The short N-terminus is required for functional expression of the virus-encoded miniature K^+ channel Kcv

Anna Moroni[a,b], Carlo Viscomi[c], Vanessa Sangiorgio[c], Cinzia Pagliuca[a], Tobias Meckel[d], Ferenc Horvath[e], Sabrina Gazzarrini[a], Paola Valbuzzi[a], James L. Van Etten[f], Dario DiFrancesco[b,c], Gerhard Thiel[d,*]

[a] *Department of Biology and Istituto di Biofisica del CNR, Università degli Studi di Milano, Via Celoria 26, 20133 Milan, Italy*
[b] *I.N.F.M. Milano University Unit, Via Celoria 26, 20133 Milan, Italy*
[c] *Department of General Physiology and Biochemistry, Università degli Studi di Milano, Milan, Italy*
[d] *Darmstadt University of Technology, Schnittspahnstrasse 3, 64287 Darmstadt, Germany*
[e] *Department of Plant Physiology, Faculty of Science, University of Szeged, P.O. Box 654, H-6701 Szeged, Hungary*
[f] *Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583-0722, USA*

Received 1 July 2002; revised 6 September 2002; accepted 6 September 2002

First published online 19 September 2002

Edited by Maurice Montal

Abstract **Kcv (K^+ *Chlorella* virus) is a miniature virus-encoded K^+ channel. Its predicted membrane–pore–membrane structure lacks a cytoplasmic C-terminus and it has a short 12 amino acid (aa) cytoplasmic N-terminus. Kcv forms a functional channel when expressed in human HEK 293 cells. Deletion of the 14 N-terminal aa results in no apparent differences in the subcellular location and expression level of the Kcv protein. However, the truncated protein does not induce a measurable current in transfected HEK 293 cells or *Xenopus* oocytes. We conclude that the N-terminus controls functional properties of the Kcv channel, but does not influence protein expression.**

Key words: K^+ channel; *Chlorella* virus PBCV-1; Kcv; HEK 293 cell

1. Introduction

Potassium channels are tetramers formed by membrane-spanning protein subunits [1–3] arranged around a central ion conducting pore that allows the selective passage of K^+ ions [4,5]. Extensive electrophysiological and biochemical analyses performed on wild-type (wt) and mutated K^+ channel proteins indicate a modular organization in which different protein regions are responsible for distinct channel properties such as ion conduction, gating, and subunit assembly [6]. The last (S5–S6) or the only (M1–M2) two transmembrane domains of voltage-gated (Kv) and inward rectifier (Kir) channels, respectively, assemble into the so-called pore region, a membrane–pore–membrane module which includes the selectivity filter, a conserved sequence that controls ion selectivity [6]. Gating of Kv channels is controlled by the voltage sensor, a charged S4 domain embedded in the membrane. The N- and C-terminal regions of K^+ channel proteins are located in the cytoplasm [6] and they serve several regulatory roles, including gating and subunit assembly [6,7].

The 'miniature' K^+ channel Kcv, encoded by the algal virus PBCV-1 (*Paramecium bursaria Chlorella* virus), is the smallest and simplest functional K^+ channel found to date [8]. The 94 amino acid (aa) Kcv protein consists of two membrane-spanning domains linked by a stretch of 44 aa. Within this region is the selectivity filter THSTVGFG. Unique features of Kcv are its short cytoplasmic N-terminus (about 12 aa) and the lack of a cytoplasmic C-terminal domain.

Kcv induces currents in *Xenopus* oocytes [8] and, as reported here, in human HEK 293 cells with the typical features of a K^+ channel including ion selectivity, a moderate voltage-dependence, and a sensitivity to known channel blockers. These results indicate that, despite its small size, Kcv forms a functional K^+ selective channel. In fact Kcv may be close to the minimum size required for forming a functional potassium selective channel. If this hypothesis is correct, at least most, if not all, of the amino acids in the Kcv protein are required for the protein to function properly.

