GORK, a delayed outward rectifier expressed in guard cells of *Arabidopsis thaliana*, is a K^+-selective, K^+-sensing ion channel

Peter Ache[1], Dirk Becker[1], Natalya Ivashikina, Petra Dietrich, M. Rob G. Roelfsema, Rainer Hedrich*

Lehrstuhl für Molekulare Pflanzenphysiologie und Biophysik, Julius-von-Sachs-Institut für Biowissenschaften, Universität Würzburg, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany

Received 23 October 2000; accepted 7 November 2000

First published online 21 November 2000

Edited by Ulf-Ingo Flügge

Abstract Here we report on the molecular identification, guard cell expression and functional characterization of *At*GORK, an *Arabidopsis thaliana* guard cell outward rectifying K^+ channel. GORK represents a new member of the plant *Shaker* K^+ channel superfamily. When heterologously expressed in *Xenopus* oocytes the gene product of GORK mediated depolarization-activated K^+ currents. In agreement with the delayed outward rectifier in intact guard cells and protoplasts thereof, GORK is activated in a voltage- and potassium-dependent manner. Furthermore, the single channel conductance and regulation of GORK in response to pH changes resembles the biophysical properties of the guard cell delayed outward rectifier. Thus GORK very likely represents the molecular entity for depolarization-induced potassium release from guard cells.

Key words: Potassium transport; Guard cell; *Arabidopsis thaliana*

1. Introduction

Since the development of single cell electrophysiological techniques, the majority of patch-clamp and conventional voltage-clamp studies in plants has focused on the role of guard cell plasma membrane ion channels in stomatal closure. In 1993, Blatt and Armstrong [1] challenged impaled guard cells with abscisic acid (ABA). Thereby they recognized the inhibition of inward rectifying and activation of outward rectifying K^+ channels as an early step in ABA signaling. Simultaneous electrophysiological recordings and intracellular pH measurements showed that a drop in cytosolic pH precedes the change in the two K^+ channel activities. After the cloning of KAT1 and KST1, inward rectifying K^+ channels expressed in *Arabidopsis thaliana* and *Solanum tuberosum* guard cells, respectively [2–4], the pH-dependent modulation of this channel type has been identified as a channel intrinsic property [5–8]. In search for signal transduction elements between ABA perception and inhibition of the inward rectifying K^+ channel and activation of the outward rectifier, ABA-insensitive mutants have been investigated. Among them *abi1* was identified as a mutation in a protein phosphatase [9,10]. Electrophysiological studies on guard cells from *abi1* expressing tobacco plants revealed defects in the pH-mediated regulation of the two K^+ channels [11].

In order to distinguish on the molecular level between the individual ion channels in the plasma membrane of the guard cell and interacting factors, we have cloned and characterized *At*GORK, an outward rectifier expressed in guard cells of *A. thaliana*.

2. Materials and methods

2.1. Cloning and localization

Degenerated oligonucleotides, Kout1 (5′-CARGARGGNTAYACNTGG-3′) and Kout2 (5′-TARTCIGTYTTRTTIGGRTC-3′) were derived from an alignment of *SKOR* (stelar K^+ outward rectifier, accession number CAA11280), *PTORK* (*Populus tremula* outward rectifying K^+ channel, accession number AJ271446) and *SPORK* (*Samanea saman* pulvinus outward rectifying K^+ channel, accession number AJ299019). Using mRNA isolated from *Arabidopsis* protoplasts, in an RT-PCR approach an 998 bp cDNA fragment was amplified, subcloned into the pCRII-TOPO plasmid (Invitrogen) and verified by sequencing and database comparison. The missing 5′ and 3′ ends were obtained using the SMART-RACE procedure (Clontech).

Expression was analyzed by RT-PCR experiments on mRNA isolated from various *Arabidopsis* tissues. Using the Dynabeads mRNA direct kit (Dynal), mRNA was isolated directly from tissues, reverse transcribed (M-MLV Reverse Transcriptase, Promega) and subsequently subjected to PCR using *GORK*-specific primers (sense-primer: 5′-CCTCCTTTAATTTAGAAG-3′; antisense-primer: 5′-GCTCCATCCGATAG-3′).

2.2. Electrophysiology on single A. thaliana guard cells

2.2.1. Double-barreled voltage-clamp. Guard cells in intact leaves were impaled with double-barreled electrodes as described previously [12]. The reference electrode, an Ag/AgCl half cell connected to a glass capillary filled with 50 mM KCl, was placed on the cuticula.

