Regulation of mammalian *Shaker*-related K^+ channels: evidence for non-conducting closed and non-conducting inactivated states

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1. Using the whole-cell recording mode we have characterized two non-conducting states in mammalian *Shaker*-related voltage-gated K^+ channels induced by the removal of extracellular potassium, K_o^+.

2. In the absence of K_o^+, current through Kv1.4 was almost completely abolished due to the presence of a charged lysine residue at position 533 at the entrance to the pore. Removal of K_o^+ had a similar effect on current through Kv1.3 when the histidine at the homologous position (H404) was protonated (pH 6·0). Channels containing uncharged residues at the corresponding position (Kv1.1: Y; Kv1.2: V) did not exhibit this behaviour.

3. To characterize the nature of the interaction between Kv1.3 and K_o^+ concentration ($[K^+]_o$), we replaced H404 with amino acids of different character, size and charge. Substitution of hydrophobic residues (A, V and L) either in all four subunits or in only two subunits in the tetramer made the channel insensitive to the removal of K_o^+, possibly by stabilizing the channel complex. Replacement of H404 with the charged residue arginine, or the polar residue asparagine, enhanced the sensitivity of the channel to 0 mM K_o^+, possibly by making the channel unstable in the absence of K_o^+. Mutation at a neighbouring position (400) had a similar effect.

4. The effect of removing K_o^+ on current amplitude does not seem to be correlated with the rate of C-type inactivation since the slowly inactivating G380F mutant channel exhibited a similar $[K^+]_o$ dependence as the wild-type Kv1.3 channel.

5. CP-339,818, a drug that recognizes only the inactivated conformation of Kv1.3, could not block current in the absence of K_o^+ unless the channels were inactivated through depolarizing pulses.

6. We conclude that removal of K_o^+ induces the Kv1.3 channel to transition to a non-conducting 'closed' state which can switch into a non-conducting 'inactivated' state upon depolarization.

Voltage-gated potassium (Kv) channels are membrane proteins that regulate the flow of potassium ions across the membranes of all living cells (Hille, 1992; Chandy & Gutman, 1995). A family of nineteen genes encode these diverse proteins in mammals, the largest sub-family, Kv1, containing at least six members (Chandy & Gutman, 1995). Each protein has roughly 500–600 amino acids that are distributed over long N- and C-termini, six putative transmembrane segments (S1–S6), and a short loop between S5 and S6 (P-loop) which contributes to the formation of the pore. Functional Kv channels are tetramers (MacKinnon, 1991) with an approximate mass of 250–320 kDa and x–y dimensions of 6·5–8·0 × 6·5–8·0 nm (Li, Unwin, Stauffer, Jan & Jan, 1994; Spencer *et al.* 1997). Using structurally defined peptide antagonists of Kv channels as molecular callipers, three groups have independently revealed the existence of a shallow saucer-shaped vestibule at the external entrance to the ion conduction pathway which is ~3 nm wide and ~0·6 nm deep (Goldstein, Pheasant & Miller, 1994; Aiyar *et al.* 1995; Hidalgo & MacKinnon, 1995; Aiyar, Rizzi, Gutman & Chandy, 1996).

The external potassium concentration ($[K^+]_o$) is tightly regulated in mammals, and even minor changes in this level can induce significant alterations in the physiological function of excitable tissues, leading to epileptiform seizures, or to electrical instability following cardiac ischaemia (Heinemann, Konnerth, Pumain & Wadman, 1986; Yaari, Konnerth & Heinemann, 1986; Pardo *et al.* 1992; Leschinger, Stabel, Igelmund & Heinemann, 1993). Such pathological alterations are thought to be partly mediated by potassium-dependent K^+ conductances which modulate the firing frequency of excitable cells as a function of $[K^+]_o$ (Pardo *et al.* 1992). For example, depletion of K_o^+ has been previously reported to decrease current through the rapidly inactivating Kv1.4 channel expressed in rat hippocampal neurons (Pardo *et al.* 1992; Eder, Klee & Heinemann, 1996; Beck *et al.* 1996). Site-directed mutagenesis studies identified lysine 533, a residue located in the external vestibule, as being responsible for this exquisite sensitivity of Kv1.4 to $[K^+]_o$. Another K^+ channel, HERG, also seems to be dependent on $[K^+]_o$ (Sanguinetti, Jiang, Curran & Keating, 1995; Trudeau, Warmke, Ganetzki & Robertson, 1995; Schönherr & Heinemann, 1996).

