

ARTICLE

Investigating the role of potassium channel KAT1 in NO mediated stomatal closure

Attila Ördög, Barnabás Wodala, Ferenc Horváth*

¹Department of Plant Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

ABSTRACT KAT1, a hyperpolarization-activated, inward-rectifying K⁺ channel is one of the main transporters participating in guard cell volume regulation. The voltage-dependence of KAT1 channel is regulated by several intracellular factors, including pH, ATP and cGMP (Hoshi 1995). NO, a regulator of a branch of ABA signalling pathways in guard cells decreases the Ca²⁺-dependent inward potassium current by increasing the cytosolic free Ca²⁺ concentration. Based on the Ca²⁺-independent feature of KAT1 homotetramer protein, we aimed to examine the direct and indirect effects of NO on this channel. **Acta Biol Szeged 52(1):163-165 (2008)**

KEY WORDS

KAT1
nitric oxide
guard cell
GSNO

The volume of guard cells determines the size of stomatal apertures, thus CO₂ flow into the leaves for photosynthesis and water loss through transpiration. Guard cell expansion correlates with the potassium inward current (K_{in}) through *Shaker*-type K⁺ channels embedded in the plasma membrane. Nine *Shaker* channel genes were identified in *Arabidopsis*, and five of them, namely *KAT1*, *KAT2*, *AKT1*, *AKT2/3* and *AtKC1* were found to be inward rectifiers expressed in guard cells (Pilot et al. 2003), although the rectification properties of AKT2/3 are dependent on its phosphorylation status. *Shaker* channels are tetramer structures made up of four monomer proteins. Both homomer and heteromer channels were shown to be functional in heterologous expression systems, however AtKC1 monomer is only operable by forming a heteromeric channel with KAT1 or AKT1. Co-assembly of different monomers determines the characteristics of the channel. For instance, extracellular Ca²⁺-sensitivity is carried by AKT2/3 monomer as *akt2/3* knockout mutants lacked the voltage- and concentration-dependent Ca²⁺ block (Ivashikina et al. 2005).

Abscisic acid (ABA) closes stomata by facilitating K⁺, Cl⁻ and subsequent water efflux from guard cells. ABA elevates the concentration of cytosolic-free Ca²⁺ ([Ca²⁺]_{cyt}) by modulating the gating properties of Ca²⁺ channels on the plasma membrane, and through the indirect activation of Ca²⁺ channels of internal stores. Increasing [Ca²⁺]_{cyt} inactivates Ca-dependent K_{in} channels by a negative shift in their voltage-dependence.

Nitric oxide (NO), an element of ABA signalization has been characterized as an intracellular Ca²⁺ releasing agent by the activation of cGMP-dependent cyclic ADP-ribose synthesis (Garcia-Mata et al. 2003). Broad-range protein kinase

antagonists, like staurosporine and K252A, block the NO-dependent Ca²⁺-release, showing a possibility of regulation through phosphorylation by serine-threonine protein kinases (Sokolovski et al. 2005).

Direct (de-)phosphorylation of channel proteins might also be part of the regulation process, for instance protein phosphatase 2C, a partner of AKT2 in ABA signalling, inhibited the channel activity and increased the channel inward rectification by phosphorylation (Cherel et al. 2002).

The aim of our study is to test the effect of NO on different inward-rectifying *Shaker*-type homomer and heteromer proteins. The present work deals with the KAT1 homomer heterologously expressed in human embryo kidney (HEK293) cells.

Materials and Methods

HEK293 cell culture maintenance and transfection

HEK293 cells were used for the heterologous expression of KAT1. The cells were maintained at 37°C, 5% CO₂ in DMEM/F12, foetal calf serum (FCS), penicillin and streptomycin. The exact composition of the medium was DMEM/F12:FCS = 9:1, and the activity of penicillin and streptomycin was 10 U/ml. The plasmid pCB6-KAT1 (BD Biosciences, Clontech, USA) was used for the functional expression of the KAT1 sequence. For the cationic lipid precipitation, a MetafecteneTM Pro (Biontex Laboratories GmbH, Germany) transfection reagent was applied. The transfected cells were isolated for patch clamp recording 24 hours after transfection.