To test this hypothesis, we have started to analyze the N-terminus of Kcv. This region shows a moderate degree of hydrophobicity [8] although the region contains two charged amino acids (K^6 and R^{10}). A prediction that the N-terminus of the protein is involved in the voltage sensing of the channel is probably incorrect because the electrophysiological properties of a Kcv K^6A and $R^{10}A$ double mutant are identical to wt Kcv [9].

In this report we continue to analyze the role of the N-terminus by deleting the first 14 aa from the Kcv sequence. We present information on the intracellular location, protein expression, and whole-cell current measurements from wt and N-deleted Kcv expressed in human HEK 293 cells.

2. Materials and methods

2.1. Green fluorescent protein (GFP) fusion construct

For heterologous expression, a Kcv-GFP fusion protein was produced as follows: the *kcv* gene was amplified by PCR with primers (FOR-5′-GCTAGATCTGGATCCGTATGTTAGTGTTTAGTA-3′ and REV-5′-ATAAGAATTCTAAAGTTAGAACGATGAAGAACAC-3′) which inserted *Bgl*II and *Eco*RI restriction sites, at the 5′- and 3′-ends of *kcv*, respectively. The REV primer also led to the deletion of the *kcv* stop codon (TGA). The PCR product was inserted into a cloning vector (PCR2.1 TOPO, Invitrogen) and sequenced. *Kcv*

*Corresponding author. Fax.: (49)-6151-164630.
E-mail address: thiel@bio.tu-darmstadt.de (G. Thiel).

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)03397-5

was then cloned in frame into the *Bam*HI and *Xho*I sites of the pEGFP eukaryotic expression vector (Clontech) upstream of the GFP gene.

2.2. Construction of the deletion mutant ΔN-GFP

A channel protein lacking the 14 N-terminal amino acids (ΔN) was made by PCR amplifying *kcv* with the following primers: N-del FOR 5′-GTCGGATCCCCATTCATGATACATCTC-3′ and REV 5′-ATAAGAATTCTAAAGTTAGAACGATGAAGAACAC-3′. The PCR product was validated by sequencing and inserted into the pEGFP-N2 vector upstream of the GFP gene as described above (ΔN-GFP). The result is that Met^{15} in the wt Kcv protein becomes the N-terminal aa in ΔN-GFP.

2.3. Transfection of mammalian cell lines

For functional expression the Kcv-GFP and ΔN-GFP constructs were transfected with 23 μg/ml DNA into modified human HEK 293 cells (Phoenix) [10]. The empty plasmid (pEGFP-N2) or the plasmid containing the GFP gene only were used for mock transfection of control cells. HEK 293 cells were transiently transfected using a standard calcium phosphate protocol, as previously described [11].

2.4. Confocal microscopy

HEK cells were viewed and documented with a Leica TCS SP spectral confocal microscope equipped with an argon–krypton laser (Leica Microsystems Heidelberg GmbH, Germany). Images were acquired with a HCX PL APO 63x/1.2w objective. EGFP was excited with the 488 nm argon laser line, and confocal sections were collected using a 505–555 nm emission setting.

2.5. Western blot analysis

Western blot analysis was performed on total protein extracts as follows: 2 days after transfection, cells (from a 35 mm plate) were rinsed twice with phosphate-buffered saline, disrupted at 4°C with lysis buffer (Tris–HCl 200 mM pH 6.8, SDS 2%) and harvested by scraping. After sonication (four times, each 10 s, 4°C), nuclei and cell debris were removed by low speed centrifugation (15 000×*g*, 5 min at 4°C).

The protein content of each sample was determined by the Markwell assay [12].

Equal amounts of protein (4 μg) were solubilized with the following buffer: H_3PO_4 20 mM, SDS 4%, β-mercaptoethanol 3%, glycerol 20%, EDTA 1 mM (titrated with Tris to pH 2.4) for 1 h at 30°C. After addition of 0.004% bromophenol blue, protein samples were separated on an 18% SDS–polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane (0.2 μm, Amersham) for 2 h at 250 mA. The membrane was submerged overnight at room temperature in bovine serum albumin 3% in TBS+Tween-20 0.2% and then washed for 3 min in TBS, 5 min in TBS+Tween-20 0.01%, 15 min in TBS+Tween-20 0.01%, and 5 min in TBS. The membrane was incubated with GFP monoclonal antibody (1:500, Clontech) for 2 h at room temperature, washed as above, and incubated with an anti-mouse horseradish peroxidase conjugate (1:10 000, Sigma) for 1 h at room temperature. Immunodetection was performed with the ECL Western blot detection system from Amersham.

