J. Membrane Biol. 201, 157–165 (2004)
DOI: 10.1007/s00232-004-0713-8

The Journal of
Membrane
Biology

Orientation of *Arabidopsis thaliana* KAT1 Channel in the Plasma Membrane

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Received: 4 May 2004/Revised: 18 August 2004

Abstract. The *Arabidopsis thaliana* KAT1, an inward-rectifying potassium channel, shares molecular features with the *Shaker* family of outward rectifier K^+ channels. The KAT1 amino-acid sequence reveals the presence of a positively charged S4 and a segment containing the TXGYGD signature sequence in the pore (P) region. To test whether the inward-rectifying properties of KAT1 are due to reverse orientation in the membrane, such that the voltage sensor is oriented in the opposite direction of the electric field compared with the *Shaker* K^+ channel, we have inserted a flag epitope in the NH_2 terminus or the S3–S4 loop. The KAT1 and tagged constructs expressed functional channels in whole cells, *Xenopus* oocytes and COS-7. The electrophysiological properties of both tagged constructs were similar to those of the wild type. Immunofluorescence with an antibody against the flag epitope and an anti-C terminal KAT1 determined the membrane localization of these epitopes and the orientation of the KAT1 channel in the membrane. Our data confirm that KAT1 in eukaryotic cells has an orientation similar to the *Shaker* K^+ channel.

Key words: Potassium channel — Inward rectifier — Membrane orientation — *Arabidopsis thaliana*

Introduction

KAT1 and AKT1 were the first inward-rectifier channels cloned from the plant *Arabidopsis thaliana* (Anderson et al., 1992; Sentenac et al., 1992). The *KAT1* gene is expressed in guard cells and likely to play a role in guard cell K^+ uptake, but is not essential for stomatal opening (Nakamura et al., 1995; Roelfsema & Prins, 1997; Szyroki et al., 2001). The deduced peptide sequence of KAT1 renders a protein of 677 amino acids. Based on hydropathy plots Anderson et al., proposed the presence of six transmembrane domains plus a pore-forming region (P region). Examination of the primary amino-acid sequence also revealed the presence of an S4 transmembrane segment containing regularly spaced basic residues and the TXGYGD signature sequence in the P region (Doyle et al., 1998), indicating that KAT1 shares molecular features with the *Shaker* family of outward-rectifier K^+ channels. Downstream of the S6 transmembrane segment, a putative cyclic nucleotide binding region is present in KAT1 (Schroeder, Ward & Grassman, 1994). KAT1 also displays properties of the HCN family hyperpolarization-activated cyclic nucleotide-gated channels, (Clapham, 1998) and the sea urchin sperm channel (Gausset al., 1998).

Several hypotheses have been proposed to explain the inward rectification in KAT1 channels. The most plausible one is that voltage-dependence in KAT1 arises as a consequence of the presence of a voltage-sensor element in the channel-forming protein, most probably the S4 segment (Marten & Hoshi 1998; Zei & Aldrich, 1998; Cosmelli et al., 2000; Latorre et al., 2003a, b;). The topology of the KAT1 channel has already been determined in *E. coli*. In this study, the KAT1 was shown to be functional by complementation assays (Uozumi et al., 1998). Recent work on the topotogenic function of each of the KAT1 six transmembrane regions has used individual and combined transmembrane segments to evaluate their preference of orientation and coordinated insertion into the endoplasmic reticulum (ER). It was demonstrated that the four hydrophobic segments SI, S2, S5 and S6 are inserted sequentially in the ER membrane, whereas the segments S3 and S4

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integrated into the membrane only after specific interaction between the two segments (Sato et al., 2002, 2003). To determine the orientation of KAT1 in the plasma membrane of eukaryotic cells and as a complementation to previous studies, we have used a methodology that has been successful in determining the topology of the *Shaker* K^+ channels (Shih & Goldin, 1997), the large conductance Ca^{2+}-activated channel (Meera et al., 1997) and the β-adrenergic receptor (Wang et al., 1989). In this approach, we have inserted a flag epitope consisting of eight amino acids (DYKDDDDK) in the NH_2 and the S3–S4 linker. These two flag epitopes are expected to be on opposite sides with respect to the membrane. To locate the carboxy terminus, we have used an anti-KAT1 C-terminal antibody. The wild-type KAT1 and the tagged channels were expressed in whole cells, *Xenopus* oocytes and COS-7 cells, and their function was examined by cut-open oocyte voltage clamping and whole-cell patch-clamp, respectively. The tagged KAT1 constructs expressed functional channels and their electrophysiological properties were similar to those of the wild type. Immunofluorescent staining with a monoclonal antibody against the epitope Flag and the anti C-terminal KAT1 antibody ascertained the membrane localization of these epitopes. In this work we show that in *Xenopus laevis* oocytes and COS-7 cell membranes, the KAT1 channel is oriented with both NH_2 and COOH termini on the intracellular face of the membrane, and the S3–S4 linker is oriented toward the extracellular side. Our results confirm that in these cells, KAT1 has a similar orientation to that of the *Shaker* K^+ channel.