In the present study, we have examined the $[K^+]_o$ dependence of four Kv channels, Kv1.1–1.4. Of these four cloned channels, removal of external potassium (K_o^+) only suppressed current through Kv1.3 and Kv1.4. Using site-specific mutagenesis in combination with electrophysiological and pharmacological approaches, we have identified the residues in Kv1.3, the voltage-gated K^+ channel expressed in human T lymphocytes (Grissmer *et al.* 1990), that are responsible for this $[K^+]_o$ dependence. We provide evidence that the removal of K_o^+ shifts the channel into a non-conducting 'closed' state which can transition into a non-conducting inactivated state following repeated depolarizations. Some of these results have been reported in preliminary communications (Grissmer, Nguyen, Aiyar & Chandy, 1994; Jäger *et al.* 1997*a*, *b*).

METHODS

Cell lines stably expressing Kv1.1, Kv1.2 and Kv1.3

NIH3T3 and L929 cells stably transfected with mouse Kv1.1 and Kv1.3 (mKv1.1 and mKv1.3; Grissmer *et al.* 1994) were obtained from Dr D. Auperin and Dr D. Hanson (Pfizer Inc., Groton, CT, USA). B82 mouse fibroblasts stably transfected with rat Kv1.2 (rKv1.2; NGK1) were obtained from Dr Higashida (Neuroinformation Research Institute, Kanazawa University School of Medicine, Kanazawa, Japan). Cells were maintained in a culture medium of Dulbecco's modified Eagle's medium (DMEM) supplemented with 580 mg l^{-1} L-glutamine and 10% heat-inactivated fetal calf serum in a humidified, 5% CO_2 incubator at 37 °C.

Cells used for cRNA injection

Rat basophilic leukaemia cells (RBL) were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in a culture medium of Eagle's minimal essential medium (EMEM) supplemented with 1 mM L-glutamine and 10% heat-inactivated fetal calf serum in a humidified, 5% CO_2 incubator at 37 °C. Cells were plated to grow non-confluently onto glass 1 day prior to use for injection and electrophysiological experiments. Some experiments were performed on *Xenopus* oocytes as described earlier (Aiyar *et al.* 1995, 1996).

Solutions

Experiments were done at room temperature (21–25 °C). Cells measured in the whole-cell configuration were normally bathed in mammalian Na^+ solution containing (mM): 160 NaCl, 4·5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, adjusted to pH 7·4 with NaOH, with an osmolarity of 290–320 mosmol l^{-1}. A simple syringe-driven perfusion system was used to exchange the bath solutions in the recording chamber. The internal pipette solution for the whole-cell recordings contained (mM): 134 KF, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 EGTA, adjusted to pH 7·2 with KOH, with an osmolarity of 290–320 mosmol l^{-1}. *Xenopus* oocyte bathing solution (ND96) contained (mM): 96 NaCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, with various K^+ concentrations, adjusted to pH 7·6 with NaOH.

Chemicals

CP-339,818 is a dihydroquinoline antagonist of the Kv1.3 channel that preferentially binds to the C-type inactivated conformation (Nguyen *et al.* 1996). This compound was obtained from Dr Doug Hanson (Pfizer Inc., Groton, CT, USA) and was dissolved in DMSO to make a stock solution (10 mM) from which final dilutions were made. The stock solution was stored at 4 °C and protected from light. The final DMSO concentrations diluted in the external solutions were $< 0{\cdot}1$%. Fluorescein-dextran (MW, 10 000) was obtained from Molecular Probes and from Sigma. DMEM and EMEM were obtained from Gibco BRL. Glutamine was obtained from Biochrom (Berlin, Germany).

cRNA synthesis

All the mutant and wild-type mouse Kv1.3 clones, as well as the mouse Kv1.4 clone, used in this study have been previously described (Aiyar *et al.* 1995, 1996; Wymore *et al.* 1996). Each Kv1.3 construct was linearized with *Eco*RI (the Kv1.4 construct was linearized with *Bgl* II) and *in vitro* transcribed with the SP6 Cap-Scribe System (Boehringer Mannheim). The resulting cRNA was phenol/chloroform extracted and stored at −75 °C.