2.6. Electrophysiology

Experiments were performed on cells incubated after transfection at 37°C in 5% CO_2 for 1–5 days. On the day of the experiment, cells were dispersed by trypsin, plated at a low density on 35 mm plastic Petri dishes and allowed to settle for 2–4 h; dishes were then placed on the stage of an inverted microscope and single cells patch-clamped in the whole-cell configuration according to standard methods [13] using an EPC-7 patch Clamp amplifier under control of pCLAMP 5.5 software (Axon Instruments, USA). The currents were digitized at 5 kHz and low pass filtered at 1–2 kHz. Membrane currents were analyzed from raw data without any leak subtraction. Cells were perfused at room temperature (24–26°C) with a control solution containing (mM): choline-Cl, 110; KCl, 30; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES-NaOH, 5 (pH 7.4). Fast delivery of the solution was obtained by means of a perfusion pipette positioned on top of the cell under study, which allowed relatively rapid (< 1 s) solution changes. Whole-cell pipettes contained (mM): NaCl, 10; KCl, 130; EGTA, 1.0; $MgCl_2$, 0.5; ATP (Na salt), 2; GTP (Na salt), 0.1; phosphocreatine, 5; HEPES-KOH, 5 (pH 7.2).

3. Results and discussion

The consequences of removing the N-terminus from the Kcv protein (ΔN) were first analyzed by expressing the channel in *Xenopus* oocytes. In this system expression of the Kcv protein produces a specific, previously characterized, current [8,9]. ΔN expression did not produce any currents that differed from control, water-injected oocytes (results not shown). There are two explanations for this result: (i) the truncated protein does not associate with the cell membrane or (ii) the protein does not form a functional channel. To distinguish between these two possibilities we fused Kcv and ΔN genes to GFP. This allowed us to compare the intracellular location, as well as the expression level and functional properties of the two chimeras in HEK 293 cells.

3.1. Intracellular location of GFP-tagged Kcv in HEK cells

Examination of HEK 293 cells with a confocal microscope indicates that the level of expression and the subcellular location of Kcv-GFP and ΔN-GFP are similar (Fig. 1). In both cases, the fluorescent signal was excluded from the cell nucleus and was confined primarily to membranes forming reticular tubular structures, probably the endoplasmic reticulum (Fig. 1A,B). In contrast, fluorescence was distributed throughout the cytoplasm and the nucleus in cells transfected with GFP alone (Fig. 1C). This distribution is typical for GFP in its soluble form (see also [14]). Although there was no preferential location of the fusion proteins in the plasma membrane, careful examination of enlarged images revealed fluorescence signals in the plasma membrane with both constructs (Fig. 1D,E).

We also compared expression of Kcv-GFP and ΔN-GFP in HEK cells by Western blot analysis using a GFP antibody.