Materials and Methods

Antibodies

The rabbit anti-C terminal KAT1 (aa528–677) antibody was kindly provided by Dr. Frederic Gaymard, Laboratoire de Biochimie et Physiologie Moleculaire de Plantes. Montpellier. France (Gaymard et al., 1996). The mouse anti-Flag antibody was from Sigma, (St Louis, MO), and the fluorescein-5-isothiocyanate-conjugated goat anti-mouse secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz CA)

Construction of the epitope insertion mutations

The KAT1 potassium channel coding region (Accession number M86990) from pGEMHE was transferred to pALTER-1 for the generation of oligonucleotide-directed mutants according to the Altered sites II in vitro Mutagenesis Systems protocol (Promega Biotech, Madison, WI). All the oligonucleotides for mutagenesis contained the same 24 nucleotides encoding flag (GACTATAAAGACGACGATGACAAA), flanked by 15–20 nucleotides of potassium-channel sequence corresponding to sequences extending 5′ and 3′ from the inserted site within the potassium channel region. The flag epitope inserted into KAT1 between the amino acids 6 and 7 N-terminal produced the mutant KAT1Nt-flg, and between the amino acids 155 and 156 (S3-S4 linker) produced the mutant KAT1S3-flg. For the annealing reaction, the mutagenic oligonucleotides were phosphorylated with T4 polynucleotide kinase, mixed with alkaline denaturated pALTERKAT1 DNA template and the antibiotic repair oligonucleotide. The mutant strand was synthesized in vitro using T7 DNA polymerase and T4 DNA ligase. A small volume from the mutagenesis reaction was used to transform ES 1301 mutS competent cells. Control and mutant DNAs obtained were transformed into JM109. Individual colonies were picked and grown in 5 ml cultures from which DNA was purified using the Quiagen miniprep DNA purification system. Isolates containing the flag epitope were verified by dideoxy DNA sequencing with Sequenase (United States Biochemical). For transfection studies the *KAT1* coding region was sub-cloned into pCi at the NotI site (Invitrogen).

Expression in *Xenopus* Oocytes and Immunofluorescent Staining

Plasmid DNA was linearized with Xba I and RNA was transcribed in vitro using the T7 mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX). Stage V oocytes were removed from adult female *X. laevis* frogs, prepared as previously described and incubated in ND-96 media (Gonzalez et al., 2000). 50 nl of RNA (~50 ng) was injected into each oocyte. After 24–48 hours of incubation at 18°C in ND96, potassium currents were recorded at 22°C. For extracellular staining, whole intact oocytes were incubated for 1 h at 18°C with the primary antibody in ND96, with 1% BSA. Oocytes expressing wild-type KAT1 were incubated with rabbit anti-C terminal KAT1 antibody. Oocytes expressing the mutants KATNt-flg, or KATS3-flg were incubated with mouse anti-Flag antibody. After the incubation with the primary antibody, oocytes were washed five times in ND96 and incubated with fluorescein-5-isothiocyanate-conjugated (FITC) anti-rabbit secondary antibody or FITC anti-mouse secondary antibody. Images were generated on a confocal laser scanning imaging system. For intracellular staining, the intact oocytes expressing wild-type KAT1, KATNt-flg or KATS3-flg were fixed in 3.7% formaldehyde in ND96 and embedded in Tissue-Tek Oct Compound (Baxter Scientific Products, Salt Lake City, UT). A Reichter Jung Cryocut 1800 cryostat was used to cut 14 μm sections from the OCT blocks, which were mounted on slides. The fluorescence labeling with FITC was done as described for the intact oocytes, except that after the labeling reaction the sections were treated with 3% Evans blue in PBS to minimize the autofluorescence of the oocytes sections (Becker, DeIoannes & Izquierdo, 1993). The fluorescence labeling was visualized by confocal microscopy.

Expression in COS-7 Cells and Immunofluorescence Staining

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco-BRL), 50 U/ml penicillin, 50 U/ml streptomycin (Gibco-BRL) in a humidified incubator at 37°C under 5% CO_2. Cells were seeded at 20% confluence and transfected either by the Lipofectin Reagent (BRL Bethesda Research Laboratories) or calcium phosphate precipitation method according to Dreyer et al. (2001). Briefly, the day before transfection, cells were trypsinized and transferred to 35-mm dishes (20,000 cells per dish). Per dish, 80 μl HBS 2 × (280 mM NaCl, 50 mM HEPES, 15 mM Na_2HPO_4, pH 7.15) were gently mixed

with 80 μl TE-$CaCl_2$ (1 mM Tris-HCl, pH 8, 1 mM EDTA, 250 mM $CaCl_2$) containing 1–2 μg of pCiKAT1, pCiKATNt-flg or pCiKATS3-flg. The DNA was added to cells in their culture medium. After 8 h, the transfection medium was removed, the cells were rinsed twice with DMEM, fresh medium was added and cells were incubated at 37°C. Electrophysiological measurements were performed 3 or 4 days later. Inmunofluorescence was done 4 or 5 days later.

For immunofluorescence analysis, cells were cultured on glass cover slips that had been coated with 10 μg/ml poly-L-lysine. For staining, cells attached to cover slips were washed 3 times in PBS, then fixed with freshly prepared 4% paraformaldehyde in PBS. Fixed cells were washed 5 times in PBS and permeabilized with either 0.2% Triton in PBS or MetOH at −20°C for 10 min. The nonspecific protein binding sites were blocked by incubation in PBS containing 0.2% gelatin (gelatin-PBS). Cells were then incubated with the primary antibody diluted in gelatin-PBS at room temperature for 1 h, washed with gelatin-PBS six times for a total of 30 min, and incubated in the appropriate secondary antibody for 1 h. After washing as described above, cells were subsequently mounted in Gelvatol (10% glycerol, Dabco, azide 0.1%) and viewed on a Zeiss epifluorescence microscope or confocal microscope.