2.2.2. Patch-clamp recordings. Guard cell protoplasts were isolated as described before [13]. Solutions: The standard pipet solution (cytoplasm) contained 150 mM K-gluconate, 2 mM $MgCl_2$, 2 mM MgATP, 10 mM HEPES/Tris pH 7.4. Cytosolic Ca^{2+} was buffered with 10 mM EGTA or with 5 mM EGTA in addition to 3 mM $CaCl_2$. The bathing medium contained 30 mM K-gluconate, 1 mM $CaCl_2$, and 10 mM MES/Tris pH 5.6. All solutions were adjusted to 540 mosmol kg^{-1} using D-sorbitol.

2.3. Heterologous expression in Xenopus oocytes

For functional analysis, GORK cRNA was prepared using the mMESSAGE mMACHINE™ RNA Transcription kit (Ambion). Oocyte preparation and cRNA injection have been described elsewhere [14]. In two-electrode voltage-clamp studies oocytes were perfused with K-gluconate containing solutions, based on Tris/MES buffers. The standard solution contained 10 mM Tris/MES pH 7.4, 1 mM

*Corresponding author: Fax: (49)-931-8886157.
E-mail: hedrich@botanik.uni-wuerzburg.de

[1] These authors contributed equally to this study

0014-5793/00/$20.00
PII: S0014-5793(00)02248-1

$CaCl_2$, 1 mM $MgCl_2$ and 30 mM K-gluconate. Osmolarity was adjusted to 220 mosmol kg^{-1} using D-sorbitol and the ionic strength was adjusted with Li-gluconate. Voltage-dependent activation of GORK was recorded using single-pulse protocols. Starting from a holding potential (V_H) of −100 mV, pulses were applied to various test voltages as indicated in the figure legends.

Single channel analysis was performed in the cell-attached configuration of the patch-clamp technique applied to GORK expressing *Xenopus* oocytes. Current fluctuations were recorded with identical pipet and bath solutions composed of 30 mM K-gluconate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and either 10 mM MES/Tris pH 5.6 or 10 mM Tris/MES pH 7.4. The solutions were adjusted to 220 mosmol kg^{-1} using D-sorbitol.

3. Results and discussion

3.1. Identification of the outward rectifier in guard cells

To prove whether the depolarization-activated outward K^+ current exhibits the same electrophysiological fingerprint in turgid guard cells of intact *Arabidopsis* leaves and isolated, turgorless protoplasts thereof, we studied the voltage dependence and kinetics of the outward rectifier in the two experimental systems. Single guard cells in their natural environment of an intact leaf were impaled with double-barreled microelectrodes. With the membrane potential clamped to −80 mV, 2 s voltage pulses were applied to hyperpolarizing and depolarizing voltages. With negative-going voltages inward rectifying K^+ currents similar to those described for guard cells as well as the gene product of *KAT1* when expressed in *Xenopus* oocytes were elicited (data not shown, cf. [13]). Upon application of depolarizing voltage pulses time- and voltage-dependent outward currents were elicited (Fig. 1A). In addition we enzymatically isolated guard cell protoplasts from epidermal peels and applied the whole-cell and outside-out patch configuration of the patch-clamp technique and tested whether the lack of turgor alters the K^+ channel properties of guard cells. In response to depolarizing membrane potentials the voltage- and time-dependent properties of the K^+ channels remained unaffected by the loss of turgor (Fig. 1B). Similar to the macroscopic K^+ currents, single 20 pS, K^+-selective channels were elicited at depolarizing voltages (Fig. 1C,D).

3.2. Molecular characterization of GORK

We isolated mRNA form guard cell protoplasts to screen for the gene, encoding the outward rectifier. So far two *Arabidopsis* genes, *SKOR* and *KCO1*, encoding depolarization-activated K^+ channels have been described [15,16]. KCO1, however, very likely represents a vacuolar K^+ channel (own unpublished data), since the plasma membrane K^+ currents in protoplasts isolated from *KCO1* knockout plants were not different from wild type *Arabidopsis*. Furthermore, green fluorescence appeared in the vacuolar membrane of cultured cells, transformed with a fusion between *KCO1* and *GFP* (35S-*KCO1-GFP*). We therefore focused on the *SKOR* family and designed the degenerated primers Kout1 and Kout2. Using Kout1 and Kout2 in PCR experiments we could amplify a fragment of 998 bp with 69% homology to SKOR cDNA.