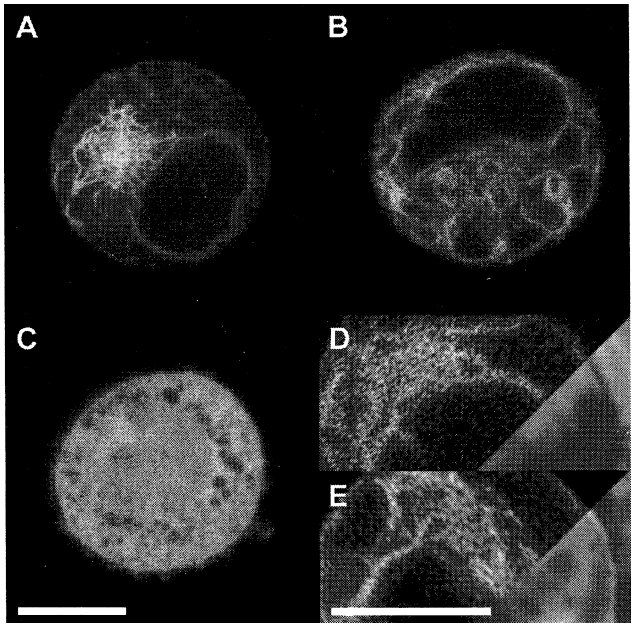

Fig. 1. Confocal images in the equatorial plane of human HEK 293 cells transfected with Kcv-GFP (A), ΔN-GFP (B) and GFP (C). D and E show enlargement of fluorescent and transmission images of the plasma membrane region from cells transfected with Kcv-GFP (D) and ΔN-GFP (E). The transmission images (lower right corners) complement the respective fluorescent images. Scale bar 5 μm.

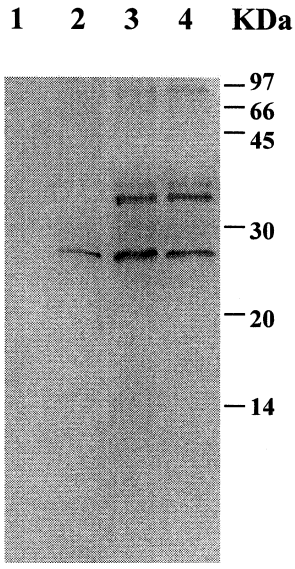

Fig. 2. Western blot analysis performed on total proteins extracted from HEK 293 cells with an anti-GFP antibody. Lane 1: control untransfected cells; lane 2: cells transfected with GFP; lane 3: cells transfected with ΔN-GFP; lane 4: cells transfected with Kcv-GFP. Molecular weights are indicated on the right side of the figure.

Fig. 2 shows that the GFP antibody recognized two major proteins in both ΔN-GFP (lane 3) and Kcv-GFP (lane 4) transfected cells. The slower migrating protein runs at the predicted molecular weight (37 kDa) of a monomer of the fusion protein. The faster migrating protein has a calculated molecular weight of 27 kDa (the predicted molecular weight of GFP) and the protein migrates at the same rate as GFP alone. Most importantly the expression levels of the two constructs are similar in both ΔN-GFP and Kcv-GFP-transfected cells.

3.2. Kcv currents in mammalian cells

Although the GFP chimeras were not preferentially targeted to the plasma membrane, the following electrophysiological experiments indicate that some of the proteins reached the outer membrane. Fig. 3 shows representative whole-cell currents recorded in HEK 293 cells from a control (GFP, Fig. 3A) and two Kcv-GFP-transfected cells (Fig. 3B,C). The cells were bathed in a solution containing 140 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES-NaOH/pH 7.4. Steps to voltages between +60 mV and −140 mV followed by a step to −80 mV were applied from a holding potential of −20 mV. The currents measured in Kcv-GFP-transfected cells (Fig. 3B,C) were significantly larger than the control current at all voltages. In the few cells that had high expression levels (≥ 2 nA at −100 mV, Fig. 3C), the current exhibited two kinetic components, a dominant instantaneous component and a much smaller time-dependent component, whose relaxation was clearly visible in the tail currents at −80 mV. The current–voltage relation of instantaneous and steady state currents is shown in Fig. 3E. Currents with the same features have been described for Kcv channels expressed in *Xenopus* oocytes [8,9].

More frequently Kcv-GFP-transfected cells exhibited smaller currents (< 2 nA at −100 mV) as shown in Fig. 3B. These cells produced instantaneous currents with voltage-dependence similar to those with higher expression levels, although the time-dependent relaxation was less apparent (compare Fig. 3B with Fig. 3C). A key feature of the instantaneous *I*/*V* relation in both low- and high-expressing cells (Fig. 3D,E) was a voltage-dependent current reduction at extreme positive and negative voltages.