Inmunofluorescent-labeled intact and sectioned oocytes as well as Cos-7 cell were viewed with a confocal laser scanning microscope using 4 × or 20 × objectives. Specific fluorescence was selected with laser specifications to visualize fluorescein. Images were recorded at the same settings of laser power and photo-multiplier sensitivity. Images were processed with Adobe Photoshop (ADOBE Systems, Mountain View, CA) with identical values for contrast and brightness.

Electrophysiology

Macroscopic currents in injected oocytes were measured using the cut-open oocyte voltage clamp (Taglialatela & Stefani, 1993; Latorre et al, 2003b). The external solution contained 110 mM K-methanesulfonate (KMES), 2 mM $CaCl_2$, 10 mM HEPES, pH 7. The internal solution was the same except that $CaCl_2$ was replaced with EGTA-K. Currents were measured in response to voltage steps from a holding potential of 0 mV to −200 mV in 5 mV increments followed by a pulse to −30 mV. The conductance-voltage curve was obtained using tail-current measurements. The tail currents were fitted with a single exponential and the time zero amplitudes were extrapolated. The relative conductance, G/G_{max} data, were fitted using a Boltzmann function:

$$G = G_{max}/(1 + e^{zF(V1/2-V)/(RT)}) \quad (1)$$

where z is the apparent number of gating charges and provides a measure of the voltage sensitivity of the channel and the $V_{1/2}$ is the half-activation voltage and is proportional to the energy difference between open and closed states. R and T have their usual meaning.

The macroscopic currents from COS-7 control and transfected cells were recorded using whole-cell patch-clamp techniques. The external solution was (in mM) 150 K-gluconate, 20 KCl, 1 $CaCl_2$, 2.5 $MgCl_2$, 10 HEPES, pH 7.2. The pipette solution composition was (in mM) 114 K-gluconate, 20 KCl, 2 $MgCl_2$, 1 $CaCl_2$ 10 EGTA, 10 HEPES pH 7.3 and 4 ATP. Currents were elicited in response to voltage steps from a holding potential of 0 mV to voltage ranging from −200 to −320 mV in 20 mV increments. The data for G/G_{max} analysis in the case of COS-7 cells were obtained from the steady-state currents at the end of the hyperpolarizing pulse, and fitted using Eq. 1. In both oocytes and transfected COS-7 cells the currents recorded were analyzed using pClamp Software (Axon Instruments, Inc. Union City CA).

Results

Insertion of Flag Epitopes Preserves the Overall KAT1 Topology

To distinguish between possible topological models of KAT1 channels, the hydrophilic epitope flag (DYKDDDDK) was introduced in two critical positions of the KAT1 channel-forming protein. The flag epitope inserted between the amino acids T6 and R7 produced KATNt-flg, while KATS3-flg was obtained inserting the flag epitope between the amino acids T155 and A156. These two positions helped us determine which side of the cellular membrane the amino terminus and the S3-S4 linker are facing. A third position of the protein, the carboxy terminal KAT1 was localized using a rabbit anti-C terminal KAT1 antibody, elicited against the amino-acid residues 528–677 (Gaymard et al., 1996)

In silico hydropathy analysis of KAT1 wt KATNt-flg and KATS3-flg showed that the insertion of the hydrophilic epitope flag preserves the overall KAT1 topology (*not shown*). Upon expression of the wild-type KAT1 or the tagged channels, the flag epitopes were localized using specific anti-flag antibodies and the C-terminal KAT1 using the anti-C terminal KAT1 antibody.

Insertion of Flag Epitopes Results in Functional Channels

To determine whether the insertion of the flag epitopes resulted in functional channels in oocytes, currents were measured using vaseline gap cut-open oocyte voltage-clamp. As can be appreciated in Fig. 1*J*, *K*, *L*, the constructs expressed inward currents that were completely absent in non-injected oocytes (*data not shown*). Functional expression was considered a strong indication that the epitope insertion did not lead to changes in the overall membrane topology or channel function. Fig. 2 is a clear indication that the voltage-sensing mechanism of the tagged channels did not suffer appreciable modifications with the insertion of the flag epitope. The slopes of G/G_{max}-V curves of the constructs are comparable to that of the wild-type KAT1 channel. The KATS3-flg channel shows no changes in voltage dependence (z = 1.05 ± 0.3) compared with the wild-type KAT1 (z = 1.12 ± 0.09). However, KAT1Nt-flg channels are slightly more voltage-dependent (z = 1.22 ± 0.15) but their G/G_{max}-V curve is left-shifted by 22.17 mV when compared to the wild-type channel G/G_{max}-V curve ($V_{1/2}$ = −148.5 ± 7.9 mV).