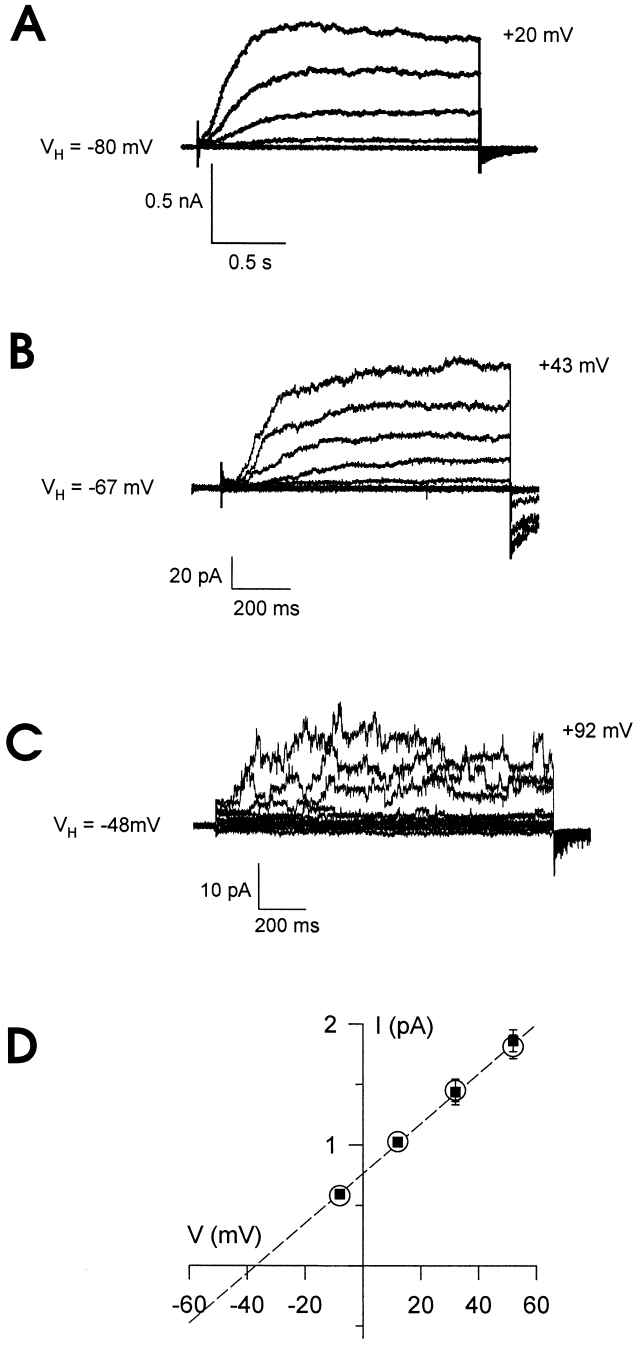


Fig. 1. Voltage- and time-dependent properties of the outward rectifying K^+ channel in *A. thaliana* guard cells. A: Guard cells in intact leaves were impaled with double-barreled electrodes. From a holding potential of −80 mV slowly activating outward currents were elicited by 2 s voltage pulses to +20 mV in 20 mV increments. B: Whole-cell patch-clamp recordings from *Arabidopsis* guard cell protoplasts in the presence of 30 mM K^+ and pH 5.6 in the bath. Starting from a holding potential of −67 mV, pulses between −117 mV and +43 mV in 20 mV increments elicited K^+ currents upon depolarization. C: Time- and voltage-dependent openings of single channels in outside-out patches, excised from guard cell protoplasts. Representative currents obtained by changing the membrane voltage from a holding potential of −48 mV in 20 mV decrements from +92 to −108 mV, followed by a pulse to −88 mV. D: Single channel conductance of the guard cell outward rectifier. Open channel conductance (20 pS) in the outside-out configuration and in the presence of 30 mM K^+ in the bath and 150 mM K^+ in the pipet. Changing the pH in the bathing medium from pH 5.6 (○) to pH 7.0 (■) did not affect the unitary conductance. Note, that the single channel current reversed closely to E_K (= −41 mV). Error bars represent the standard deviation.