As expected for a K^+ selective channel, the current reversal potential shifted from 0 at 140 mM K^+ to −29 at 30 mM K^+ and −68 mV at 3 mM K^+ (data not shown). Fig. 3F shows the linear relationship obtained by plotting the reversal voltages over the logarithm of external K^+. The calculated slope of −54.3 mV for 10-fold increases in K^+ concentration agrees well with the predicted value for a K^+ selective channel (−58 mV), according to the Nernst equation [6].

As shown in Fig. 4, there was no difference in the currents produced from control (GFP) and ΔN-GFP-transfected cells. Depolarizing voltage steps evoked in control cells the slow activating K^+ outward rectifier previously described in HEK 293 cells [15]. Similar currents were obtained in HEK 293 cells transfected with ΔN-GFP (Fig. 4A). This result is summarized as the mean instantaneous *I*/*V* relationship of GFP and ΔN-GFP-transfected cells, showing no differences in current amplitudes as well as in the reversal voltages.

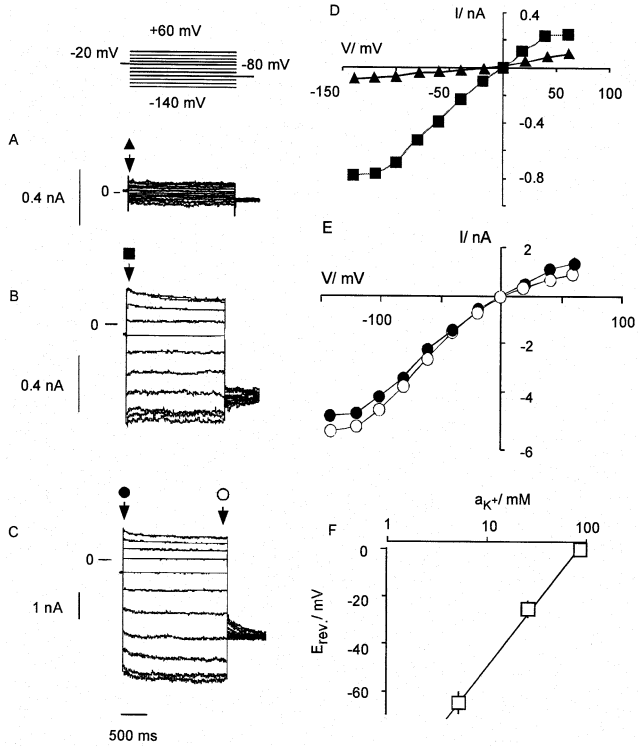

Fig. 3. Kcv-GFP currents in HEK 293 cells. Currents were recorded in 140 mM KCl from one control cell transfected with GFP only (A) and from two cells transfected with Kcv-GFP expressing low (B) and high (C) current levels. Voltage steps were applied from a holding voltage of −20 mV to test voltages as indicated. D: *I*/*V* relation of instantaneous currents from control (▲) and Kcv-GFP-transfected cells with low expression (■). E: *I*/*V* relations of instantaneous (●) and steady state currents (○) from a Kcv-GFP-transfected cell with high expression. Data sampled at times indicated by arrows (here and following figures). F: Dependence on extracellular K^+ of reversal potential of Kcv-GFP currents. The measured reversal potentials (mean of eight cells ± S.E.M.) were plotted as a function of the extracellular K^+ activity (a_{K^+}) with activity coefficients, δ: 0.92 (6 mM), 0.85 (30 mM), and 0.61 (140 mM) [17]. The regression line has a slope of 54 mV for a 10-fold increase in K^+ activity.

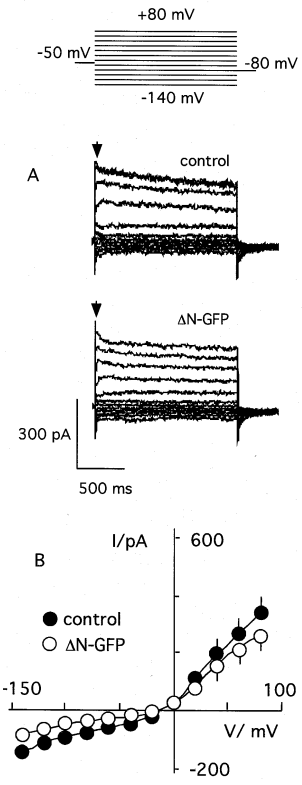

Fig. 4. Cells transfected with ΔN-GFP fail to express a Kcv like conductance. A: Representative current responses to the indicated voltage protocol recorded in 30 mM K^+ from HEK 293 cells transfected with GFP (control) and ΔN-GFP. B: Mean *I/V* relation of 15 GFP and 10 ΔN-GFP cells (±S.E.M.).