Likewise, to determine whether the insertion of the flag epitopes resulted in functional channels in COS-7 cells, currents were measured using the patch-clamp technique in the whole-cell configuration. As

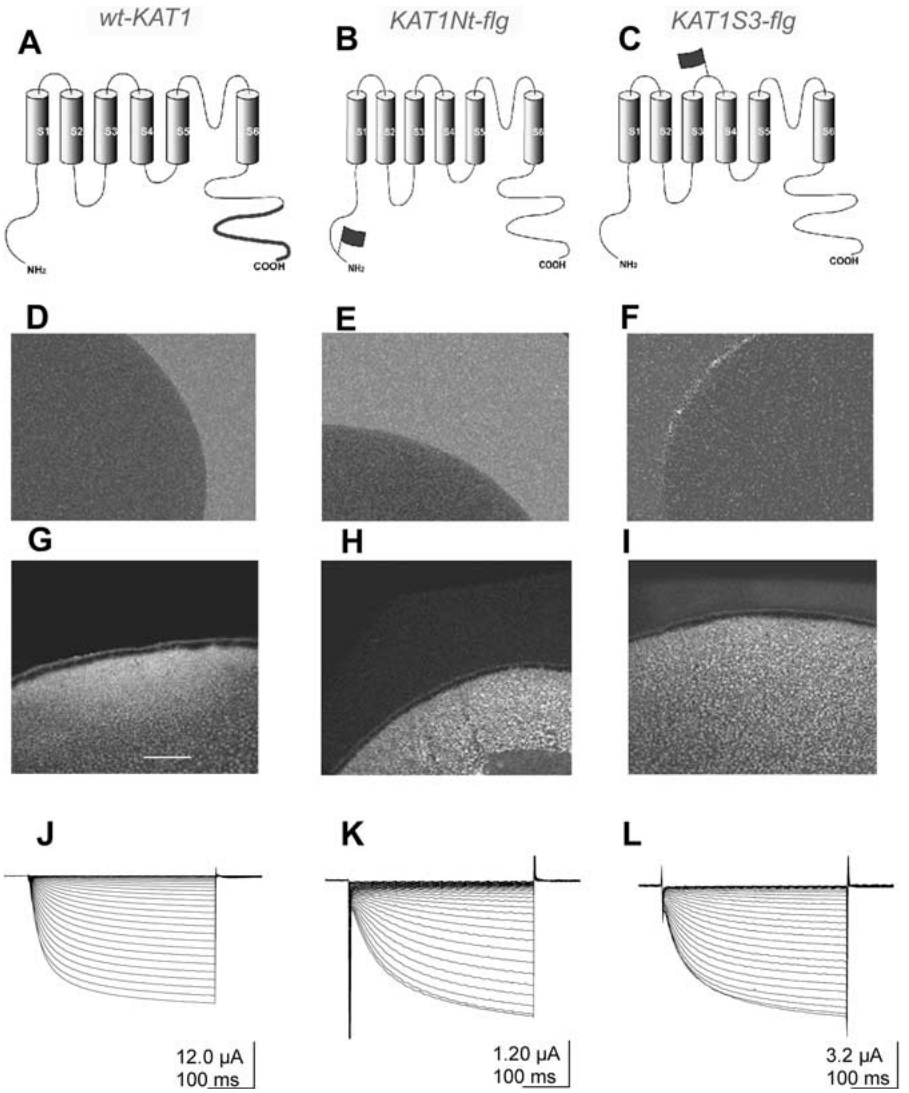

Fig. 1. Confocal fluorescent microphotographs of intact and sectioned oocytes and current records of oocytes expressing wild-type KAT1 or tagged channels KAT1Nt-flg and KAT1S3-flg. *A*, *B* and *C* show schematic diagrams indicating the region in which each flag insertion is located or the domain of the protein used to develop the anti-C terminal antibody. Oocytes expressing the wild-type KAT1 were labeled with the anti-C terminal KAT1 antibody (*D* and *G*). Oocytes expressing the mutants KAT1Nt-flg (*E* and *H*) and KAT1S3-flg (*F* and *I*) were labeled with the anti-flag antibody. Microphotographs *D*, *E* and *F* show intact oocytes labeled on the extracellular side of the membrane. Microphotographs *G*, *H* and *I* show intracellular labeling; sectioned oocytes. Bar: 250 µm. All injected RNAs produced robust inward potassium currents. Functional expressions of wt and flag-tagged clones in oocytes were measured with the cut-open oocyte voltage-clamp technique. *J*, *K*, *L* show the current records from the oocytes expressing wild-type KAT1, KAT1Nt-flg and the KAT1S3-flg respectively.

can be appreciated in Fig. 3*J*, *K*, *L*, the constructs expressed inward currents that were completely absent in control cells (*data not shown*). We also characterized the voltage dependence of the conductance and the tagged channels showed G/G_{max}-V curves comparable to that of the wild-type channel (Fig. 4). As found in oocytes, none of the flag insertions appeared to have deleterious effects on the voltage-sensing machine of the KAT1 channel. The expression of the tagged channels induced robust hyperpolarizing currents equivalent to wild type levels in *X. laevis* oocytes as well as in Cos-7 cells, as shown in Figs. 1 and 3. However, the tagged channels KAT1Nt-flg expressed in oocytes or in Cos-7 cells show the largest shifts of $V_{1/2}$, −25 mV, −83 mV respectively.