Injection of cRNA into RBL cells

cRNA was diluted with a fluorescent fluorescein isothiocyanate (FITC) dye (0·5% FITC-dextran in 100 mM KCl) to a final concentration of 1 $\mu g\ \mu l^{-1}$. Injection capillaries (Femtotips, Eppendorf, Germany) were filled with the cRNA–FITC solution and RBL cells were injected using an Eppendorf microinjection system (Micromanipulator 5171 and Transjector 5246) as previously described (Ikeda *et al.* 1992; Rauer & Grissmer, 1996; Nguyen *et al.* 1996). RBL cells were chosen because they lack endogenous Kv channels (McCloskey & Cahalan, 1990). Fluorescent cells were visualized 2–6 h later and electrical currents measured using the patch clamp method.

Electrophysiology

Experiments on the cell lines or injected RBL cells were carried out using the whole-cell or the inside-out patch recording mode of the patch clamp technique (Hamill *et al.* 1981; Grissmer & Cahalan, 1989*a*, *b*; Grissmer, Lewis & Cahalan, 1992; Grissmer, Nguyen & Cahalan, 1993; Hanselmann & Grissmer, 1996). Electrodes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK) in two stages, coated with Sylgard (Dow Corning), and fire-polished to resistances of 2·5–4 MΩ measured in the bath. Membrane currents were recorded with an EPC-9 patch clamp amplifier (HEKA elektronik, Lambrecht, Germany) interfaced to a Macintosh computer running acquisition and analysis software

(Pulse and PulseFit). Capacitative and leak currents were subtracted using the $P/10$ procedure. Series resistance compensation (80%) was employed if the current exceeded 1 nA. The holding potential in all experiments was −80 mV.

For the *Xenopus* oocyte experiments we used the two-electrode voltage clamp technique to measure membrane currents (Stühmer, 1992). Data were recorded with a two-electrode voltage clamp amplifier (Warner Instruments, Hamden, CT, USA). The command input of the amplifier was controlled by an IBM-compatible computer using pCLAMP software (Axon Instuments). Capacitative and leak currents were subtracted before analysis using the $P/4$ procedure.

RESULTS

$[K^+]_o$ dependence of Kv1.1–Kv1.4

Pardo *et al.* (1992) reported that the removal of K_o^+ abolished current through the rat Kv1.4 channel, and showed that the presence of a charged lysine residue (K533) at the outer mouth of the pore was responsible for this effect. As a first step in our study of this phenomenon, we examined the $[K^+]_o$ dependence of three *Shaker*-related mouse clones, mKv1.1, mKv1.3, mKv1.4, and one rat clone, rKv1.2. Whole-cell currents were elicited with 200 ms depolarizing voltage steps from a holding potential