Replacing external K^+ with Na^+ significantly reduced the inward current of Kcv-GFP-transfected cells (Fig. 5A) and produced a concomitant negative shift of the reversal voltage by 61 mV (Fig. 5C) (mean shift 55 ± 5 mV, $n = 11$). The same cation substitution (Fig. 5B) had a minimum effect on the *I/V* relationship of cells transfected with ΔN-GFP, shifting the reversal potential by only 4 mV (Fig. 5D) (mean 3.6 ± 1 mV, $n = 10$).

Detection of Kcv-specific currents in the Kcv-GFP-transfected cells indicates that at least some Kcv-GFP protein reaches the plasma membrane. Because Kcv-GFP and ΔN-GFP are distributed (Fig. 1) and expressed (Fig. 2) similarly in the transfected cells, we assume that ΔN-GFP is also incorporated into the plasma membrane. The lack of currents produced by this chimera agrees with the results obtained in *Xenopus* oocytes, and suggests that the Kcv N-terminus is required to form a functional channel.

3.3. Is the N-terminus a regulatory domain?

Examination of the Kcv N-terminal region by computer analysis identified one possible regulatory sequence. The TRTE amino acid sequence (aa 9–12) resembles a casein protein kinase 2 target site [16]. Accordingly, we created a T^9A substitution in Kcv that destroys this putative phosphorylation site. However, expression of this mutant and wt Kcv in *Xenopus* oocytes did not produce any detectable differences in K^+ conductance. Furthermore, no electrophysiological differences were detected in the responses of the two channels to specific and non-specific protein kinase inhibitors (results not shown).

4. Conclusions

The results presented in this manuscript establish that the *Chlorella* virus PBCV-1-encoded Kcv protein forms a functional K^+ channel in human HEK 293 cells. The properties of the channel in these cells, including kinetics, ion selectivity and voltage-dependence, are similar to those recorded in *Xenopus* oocytes [8,9]. Therefore, the Kcv mRNA can be translated and the nascent protein delivered to the plasma membrane in at least two eukaryotic systems. The results also imply that the properties of the Kcv channel, which are similar in oocytes and HEK 293 cells, are independent of host endogenous channel subunits or cell-specific factors, such as regulatory proteins.

The results also establish that the Kcv N-terminus, albeit short, is essential for the channel to function. As inferred from the Western blot experiments and from the microscopic analysis of the GFP chimeras, deletion of the first 14 aa does not alter the synthesis of the protein nor does it affect the subcellular location of the channel. However, electrical recordings in ΔN-GFP-transfected cells failed to detect a Kcv-specific current. We conclude that for some unknown reason the N-terminal deleted channel is in a non-functional state.

Finally, we demonstrate that several point mutations (K^6A, T^9A, $R^{10}A$) in the N-terminal domain have no effect on the Kcv electrophysiological properties in *Xenopus* oocytes ([9] and this paper). Notably these alterations, which would be expected to produce a dramatic impact on the electrostatic properties of the N-terminus, have no effect on channel function. This suggests some structural flexibility of the N-terminus in performing its essential function in Kcv.