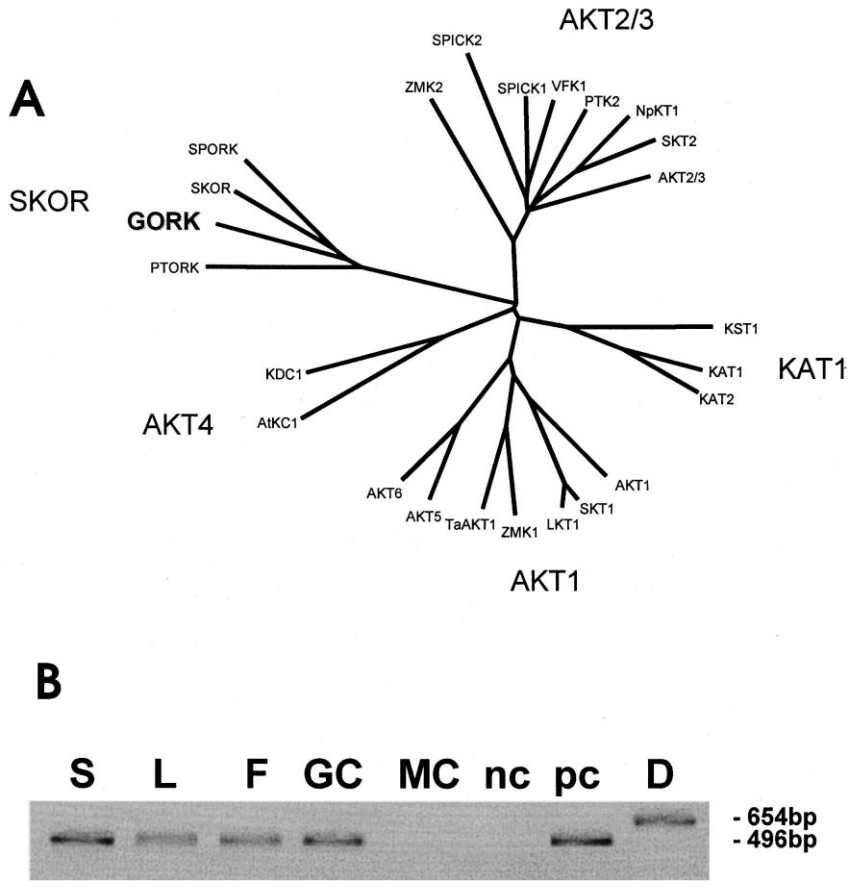


Fig. 2. Molecular biology of GORK. A: Phylogenetic tree, demonstrating that GORK (accession number AJ279009), together with SKOR, PTORK and SPORK groups into a new family of *Shaker*-like plant potassium channels. Sequence alignments were performed with Clustal X [24] and trees were drawn using TreeView [25]. B: Localization of GORK mRNA. RT-PCR analyses on mRNA derived from *Arabidopsis* stem (S), leaves (L), flowers (F), guard cell protoplasts (GC), and mesophyll protoplasts (MC). Negative control (SKOR cDNA, nc), positive control (GORK cDNA, pc) as well as signals obtained with *Arabidopsis* genomic DNA (D) demonstrate the specificity of the *GORK* primers. Note, that primers did not cross-react with *SKOR* transcripts, although GORK cDNA shares 67% identity with SKOR cDNA.

Based on RACE techniques we were able to clone the respective 5′ and 3′ terminal regions of this *SKOR*-like K^+ channel sequence. In the evolutionary tree of known plant K^+ channels (Fig. 2A), GORK groups in a separate branch together with SKOR, PTORK and SPORK. Based on an alignment of the deduced protein sequences, the overall homology between the SKOR and GORK from *A. thaliana* was 73%, for the homolog from *S. saman* 75% and 70% for the one from *P. tremula x tremuloides*.

To identify the expression pattern of *GORK* in the shoot we performed an RT-PCR analysis on mRNA isolated from guard cell protoplasts, mesophyll protoplasts and various tissues. A *GORK*-specific signal was obtained in all guard cell-containing tissues (Fig. 2B). Note the lack of a signal with mesophyll cells.