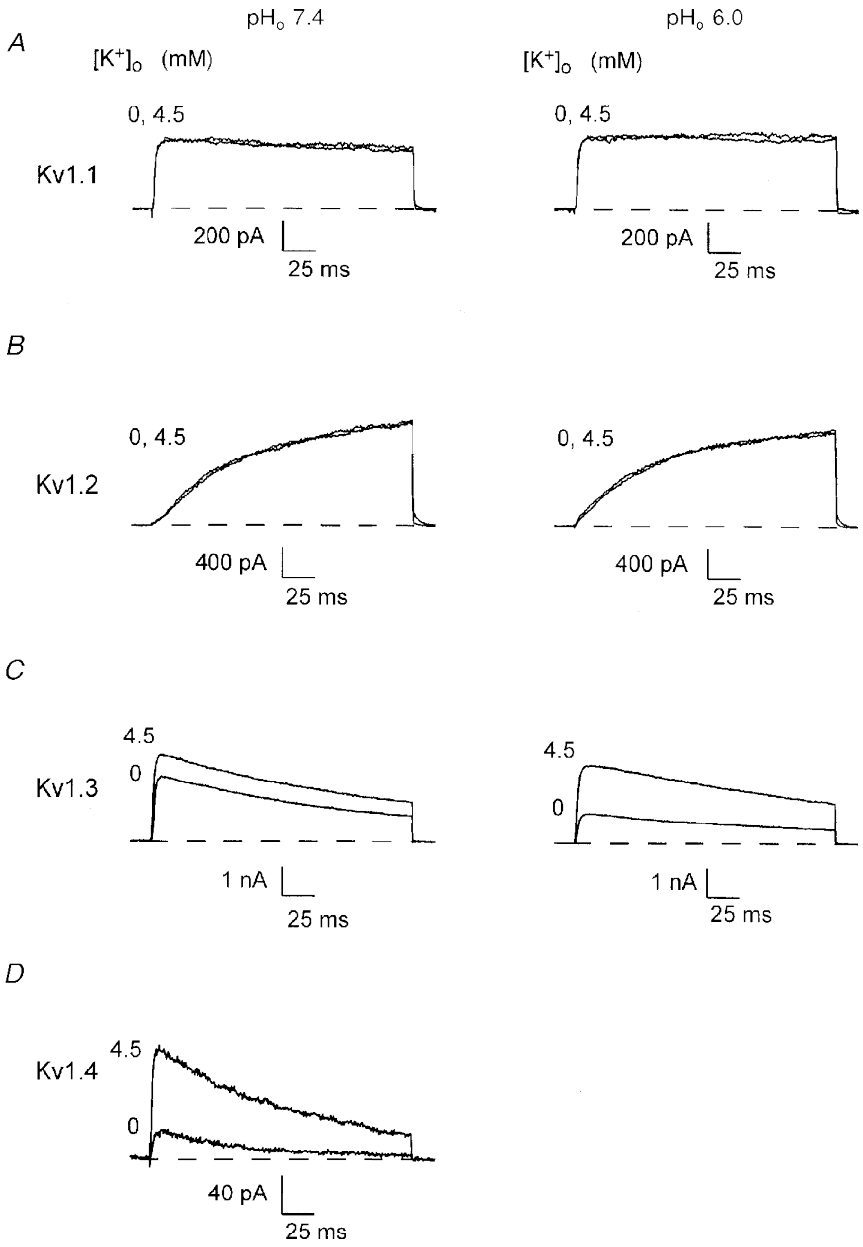


Figure 1. Effect of reducing $[K^+]_o$ and pH_o on current through voltage-gated *Shaker*-related potassium channels

Current was elicited by 200 ms depolarizing voltage steps from −80 to 40 mV from a holding potential of −80 mV. Currents are shown in the presence and absence of extracellular K^+ at pH_o 7·4 (left) and at pH_o 6·0 (right).

Table 1. Ratio of peak current amplitude in the absence to that in the presence (4·5 mM) of K_o^+ at pH_o 7·4 and pH_o 6·0

	pH_o 7·4	pH_o 6·0
H404Y	0·93 ± 0·01	0·97 ± 0·03
H404V	1·03 ± 0·03	1·01 ± 0·03
H404R	0·11 ± 0·01	n.d.
H404N	0·05 ± 0·02	0·04 ± 0·01
H404S	0·51 ± 0·07	0·62 ± 0·04
H404A	0·98 ± 0·03	0·96 ± 0·04
H404L	0·88 ± 0·01	0·87 ± 0·05
H404T	1·04 ± 0·03	0·98 ± 0·03

Values given are means ± S.D. for at least 3 experiments each. n.d., not determined.

of −80 to 40 mV in the presence (4·5 mM) and absence of K_o^+, and at either pH_o 7·4 or pH_o 6·0. The results of these experiments are shown in Fig. 1. Removal of K_o^+ had no effect on current through Kv1.1 (Fig. 1*A*) and Kv1.2 (Fig. 1*B*) at either pH_o. In contrast, current through Kv1.3 (Fig. 1*C*) was reduced by ~25% in the absence of K_o^+ at pH_o 7·4 (Fig. 1*C*, left), and this effect was much more pronounced at pH_o 6·0 (Fig. 1*C*, right). The suppression of the Kv1.4 current upon removal of K_o^+ was even more dramatic, being almost complete at pH_o 7·4 (Fig. 1*D*). These effects were readily reversible upon external addition of K^+.