Patch clamp recordings in HEK293 cells

Dishes with transfected cells were placed on a stage of an inverted microscope and single cells were patch-clamped in

*Corresponding author. E-mail: horvathf@bio.u-szeged.hu

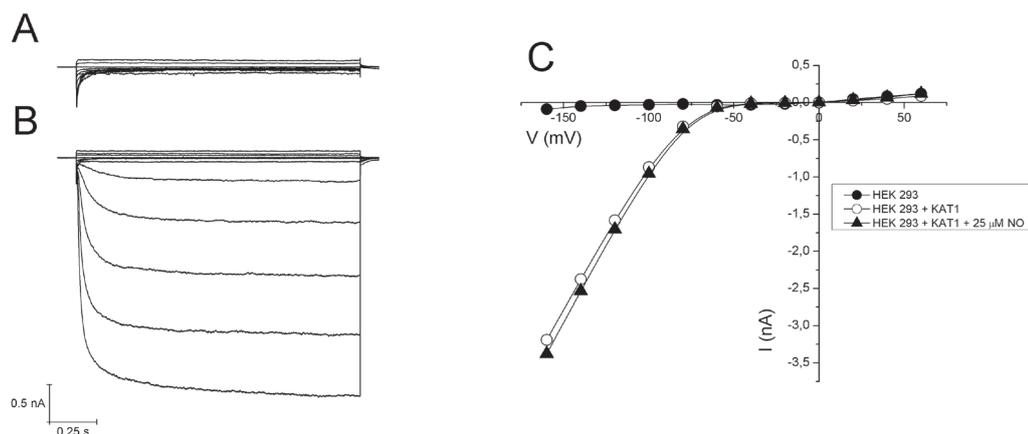


Figure 1. Whole-cell recordings in HEK293 cells before (A) and after the transfection with KAT1, an inward rectifying potassium transporter gene (B). HEK293 cells show no inward rectified potassium currents even at -160 mV clamping potential. Transfected cells display a K^+ current amplitude of -3.5 nA in response to -160 mV clamping potential. The inward current is time- and voltage-dependent, and fully activated after some hundreds of milliseconds. Endogenous outward current activations detected in the first 100 ms of test pulses are the characteristics of HEK293 whole-cell currents. (C) Steady-state current-voltage relationship in HEK293 host cell in a KAT1 transfected cell and in KAT1 transfected cell with NO treatment. Current values were taken from the average of whole-cell currents in the last 0.1 s of test pulses.

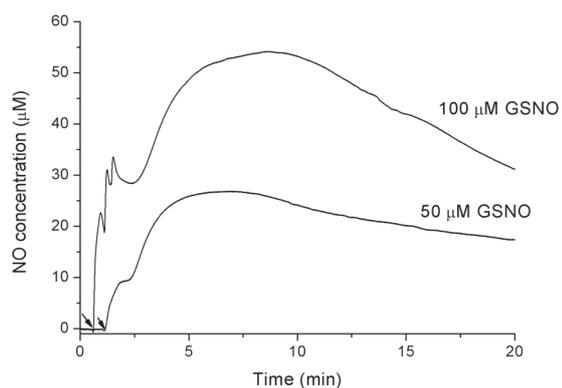


Figure 2. Time course of NO concentration generated by photolytic decomposition of GSNO. Light intensity was set to $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ by an external halogen lamp. GSNO was administered to Petri dishes containing 2 ml of bath solution at times indicated by arrows to a concentration of 50 and 100 μM , respectively.

the whole-cell configuration according to standard methods using an EPC-10 patch clamp amplifier (HEKA, Lambrecht, Germany). Data acquisition and analysis were performed using PatchMaster software (HEKA, Lambrecht, Germany). Whole-cell currents were obtained by applying a standard voltage protocol, which involved clamping the cells for 1 s from a holding potential of 0 mV, to test voltages between +60 mV to -160 mV with 20 mV steps.