Immunodetection of Flag and C-Terminal Epitopes Elucidates the Orientation of KAT1 in the Cytoplasmic Membrane

Representative images of antibody-labeled oocytes expressing the wild-type KAT1 channels are shown in

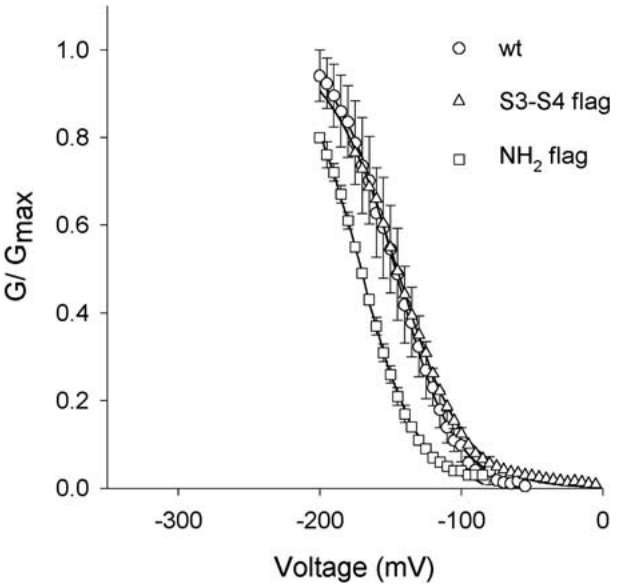

Fig. 2. Voltage-gating curves obtained from oocytes expressing wild-type KAT1, KAT1Nt-flg or KAT1S3-flg. The voltage activation data was fitted using Eq. 1. Values for $V_{1/2}$ (in mV) and z are: -148.5 ± 7.94 and 1.12 ± 0.15 for wild-type KAT1 ($n = 4$); -146.67 ± 7.51 and 1.05 ± 0.30 for KAT1S3-flg ($n = 3$); and -170.67 ± 1.15 and 1.22 ± 0.09 for KAT1Nt-flg ($n = 3$).

Figs. 1*D* and *G*, and while *E*, *H* and *F*, *I* show representative images of oocytes expressing KAT1Nt-flg and KATS3-flg respectively. Only cells expressing KATS3-flg show a measurable fluorescence due to the binding of the anti-flg antibody followed by FITC secondary antibody under non-permeabilizing conditions (*compare* Fig. 1*F* with 1*D* and 1*E*). Under permeabilizing conditions cells expressing the wild-type channel and the tagged channels were abundantly labeled, Figs. 1*G*, *H*, and *I*. The fluorescence produced due to the expression of the wild-type and tagged channels is clearly observed at the membrane. The fluorescence at the interior of the oocyte is due mainly to autofluorescence, since non-injected oocytes show a similar level of cytoplasmic fluorescence without the membrane labeling (*data not shown*). Representative images of antibody-labeled Cos-7 cells expressing wild-type KAT1 channels are shown in Fig. 3*D* and *G* while Figs. *E*, *H* and *F*, *I* show representative images of Cos-7 cells expressing KAT1Nt-flg and KATS3-flg, respectively. Although the fluorescence observed in permeabilized cells was high in all cases, in Cos-7 cells expressing the tagged channels it was even higher, concomitantly with slight changes in cell morphology (*compare* Fig. 3*H* and *I* with *G*. This change in morphology seems to indicate accumulation of tagged unfolded proteins in the endoplasmic reticulum and possible aggresomes, which have been proposed to form in the presence of misfolded proteins (Johnston, Ward & Kopito, 1998).

The fact that an epitope at the S3-S4 linker is accessible for antibody binding in non-permeabilized oocytes and COS-7 cells (Fig. 1*D*, *E*, *F* and Fig. 3*D*, *E*, and *F*) demonstrated that in KAT1 channels this loop is facing the external side of the cellular membrane. On the other hand, the N and the C termini were only accessible in permeabilized cells, indicating that they are cytoplasmic (Figs. 1*G*, 1*H* and 3*G*, 3*H*).

Discussion

Plant cells have K^+ channels showing similarities to the animal *Shaker* K^+-channel family amino-acid sequence, the hydrophobic domains (S1, S2, S3, S5, S6), a putative S7, an amphipatic S4 domain involved in voltage sensing and a hydrophobic region between S4 and S5, called P (pore) (Very & Sentenac, 2003; Jiang et al., 2003). Some of these plants' *Shaker* channels, like the KAT1 channel, are hyperpolarization-activated inward rectifiers. KAT1 hydropathy profiles predict six membrane-spanning regions, in agreement with the method of Hopp and Woods, with a window of 12 or 13 amino acids (Anderson et al., 1992; von Heijne, 1992, Claros & von Heijne, 1994). Which region of the protein confers the KAT1 channel its inward rectification properties is still an open question. The experimental evidence strongly suggests that the KAT1 voltage dependence is a consequence of the movement of an intrinsic voltage sensor, most probably in segment S4 (Marten & Hoshi, 1998; Zei & Aldrich, 1998; Cosmelli et al., 2000; Latorre et al., 2003a, b). Previous results show that KAT1 as well as the hyperpolarization-activated, cyclic nucleotide-gated spHCN and the MVP, a methanoccocal voltage-gated K^+ channel, open when the S4 domain moves inwardly (Cosmelli et al., 2000; Männikö et al., 2002; Latorre et al., 2003a, b; Sesti et al., 2003).