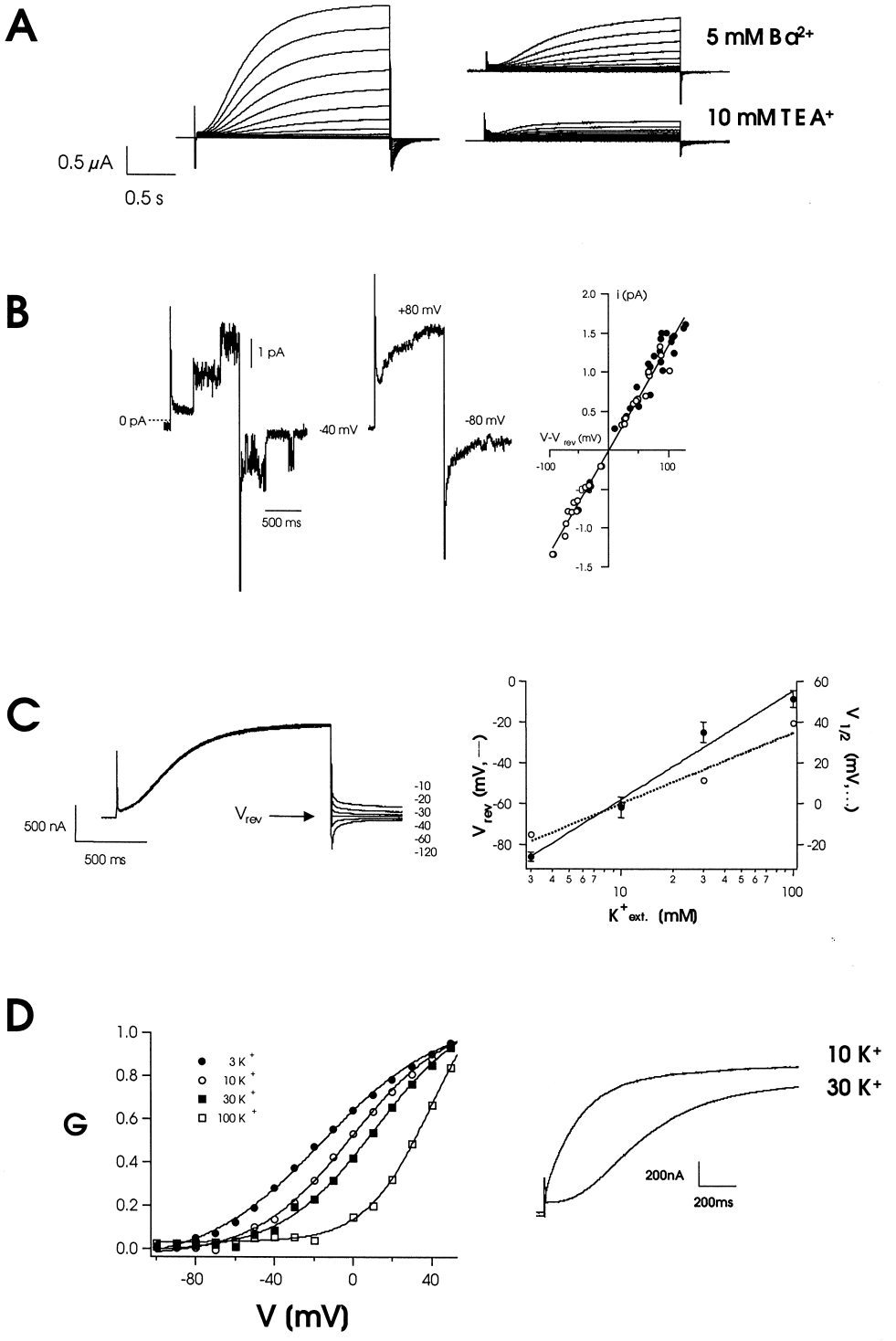
3.3. Expression in Xenopus oocytes

To prove whether *GORK* is indeed encoding an outward rectifying K^+ channel with properties similar to those recorded from guard cells and protoplasts thereof, we injected frog oocytes with GORK cRNA. 1–3 days following injection, depolarization-activated outward currents appeared (Fig. 3A). In the presence of 30 mM KCl or K-gluconate outward currents were elicited at membrane potentials positive of −20 mV with features similar to the outward rectifier in guard cells (Fig. 1). Upon application of the K^+ channel blockers TEA^+ and Ba^{2+} these currents were largely suppressed (Fig. 3A, right).