Borosilicate glass pipettes (HILGENBERG, Malsfeld, Germany) were used for patch clamp recordings. The standard pipette solution contained 130 mM potassium D-gluconate, 10 mM NaCl, 5 mM HEPES, 5 mM EGTA, 0.1 μM CaCl_2 ,

2 mM MgCl_2 , 2 mM ATP (Na salt), 0.1 mM GTP (Na salt), 5 mM Phosphocreatin (Na salt), pH = 7.4 (with KOH). The bath solution contained 130 mM KCl, 10 mM Choline-Cl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, pH 7.4 (with KOH). Changes in the composition of pipette or bath solutions are indicated at a given experiment.

NO measurements

Solution of NO donor S-nitrosoglutathione (GSNO) was incubated in Petri dishes under $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light (Schott KL2500, Mainz, Germany), and NO release was measured using a NO electrode (ISO-NOP, World Precision Instruments Inc, USA) dipped in the aqueous phase. The NO electrode was calibrated by adding different volumes of SNAP solution to CuSO_4 solution set to pH 4 by addition of H_2SO_4 following the manufacturer's instructions.

Results and Discussion

To examine the main features of KAT1 activity, HEK293 cells were measured in whole-cell patch clamp mode. These cells are suitable for heterologous expression of an inward rectifier, because at voltages more negative than about 0 mV, they only exhibit a very low endogenous conductance (Fig. 1A). With this low background conductance the expression of recombinant KAT1 is easily detectable. Figure 1B shows the current response of a HEK293 cell transfected with KAT1 DNA. When clamped from the holding voltage of 0 mV to a series of test voltages between +60 mV and -160 mV, the cell exhibits a large inward current. This inward current displays the typical steady state I/V relation and kinetic features of KAT1 (Fig. 1C).

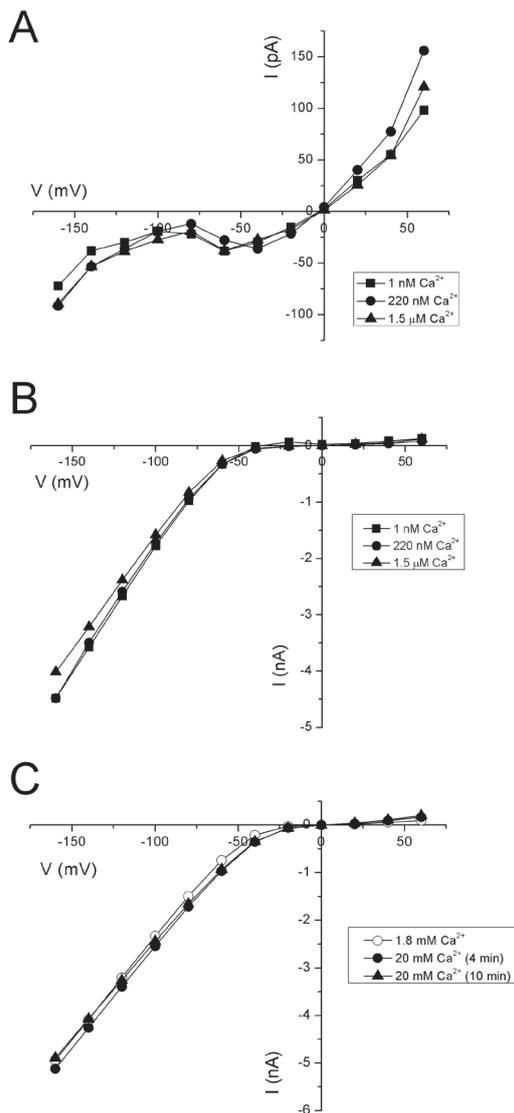


Figure 3. Different extra- and intracellular Ca^{2+} concentrations cause no change in steady-state current-voltage relationships of KAT1 transfected cells. Intracellular $[\text{Ca}^{2+}]_{\text{cyt}}$ concentration was changed using different pipette solutions in non-transfected (A), and KAT1 transfected HEK293 cells (B). Extracellular Ca^{2+} concentration was increased by a solution changer, and steady-state current-voltage relationships were determined following the indicated incubation times (C).