Histidine 404 (H404) in Kv1.3 is involved in the $[K^+]_o$ dependence

Lysine 533 (K533) in rat Kv1.4 has been previously reported to be necessary for the $[K^+]_o$ dependence of this rapidly inactivating channel (Pardo *et al.* 1992). The alignment of the P-region, shown in Fig. 2, highlights the residues in mKv1.1 (Y), rKv1.2 (V) and mKv1.3 (H) that are in positions homologous to K533 in rat Kv1.4. These residues, located in the outer vestibule (Aiyar *et al.* 1995, 1996; Guy & Durell, 1996), participate in the external TEA-binding site (Kavanaugh *et al.* 1992). Protonation of H404 can influence TEA-binding (Kavanaugh *et al.* 1992) and might also account for the increased sensitivity of mKv1.3 to the removal of K_o^+ at pH_o 6·0, while the presence of uncharged residues at the homologous position in mKv1.1 and rKv1.2 may underlie their insensitivity to changes in $[K^+]_o$. To test this idea, we replaced H404 in Kv1.3 with amino acids of different physical character. Replacement of H404 with hydrophobic or aromatic residues (V, A, L and Y) resulted in channels that were unaffected by removal of K_o^+ (Fig. 3), a behaviour reminiscent of mKv1.1 and rKv1.2. In contrast, substitution of H404 by the nitrogen-containing residues R and N enhanced sensitivity to removal of K_o^+; this was similar to the behaviour observed for mKv1.4 (Fig. 3). Substitution with non-nitrogen-containing polar residues (H404T and H404S) resulted in channels which were either insensitive or mildly sensitive to removal of K_o^+ (Fig. 3). The results are summarized in Table 1. The data are consistent with the notion that the presence of charged nitrogen-containing residues at the external entrance to the ion conduction pathway render Kv channels sensitive to the removal of K_o^+.

In order to determine whether all four H404 residues were required in the Kv1.3 tetramer for $[K^+]_o$ dependence, we generated a dimer containing one wild-type (WT) and one mutant (H404V) subunit. The tetramer should, therefore, contain two histidines and two valines at position 404. If the two histidines are sufficient to confer $[K^+]_o$ dependence, then removal of K_o^+ should result in the suppression of the Kv1.3 current. In contrast, if more than two histidines are required for this effect, the WT–H404V dimer should be insensitive to the absence of K_o^+. Results presented in Fig. 4 clearly indicate that more than two H404 residues are required to make the channel susceptible to the removal of K_o^+. Current through the WT–H404V dimer was unaffected by the removal of external potassium.

$[K^+]_o$ dependence and C-type inactivation

The H404 mutations that alter $[K^+]_o$ dependence (Fig. 3) also alter the rate (τ_h) of C-type inactivation (Nguyen *et al.* 1996). Are these two behaviours linked? Three lines of evidence indicate that these processes are not related. First, the slowly inactivating G380F mutant channel (τ_h, ~1400 ms; Nguyen *et al.* 1996) exhibited the same degree of $[K^+]_o$ dependence as the WT Kv1.3 channel (Fig. 5). As shown in Fig. 5, removal of K_o^+ caused a 30% reduction in the peak current through the G380F channel, and this effect was much more pronounced at pH_o 6·0. Second, the H404L mutant, which inactivates (τ_h, ~220 ms; Nguyen *et al.* 1996) at roughly the same rate as the WT

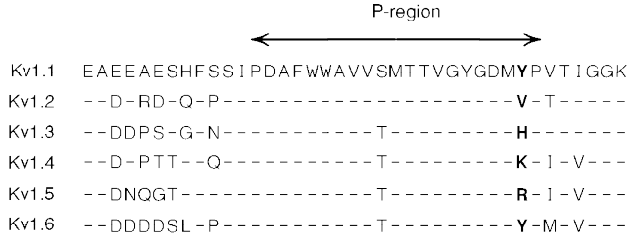


Figure 2. Putative pore region of voltage-gated *Shaker*-related potassium channels
The bold residues represent position 404 in Kv1.3, which is homologous to position 533 in Kv1.4.