In the cell-attached configuration of the patch-clamp technique applied to GORK cRNA-injected oocytes, single K^+ channels were activated by depolarization (Fig. 3B, left). In response to a subsequent hyperpolarizing step to −80 mV the closure of single channels could be resolved. The ensemble average of the single channel currents elicited by 20 voltage pulses (Fig. 3B, middle) showed activation and deactivation kinetics similar to the macroscopic GORK currents (Fig. 3A). From the voltage-dependent open channel amplitudes measured in eight cell-attached patches a single channel conductance of 13.4 ± 1.6 pS was calculated (Fig. 3B, right). This value is in the same order of magnitude as those obtained for the outward rectifier in guard cells from *Arabidopsis* (Fig. 1D), *Vicia faba* [17] and *S. tuberosum* (15 pS, own unpublished result). As in *Arabidopsis* guard cells (cf. Fig. 1D), no significant pH-sensitivity of the single channel conductance between pH 5.6 (13.2 ± 1.8 pS, $n = 3$) and pH 7.4 (13.6 ± 1.7 pS, $n = 5$) was observed in GORK expressing oocytes (Fig. 3B, right).

3.4. GORK a K^+-selective, K^+-sensing ion channel

In the presence of 30 mM K^+, tail currents reversed direc-



tion at −36 mV (Fig. 3C, left), close to the predicted Nernst potential for potassium. To explore the potassium selectivity and sensitivity of the GORK-mediated currents we performed additional measurements in 100, 10 and 3 mM external K^+. Tail-current analyses revealed a 53 mV change in the reversal potential per 10-fold change in the external K^+ concentration (Fig. 3C right), which together with the susceptibility towards K^+ channel blockers proves GORK to represent a K^+-selective channel. Alike the reversal potential the half maximal activation potential ($V_{1/2}$) shifted in a K^+-dependent manner (Fig. 3C right). When we superimposed the voltage-dependent open probability of the outward rectifier obtained at four different K^+ concentrations, $V_{1/2}$ shifted about 35 mV per 10-fold increase in K^+ concentration (Fig. 3C right and Fig. 3D, left). This behavior clearly demonstrates that in contrast to the guard cell inward rectifier [18] but well in agreement with the outward rectifier in *Arabidopsis* guard cells [19], GORK represents a K^+-sensing valve which adjusts the K^+

Fig. 3. Heterologous expression of GORK in *Xenopus* oocytes. A: GORK mediates outward currents in GORK-cRNA-injected oocytes. Left: Using the double-electrode voltage-clamp technique, time-dependent, outwardly rectifying currents were elicited upon depolarizing voltage steps between −120 mV and +60 mV in 10 mV increments, starting from a holding potential of −100 mV. These currents were never observed in control oocytes. Right: Block of outward currents in the presence of 30 mM K^+ in the bathing medium, by the externally applied K^+ channel blockers Ba^{2+} and TEA^+. B: Voltage dependence and single channel conductance of GORK. Left: Starting from a holding potential of −40 mV, depolarization to +80 mV activated single K^+ outward channels in the oocyte-attached configuration of the patch-clamp technique. Upon subsequent hyperpolarization to −80 mV, open channels mediate inward K^+ currents. The external side of the membrane was exposed to 30 mM K^+, pH 5.6. Middle: Ensemble averages of 20 single channel recordings, as shown on the left. Right: Single channel amplitudes from eight individual cell-attached measurements as a function of the driving force ($V-V_{rev}$). The straight line represents a linear fit revealing a single channel conductance of 13.4 pS in 30 mM external (pipet) K^+ solutions, pH 5.6 ($n=3$, open symbols) and pH 7.4 ($n=5$, closed symbols). C: Selectivity and voltage dependence of GORK. Left: In tail-current experiments GORK expressing oocytes were challenged with an activating pre-pulse to +60 mV. In subsequent voltage-jumps to potentials ranging from −120 mV to −10 mV tail currents were elicited which reversed direction (V_{rev}) around the predicted Nernst potential for K^+. Right: Shift in reversal potential (V_{rev}, ●) and half maximal activation potential ($V_{1/2}$, ○) in response to changes in extracellular K^+ concentration. D: Voltage-dependent gating and kinetics of GORK. Left: A Boltzmann analysis of voltage-dependent gating at various external K^+ concentrations, normalized to the conductance (G) at +60 mV. Lowering the K^+ concentration stepwise from 100 to 3 mM shifts $V_{1/2}$ negative. Right: Increasing the external K^+ concentration from 10 to 30 mM, slows down activation kinetics at +40 mV.
←

conductance of the plasma membrane to the actual K^+ gradient across. Upon reduction of the external K^+ concentration e.g. from 30 to 10 mM the sigmoidal activation kinetics, characteristic for the delayed outward rectifier, changed into an almost instantaneous activation (Fig. 3D, right).