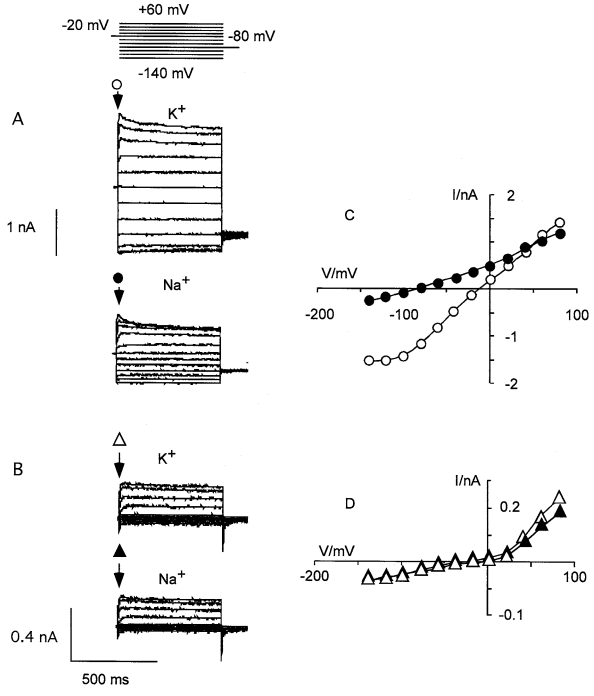

Fig. 5. Kcv channel is more permeable to K^+ than Na^+. Currents recorded in Kcv-GFP (A) and ΔN-GFP-transfected HEK 293 cells (B) in 30 mM KCl or 30 mM NaCl (voltage protocol as indicated). *I/V* relation of instantaneous currents in 30 mM KCl (open symbols) and 30 mM NaCl (closed symbols) for Kcv-GFP (C) and ΔN-GFP expressing cell (D).

Acknowledgements: We thank J. Dainty (Norwich) for help with the manuscript and Gisela Marx and Giulio Spagnol for technical support. This investigation was supported in part by bi-national traveling grant Vigoni to A.M. and G.T., Deutsche Forschungsgemeinschaft grant to G.T. and NIH Grant GM32441 to J.L.V.E.

References

[1] MacKinnon, R. (1991) Nature 350, 232–235.
[2] Yang, J., Jan, Y.N. and Jan, L.Y. (1995) Neuron 15, 1441–1447.
[3] Heginbotham, L., Odessey, E. and Miller, C. (1997) Biochemistry 36, 10335–10342.
[4] Doyle, D.A., Cabral, J.M., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. and MacKinnon, R. (1998) Science 280, 69–76.
[5] MacKinnon, R., Cohen, S.L., Kuo, A., Lee, A. and Chait, B.T. (1998) Science 280, 106–109.
[6] Hille, B. (2001) Ion Channels of Excitable Membranes, 3rd edn., Sinauer Associates, Sunderland, MA.
[7] Schulteis, C.T., Nagaya, N. and Papazian, D.M. (1998) J. Biol. Chem. 40, 26210–26217.
[8] Plugge, B., Gazzarrini, S., Nelson, M., Cerana, R., Van Etten, J.L., Derst, C., DiFrancesco, D., Moroni, A. and Thiel, G. (2000) Science 287, 1641–1644.
[9] Gazzarrini, S., Van Etten, J.L., DiFrancesco, D., Thiel, G. and Moroni, A. (2002) J. Membr. Biol. 187, 15–25.
[10] Kinsella, T.M. and Nolan, P.G. (1996) Hum. Gene Ther. 7, 1405–1413.
[11] Moroni, A., Barbuti, A., Altomare, C., Viscomi, C., Morgan, J., Baruscotti, M. and DiFrancesco, D. (2000) Pflug. Arch.-Eur. J. Physiol. 439, 618–626.
[12] Markwell, M.A.K., Haas, S.M., Bicher, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[13] DiFrancesco, D., Ferroni, A., Mazzanti, M. and Tromba, C. (1986) J. Physiol. (Lond.) 377, 61–68.
[14] Kupper, J. (1998) Eur. J. Neurosci. 10, 3908–3912.
[15] Yu, S.P. and Kerchner, G.A. (1998) J. Neurosci. Res. 52, 612–617.
[16] Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C.J., Hofmann, K. and Bairoch, A. (2002) Nucleic Acids Res. 30, 235–238.
[17] Robinson, R. and Stokes, R. (1968) Electrolyte Solutions. Butterworths Scientific Publications, London.