To explain the KAT1 inward rectification, we tested the hypothesis that the KAT1 protein is oriented with a reverse topology in the membrane, such that the voltage sensor is facing the opposite direction of the electric field in comparison to the *Shaker* K^+ channel. To determine which side of the membrane the amino terminus and the S3-S4 linker are facing, the hydrophilic epitope flag (DYKDDDDK) was introduced in two critical positions of the KAT1 channel-forming protein, one at the N-terminal and the other at the S3–S4 linker. The specific sites of insertion were chosen to avoid transmembrane regions, since insertion of a highly hydrophilic epitope into a transmembrane-spanning region may alter the tertiary structure of the protein, resulting in a non-functional channel or producing changes in the electrophysiological properties. A third region of the protein, the carboxy terminal, was localized using a rabbit anti-C terminal KAT1 (aa 528–677) antibody. The different epitopes were visualized using specific antibodies in oocytes or COS-7 cells expressing the

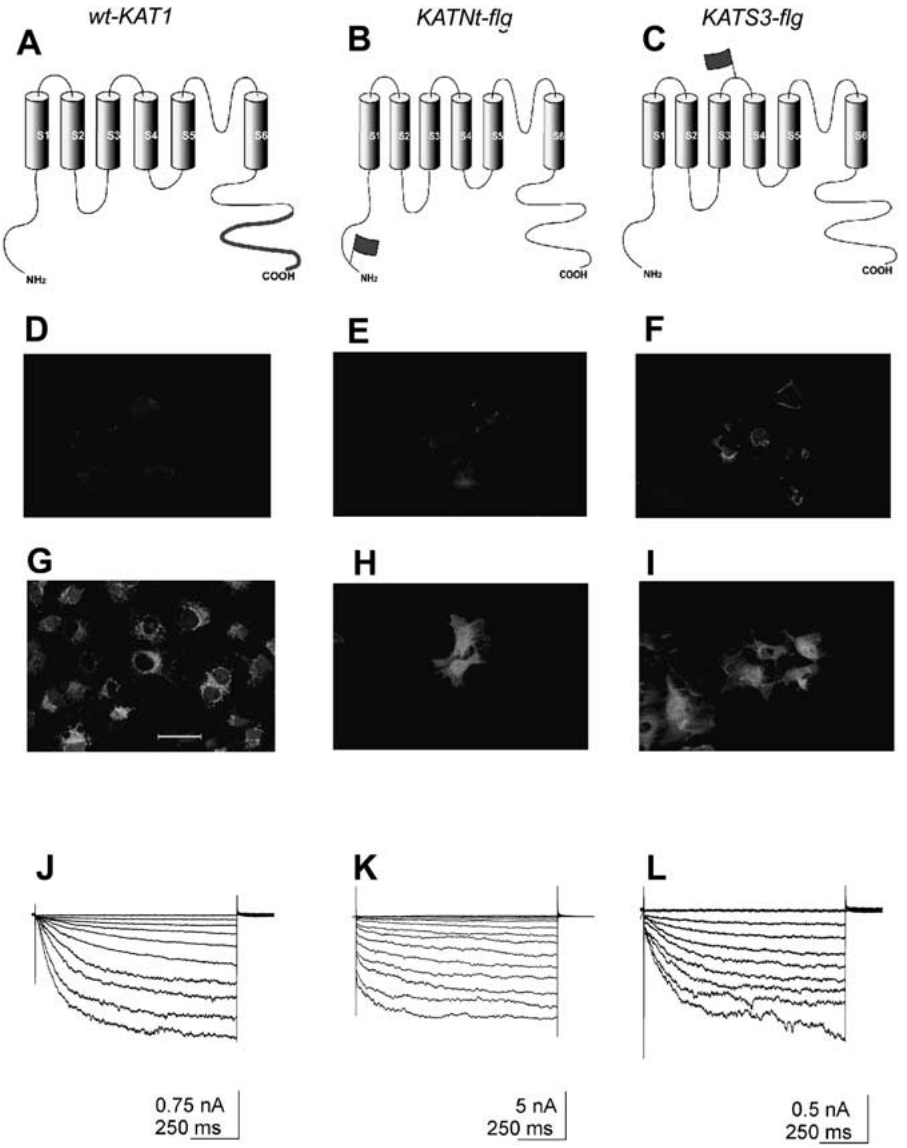

Fig. 3. Immunofluorescence labeling and current records of COS-7 transfected with wild-type KAT1 or tagged channels KAT1Nt-flg and KAT1S3-flg. Schematic diagrams indicating the region in which each flag insertion is located or the domain of the protein used to develop the anti-C terminal antibody (*A*, *B*, *C*). COS-7 cells expressing wild-type KAT1 (*D* and *G*) were labeled with the anti-C terminal KAT1 antibody. Cos-7 cells expressing KAT1Nt-flg, (*E* and *H*) or KAT1S3-flg (*F* and *I*), were labeled with the anti-flag antibody. Microphotographs of non-permeabilized COS-7 cells expresing wild-type KAT1, KAT1Nt-flg or KAT1S3-flg are shown in *D*, *E* and *F* respectively. The corresponding microphotographs of permeabilized COS-7 cells expressing wild-type KAT1, KAT1Nt-flg or KAT1S3-flg are shown in *G*, *H* and *I*, respectively. Bar: 50nm. The current records of COS-7 cells expressing wild type KAT1, KAT1Nt-flg or KAT1S3-flg are shown in *J*, *K* and *L*, respectively.