3.5. GORK and KAT1 have opposite pH dependencies

The inward and outward rectifiers of guard cells are characterized by their opposite pH dependencies [20]. To elucidate the pH dependence of GORK we co-expressed this channel type with KAT1, an *Arabidopsis* guard cell inward rectifier of known pH dependence [18], as an internal control. Oocytes expressing both guard cell K^+ channels, GORK and KAT1, were characterized by depolarization- as well as hyperpolarization-activated K^+ currents (Fig. 4). When the external pH was changed from 7.4 to 5.6 outward currents declined while inward currents increased (Fig. 4). Since the single channel conductance was not affected by physiological changes in external pH, protons do not seem to act as open channel blockers as in the case of the phloem K^+ channel AKT2/3 [21].

3.6. GORK, a new member of the 'green' Shaker K^+ channel family, encodes a delayed outward rectifier

We have shown that the outward rectifier is present and active in guard cells of intact *Arabidopsis* leaves and protoplasts thereof. The voltage-dependent regulation of this K^+ channel type in these two experimental systems was similar (Fig. 1). We therefore used mRNA from purified guard cell protoplasts to clone GORK, a *Shaker*-like plant K^+ channel. Well in agreement with the cloning strategy we found GORK mRNA in guard cell but not in mesophyll cell protoplasts. This expression pattern indicates that GORK represents the guard cell outward rectifier. Signals were, however, found in the stem and flower as well. To prove whether these signals originate from guard cells, we currently perform in situ hybridization and promoter-GUS experiments. Since patch-clamp studies identified an outward rectifying K^+ channel in mesophyll cells too [22], but mesophyll protoplasts lack GORK and SKOR transcripts (Fig. 2B and [16]), the mesophyll K^+ channel seems to be encoded by a different gene.

The extracellular K^+ concentration in the guard cell's apoplast approximates 1–2 mM when stomata are open [23]. Upon a challenge with ABA – during the process of stomatal closure – guard cells release K^+ and increase the apoplastic level of this cation up to 10 mM. We have demonstrated that

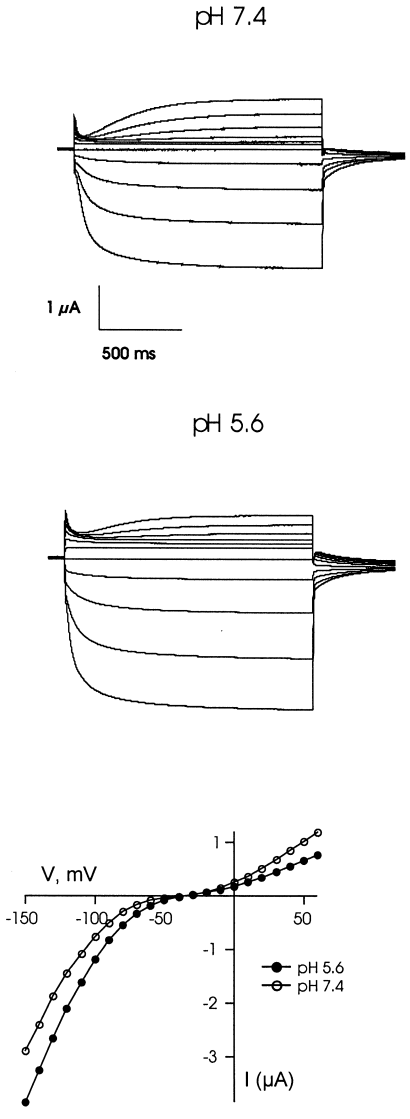


Fig. 4. Opposite pH dependence of GORK and KAT1. Co-expression of GORK and KAT1 in *Xenopus* oocytes revealed inward and outward currents with voltage pulses in the range from −160 mV to +60 mV. Decreasing the pH in the bathing medium from 7.4 (upper traces) to 5.6 (middle traces) in the presence of 10 mM K^+, externally, reduced outward currents while simultaneously increased inward currents. Lower graph: corresponding current voltage relations of steady state currents in the presence of pH 7.4 (○) and pH 5.6 (●).

the activation threshold for GORK shifts positive with an increase in the external K^+ concentration (Fig. 3C,D). In the presence of apoplastic K^+ levels characteristic for open stomata, an ABA-induced depolarization would rapidly open single, outward rectifying K^+ channels at potentials as negative as −80 mV. During depolarization of the guard cell plasma membrane induced by ABA [1], the non-inactivating GORK allows a sustained release of K^+. This K^+ efflux in the long-term will increase external K^+ and thereby shift the threshold for the voltage activation of GORK positive and slow down the activation kinetics. In line with the features of a K^+ sensor, K^+ release through GORK thus feeds back on the activation state and kinetics of this K^+ channel.

