmic side did not shift the activation potential to a more hyperpolarized potential (Fig. 2A). On average, KAT1 activated in the voltage range between −60 to −80 mV. Since hyperpolarization to potentials more negative than −150 mV were not tolerated by the Sf9 cells, we were not able to generate complete activation curves and to determine the half-activation potential. However, with respect to the degree of hyperpolarization required to induce inward-rectifying currents, the voltage threshold of KAT1 activation in Sf9 cells is 20 to 30 mV less negative than in frog oocytes (Fig. 2A).

Upon variation of the K^+ activity in the bath the reversal potentials determined by tail current experiments follow the Nernst potential for K^+ with 54.5 mV per 10 fold change in K^+ activity indicating its high selectivity for this monovalent cation (Fig. 3B). In line with this observation, Li^+ (n = 9), Na^+ (n = 6) and NH_4^+ (n = 5) were largely impermeable. In addition to po-



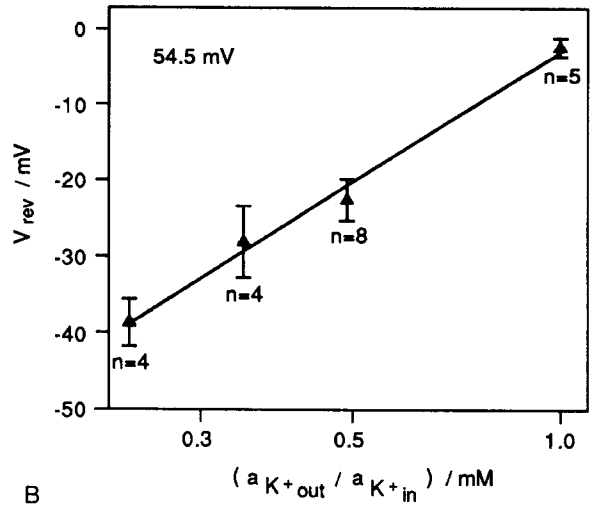


Fig. 3. Selectivity of KAT1 expressed in Sf9 cells. (A) A relative permeability of 0.4 for Rb^+ over K^+ of the inwardly rectifying currents in KAT1-expressing insect cells was calculated from the reversal potential (V_{rev} = +15 mV) of the tail currents during a double voltage-pulse experiment by using the Goldman equation. Following hyperpolarization-induced activation of KAT1, K^+ currents (tails) relax into a new steady-state upon subsequent steps to depolarized or more hyperpolarized values. The permeability towards Rb^+ was determined in the presence of 25 mM KCl, 95 mM RbCl, 5 mM $MgCl_2$, 5 mM glucose, 10 mM EGTA, 10 mM MgATP, 10 mM HEPES, pH7.2/TRIS in the pipette and 120 mM KCl, 2 mM $MgCl_2$, 5 mM glucose and 10 mM MES, pH 6.3/TRIS in the bath. In the case of Na^+, Li^+ and NH_4^+, the bath solution contained 25 mM KCl, 95 mM NaCl (or LiCl or NH_4Cl), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM MES, pH 6.3/TRIS. (B) Reversal potential V_{rev} of the voltage-dependent K^+ currents carried by KAT1 upon variation of the external K^+ activity [20]. The shift in reversal potential by 54.5 mV for a 10-fold change in the K^+ gradient indicates the selectivity of KAT1 for this monvalent cation. In order to determine the K^+ selectivity different standard bath solutions were used with respect to the KCl concentration (25, 37, 55 or 120 mM KCl). (n = number of experiments)

[1] When infected with AKT1-recombinant virus, no inward-rectifying K^+ currents were recorded in Sf9 cells. However, upon changes in the experimental conditions functional expression of AKT1 in the insect cell line was observed (H. Sentenac, pers. comm.).

tassium ions, KAT1 showed a significant permeability for Rb^+, only ($P_{Rb^+}/P_{K^+}^+ = 0.47 \pm 0.09$, $n = 4$; Fig. 3A).

In the present study, it was demonstrated that Sf9 cells represent a suitable system for heterologous expression of the cloned plant K^+ channel KAT1. As in other expression systems in Sf9 cells, KAT1 channels are characterized by high K^+ selectivity, strong rectification as well as slow hyperpolarization-dependent activation. The voltage-dependent properties of KAT1 expressed in oocytes [12,14,21,25,26] are similar to inward-rectifying K^+ channels in vivo [10]. The major difference of the in vitro studies of Schachtman et al. [21] and Hedrich et al. [12] to those reported here for Sf9 cells concerns the relative permeability of KAT1 for NH_4^+ over K^+. In contrast to the situation in oocytes but in line with results obtained in yeast [4], currents carried by NH_4^+ were not significant in Sf9 cells. The lack of NH_4^+ transport through KAT1 expressed in yeast has been erroneously related to block of the plant K^+ channel following NH_4^+ induced cytoplasmic acidification [29]. Hoshi [14], however, could demonstrate that acidification activates KAT1 through a shift of the voltage-dependence to less negative membrane potentials. In addition, the gating mechanism of KAT1 appears to be altered in Sf9 cells. With respect to the expression system the threshold potential of KAT1 activation shifted along the voltage axis. Compared to the KAT1 properties in oocytes, the activation potential of KAT1 is less negative in Sf9 cells but more negative in yeast [4,12,25,26]. Furthermore, the shift of the activation potential in insect cells is accompanied by a shift of the deactivation kinetics towards more positive potentials. This results in a slower deactivation at more hyperpolarized potentials in Sf9 cells than in oocytes [12,25,26]. Similar observations were also made for animal ion channels, e.g. the activation curve of *Shaker* K^+ channels expressed in Sf9 cells shifted towards more depolarized potentials with respect to its expression in oocytes [15]. Thus the discrepancy in permeability and voltage-dependent gating of KAT1 in the insect cell line is probably related to the expression system.

Unlike the K^+ inward rectifier in *Vicia faba* guard cells [7,22] KAT1 is insensitive towards intracellular Ca^{2+}. This behavior, however, is consistent with observations obtained from inward-rectifying K^+ channels in xylem parenchyma cells from barley, in corn coleoptiles and from KAT1-expressing oocytes [13,14,27]. Due to the latter fact, it is likely that the Ca^{2+} insensitivity is a characteristic feature of the *Arabidopsis* channel KAT1 rather than an attribute of the expression system. Nevertheless, the reliability of the data concerning properties obtained in heterologous expression systems remains uncertain until like for KST1 [18] information about the in vivo channel characteristics is available for KAT1, too.

Despite the difficulties in analyzing the biophysical properties of inward-rectifying plant K^+ channels in great detail, KAT1 expression in Sf9 cells provides a tool for reasonable functional analysis as well as isolation of μg amounts of the gene product (H. Sentenac, unpublished) for future purification, reconstitution, and crystallization of the channel protein.

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