S-nitrosylation is the reversible covalent attachment of NO to the thiol group of cysteine residues and is an ancient highly conserved cell signalling mechanism. In order to examine the possible S-nitrosylation effect of NO on KAT1 protein, 25 and 50 μM NO was applied in the bath solution by a NO-donor chemical GSNO in 50 and 100 μM under 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination (Fig. 2). Previous studies in guard cells showed that 10–20 nM NO completely inactivates the K^+ outward current, without altering its relaxation kinetics or voltage-dependence, due to the reduction of the amount of ac-

tive channels by S-nitrosylation (Sokolovski and Blatt 2004). We found, however, that NO does not modify the size and the kinetics of KAT1 current (Fig. 1C), even in a micromolar range, thus we predict that NO has no direct S-nitrosylation effect on KAT1 homotetramer channel.

NO elevates $[\text{Ca}^{2+}]_{\text{cyt}}$ by activating Ca^{2+} channels of endomembrane stores, and higher $[\text{Ca}^{2+}]_{\text{cyt}}$ inactivates Ca^{2+} -sensitive K_{in} current. In order to test the Ca^{2+} -sensitivity of the KAT1 channel, the Ca^{2+} concentration of the bath and the pipette solution was changed. The results showed that neither the intracellular, nor the extracellular Ca^{2+} altered the whole-cell KAT1 currents in HEK293 cells (Fig. 3). We concluded, that $[\text{Ca}^{2+}]_{\text{cyt}}$ elevated by NO, directly inactivates Ca^{2+} -sensitive K_{in} , but not the homotetramer KAT1 current. On the other hand, indirect regulation cannot be ruled out, as calcium-dependent protein kinase (CDPK) has been shown to phosphorylate, thus inactivate KAT1 channel in oocytes co-expressing the recombinant CDPK with KAT1 (Berkowitz et al. 2000).

NO also activates guanylate cyclase, thus elevates the cytosolic cGMP level in guard cells. KAT1 contains a cyclic nucleotide binding motif in the carboxyl domain, and its voltage-dependency has been shown to be reversibly shifted in a negative direction by the intracellular addition of cGMP (Hoshi 1995). In order to clarify, whether the NO induced cGMP levels could affect KAT1 function, further experiments are planned to measure the exact cGMP concentrations elevated by NO.

This work was supported by the Hungarian Scientific Research Fund (grant no. OTKA F 048787).

References

- Berkowitz G, Zhang X, Mercier R, Leng Q, Lawton M (2000) Co-expression of calcium-dependent protein kinase with the inward rectified guard cell K^+ channel KAT1 alters current parameters in *Xenopus laevis* oocytes. *Plant Cell Physiol* 41:785-790.
- Cherel I, Michard E, Platet N, Mouline K, Alcon C, Sentenac H, Thibaud JB (2002) Physical and functional interaction of the Arabidopsis K^+ channel AKT2 and phosphatase AtPP2CA. *Plant Cell* 14:1133-1146.
- Garcia-Mata C, Gay R, Sokolovski S, Hills A, Lamattina L, Blatt MR (2003) Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. *Proc Natl Acad Sci USA* 100:11116-11121.
- Hoshi T (1995) Regulation of Voltage-Dependence of the KAT1 Channel by Intracellular Factors. *J Gen Physiol* 105:309-328.
- Ivashikina N, Deeken R, Fischer S, Ache P, Hedrich R (2005) AKT2/3 subunits render guard cell K^+ channels Ca^{2+} sensitive. *J Gen Physiol* 125:483-492.
- Pilot G, Pratelli R, Gaymard F, Meyer Y, Sentenac H (2003) Five-group distribution of the shaker-like K^+ channel family in higher plants. *J Mol Evol* 56:418-434.
- Sokolovski S, Blatt MR (2004) Nitric oxide block of outward-rectifying K^+ channels indicates direct control by protein nitrosylation in guard cells. *Plant Physiol* 136:4275-4284.
- Sokolovski S, Hills A, Gay R, Garcia-Mata C, Lamattina L, Blatt MR (2005) Protein phosphorylation is a prerequisite for intracellular Ca^{2+} release and ion channel control by nitric oxide and abscisic acid in guard cells. *Plant J* 43:520-529.