Acknowledgements: This work was supported by grants of the Deutsche Forschungsgemeinschaft to R.H.

References

[1] Blatt, M.R. and Armstrong, F. (1993) Planta 191, 330–341.
[2] Anderson, J.A., Huprikar, S.S., Kochian, L.V., Lucas, W.J. and Gaber, R.F. (1992) Proc. Natl. Acad. Sci. USA 89, 3736–3740.
[3] Nakamura, R.L., McKendree, W.L.J., Hirsch, R.E., Sedbrook, J.C., Gaber, R.F. and Sussman, M.R. (1995) Plant Physiol. 109, 371–374.
[4] Müller-Röber, B., Ellenberg, J., Provart, N., Willmitzer, L., Busch, H., Becker, D., Dietrich, P., Hoth, S. and Hedrich, R. (1995) EMBO J. 14, 2409–2416.
[5] Hedrich, R., Moran, O., Conti, F., Busch, H., Becker, D., Gambale, F., Dreyer, I., Küch, A., Neuwinger, K. and Palme, K. (1995) Eur. Biophys. J. 24, 107–115.
[6] Hoshi, T. (1995) J. Gen. Physiol. 105, 309–328.
[7] Hoth, S., Dreyer, I., Dietrich, P., Becker, D., Mueller, R.B. and Hedrich, R. (1997) Proc. Natl. Acad. Sci. USA 94, 4806–4810.
[8] Tang, X.D., Marten, I., Dietrich, P., Ivashikina, N., Hedrich, R. and Hoshi, T. (2000) Biophys. J. 78, 1255–1269.
[9] Leung, J., Bouvier-Durand, M., Morris, P.C., Guerrier, D., Chefdor, F. and Giraudat, J. (1994) Science 264, 1448–1452.
[10] Meyer, K., Leube, M.P. and Grill, E. (1994) Science 264, 1452–1455.
[11] Armstrong, F., Leung, J., Grabov, A., Brearley, J., Giraudat, J. and Blatt, M.R. (1995) Proc. Natl. Acad. Sci. USA 92, 9520–9524.
[12] Roelfsema, M.R.G. and Prins, H.B.A. (1998) Planta 205, 100–112.
[13] Brüggemann, L., Dietrich, P., Dreyer, I. and Hedrich, R. (1999) Planta 207, 370–376.
[14] Becker, D., Dreyer, I., Hoth, S., Reid, J.D., Busch, H., Lehnen, M., Palme, K. and Hedrich, R. (1996) Proc. Natl. Acad. Sci. USA 93, 8123–8128.
[15] Czempinski, K., Zimmermann, S., Ehrhardt, T. and Müller-Röber, B. (1997) EMBO J. 16, 2565–2575.
[16] Gaymard, F., Pilot, G., Lacombe, B., Bouchez, D., Bruneau, D., Boucherez, J., Michaux-Ferriére, N., Thibaud, J.B. and Sentenac, H. (1998) Cell 94, 647–655.
[17] Schroeder, J.I., Raschke, K. and Neher, E. (1987) Proc. Natl. Acad. Sci. USA 84, 4108–4112.
[18] Brüggemann, L., Dietrich, P., Becker, D., Dreyer, I., Palme, K. and Hedrich, R. (1999) Proc. Natl. Acad. Sci. USA 96, 3298–3302.
[19] Roelfsema, M.R.G. and Prins, H.B.A. (1997) Planta 202, 18–27.
[20] Grabov, A. and Blatt, M.R. (1997) Planta 201, 84–95.
[21] Marten, I., Hoth, S., Deeken, R., Ache, P., Ketchum, K.A., Hoshi, T. and Hedrich, R. (1999) Proc. Natl. Acad. Sci. USA 96, 7581–7586.
[22] Romano, L.A., Miedema, H. and Assmann, S.M. (1998) Plant Cell Physiol. 39, 1133–1144.
[23] Felle, H.H., Hanstein, S., Steinmeyer, R. and Hedrich, R. (2000) Plant J., in press.
[24] Higgins, D.G., Thompson, J.D. and Gibson, T.J. (1996) Methods Enzymol. 266, 383–402.
[25] Page, R.D. (1996) Comput. Appl. Biosci. 12, 357–358.