wild-type KAT1 or the tagged channels. This approach exploits the fact that antibodies cannot cross cell membranes and their binding to nonpermeabilized cells is a demonstration that the epitope is extracellular. Localization of intracellular epitopes requires an appropriate method of getting the primary antibody across the membrane barrier, making the epitopes accessible only after cell permeabilization or sectioning (Shih & Goldin, 1997). This methodology has the caveat that the foreign epitopes we introduced in the protein may produce alterations of the normal topology of the channel. However, functional expression was considered a strong indication that the epitope insertion did not lead to changes in the overall membrane topology or channel function. The fact that an epitope at the S3–S4 linker is accessible for antibody binding in nonpermeabilized oocytes and COS-7 cells demonstrated that in KAT1 channels this loop is facing the external side of the cellular membrane. On the other hand, the N and

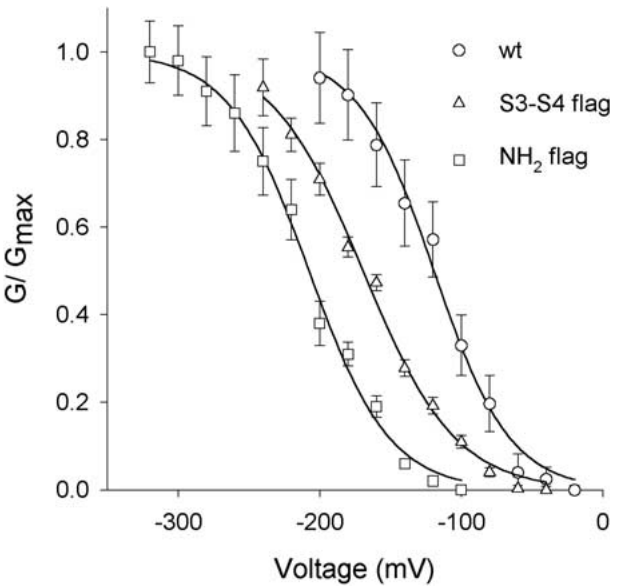

Fig. 4. Voltage-gating curves obtained from COS-7 cells expressing wild-type KAT1, KAT1Nt-flg or KAT1S3-flg. The solid lines represent the fit of the data to a Boltzmman distribution, yielding $V_{1/2} = -115 \pm 19$ mV and $z = -2.7 \pm 0.6$ for wild type KAT1 ($n = 4$), $V_{1/2} = -163 \pm 8$ mV and $z = -1.7 \pm 0.24$ for KAT1S3-flag ($n = 6$) and $V_{1/2} = -222 \pm 15$ mV and $z = -2.4 \pm 1.15$ for KAT1Nt-flag ($n = 6$).

the C termini were only accessible in permeabilized cells, indicating that they are cytoplasmic. In COS-7 cells a consistent difference in fluorescence was observed between the wild-type and tagged-channel transfectants; however, in all cases enough protein molecules reached the membrane, as proved by the functional assays. The COS-7 cells expressing the tagged channels presented a brighter and uniform fluorescence throughout the cell compared with COS-7 cells expressing the wild-type channel. This may be due to the accumulation of misfolded tagged-channel proteins. In fact, transfection of KAT1 in mammalian cells, CHO, and HeLa cells incubated at 30°C instead of 37°C, helps the expressed protein get into the membrane, thereby reaching the correct folding (Szabo et al., 2000).

As found in oocytes, none of the flag insertions appeared to have deleterious effects on the voltage-sensing machine of the KAT1 channel. The expression of the tagged channels induced robust hyperpolarizing currents equivalent to wild-type levels in *X. laevis* oocytes as well as in Cos-7 cells. Unexpectedly, the KAT1Nt-flg expressed in oocytes and KAT1Nt-flg in Cos-7 cells showed the largest shifts of $V_{1/2}$ (about −20 and −100 mV), respectively. Shifts of this magnitude imply that the perturbation of the free energy difference between open and closed states ($\Delta G = ze_0\Delta V_{1/2}$, where z is the equivalent gating charge and e_0 the electronic charge) introduced by the mutations on channel gating are modest, considering that we are changing four epitopes, one in each subunit of the KAT1 channel. The activation kinetics for the Nt-flag are markedly slower. Our results support and expand Marten and Hoshi's (1998) observation that alterations in the N-terminus significantly change the $V_{1/2}$ values, showing that not only deletions, as in Marten and Hoshi's study, but also additions to the N-terminus shift the $V_{1/2}$ values. One explanation for the largest shifts of $V_{1/2}$ between the tagged Nt-flg in oocytes versus COS-7 cells stems from the fact that even the same heterologous expression system gives inconsistent results, as has been documented by Dreyer et al. (1999).

The results shown together with the characteristics of the hydropathy plot of the amino-acid sequence of KAT1 strongly suggest that KAT1 has the same orientation, membrane topology of six membrane-spanning regions, and a pore loop characteristic of voltage-gated ion channels such as *Shaker*. The transmembrane topology of this channel was suggested previously by Uozumi et al. (1998) in *E. coli*, using a gene fusion approach that takes advantage of bacterial reporter enzymes. The authors measured the activity of β-galactosidase and of alkaline phosphatase, which is only active in the periplasm, whereas it remains inactive in the cytoplasm. They proposed that the topology of KAT1 channels is the same as that predicted for voltage-dependent K^+ channels in animal cells. The conclusion of that work was supported by the observation that KAT1 complemented the K^+ uptake efficiency in *E. coli*, indicating that the channel is inserted in the bacterial membrane with the right polarity. However, the same authors pointed out that the electrical properties induced by KAT1 channels in *E. coli* are unknown. This was our main drive to determine the KAT1 topology in animal cells. The conclusions drawn from the present results are also supported by a previous observation that cysteine-scanning mutagenesis shows that amino-acid residues in the S3–S4 near or in the N terminus of the S4 domain are accessible to cysteine reagents from the external side of the channel segment (Latorre et al., 2003b). Previous evidence for conserved membrane orientation came from a study using chimeric channels containing portions of KAT1 and the animal outward rectifying *XSha2*. These chimeric channels were capable of transporting K^+ into oocytes, suggesting that the overall membrane topology and the possible arrangements for the six membrane-spanning segments are similar (Cao et al., 1995). On the other hand, blockage studies by TEA and Cs, using KAT1 P domain mutants, (amino-acid positions H267T and E269V, which correspond to amino-acid positions 449 and 451 in *Shaker*) showed that the mutants were less sensitive to external Cs^+ and TEA blockage, indicating that the mutations are likely to be involved in external blocker interactions and may be facing

externally in KAT1, similar to *Shaker* channels (Ichida & Schroeder, 1996). Also, in vitro studies, using endoplasmic reticulum preparations, on the topogenic functions of the individual KAT1 transmembrane regions confirm the proposed topology, namely, that the N and C termini are on the same side of the membrane, opposite to the S3–S4 linker epitope (Sato et al., 2003). These studies were designed using sequential insertions of KAT1 segments into endoplasmic reticulum membranes. In contrast, our studies done on whole cells showed the expression of KAT1 in the cytoplasmic membrane, demonstrating unequivocally that this protein is inserted in the membrane with the same orientation as *Shaker* K^+ channels. Considering all these results, we can thus conclude that the inward- and outward-rectifying channels have the same orientation in the cellular membrane. We also conclude that changes in any domain of the protein will affect the behavior of the channel.

Many studies have been done pointing to specific regions of the channels as being responsible for voltage dependence of gating. On the basis of the crystal structure of the depolarization-gated K^+ (KvAP) channel, it has been proposed that the gating charges are carried in a helix-turn-helix structure formed by a stretch of S3 and S4, a structure called "the paddle". During channel activation the paddles undergo a displacement of $\sim$2 nm, which in turn opens the pore by pulling on the S4–S5 linker (Jiang et al., 2003). We have shown that adding a hydrophilic epitope to the N terminal of the KAT1 channels produced a large shift in the G/G_{max} vs voltage curve in the channels expressed in oocytes as well as in COS-7 cells. In addition, the same epitope inserted in the S3–S4 linker produced the same effects but to a lesser extent. According to the same model, the predicted topology of KvAP localizes the S3–S4 linker inside the membrane, and upon depolarization the movement of the "paddle" produces a displacement sufficient to expose the S3–S4 linker to the outside of the membrane, leading to the opening of the channel. The localization of the KAT1 S3–S4 linker obtained by our immunofluorescence experiments differs from the localization of KvAP (Jiang el al., 2003). Should this model apply to the *Shaker* and KAT1 channels due to the structural similarities, only channels in the open state would be observed by immunofluorescence. However, since KAT1 is a voltage-gated channel, in absence of electrical perturbation the channels that are detected by immunofluorescence in the oocytes and COS-7 cells would be in a closed state, exposing the linker S3–S4 to the outside. It has been speculated that if the crystal structure of KAT1 were similar to that of KvAP, channel activation should be controlled by a mechanism by which the channel opens by moving the paddles in reverse (Latorre et al., 2003b). As the authors themselves noted, it is not known to what extent the position of the helices in the crystal deviate from that in the membrane, nor to what extent they move upon gating (Jiang et al., 2003). This discrepancy in itself demonstrates that more experimental evidence is needed to infer that the model proposed for KvAP is applicable to KAT1.

The authors acknowledge Dr Ramon Latorre's contribution to this work, which was originated in his laboratory. The electrophysiology studies on COS-7 cells were done in Dr. Juan Bacigalupo's laboratory. We thank Dr. Jean-Baptiste Thibaud for the generous gift of the pCIKAT1 construct, for his valuable help to improve the transfection protocol in COS-7 cells, for critical reading of the manuscript and lively discussions; Dr. Martin Wallner for helpful hints throughout this work; Dr. Maria Ines Becker for valuable advice and for allowing the use of the fluorescence microscope, and Drs. Cecilia Vergara, Lee Meisel, and Daniel Wolff for critical reading of the manuscript. Supported by Fondecyt grants 1000890 and 1030830, and a Presidential Chair in Science (RL) and by Mideplan ICM P-99–031–F to the CBB Millenium Institute, Faculty of Sciences, University of Chile (CVM and RD). Institutional support to the Centro de Estudios Cientificos (CECS) from Empresas CMPC is gratefully acknowledged. CECS is a Millennium Science Institute and is funded in part by grants from Fundación Andes and the Tinker Foundation.

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