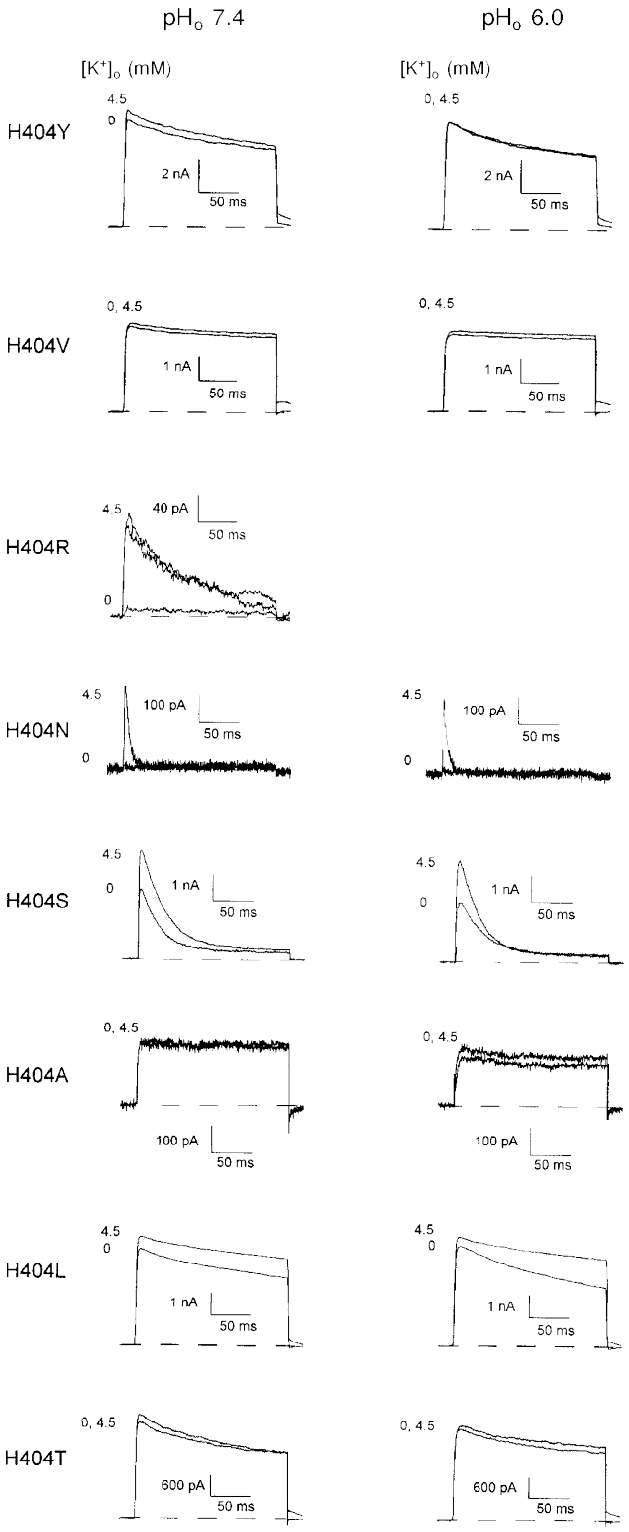


Figure 3. Effect of reducing $[K^+]_o$ and pH_o on current through different H404 mutant Kv1.3 channels

Current was elicited as described in the legend to Fig. 1 and in identical solutions except for the H404R experiment in which current traces are shown before, during, and after the removal of K_o^+.

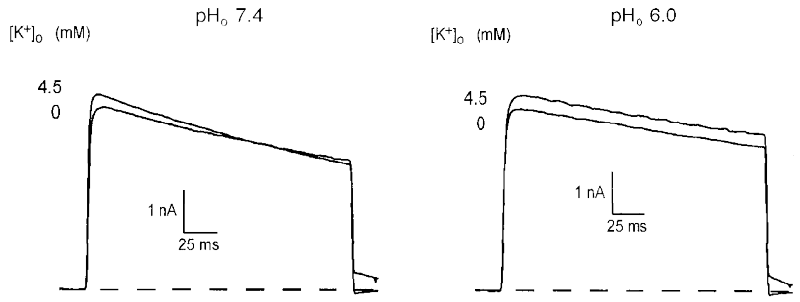


Figure 4. Effect of reducing $[K^+]_o$ and pH_o on current through a dimer of WT–H404V mutant Kv1.3 channels

Current was elicited as described in the legend to Fig. 1 and in identical solutions.

channel (τ_h, ~230 ms; Nguyen *et al.* 1996), and the H404S mutant, which inactivates approximately five times faster (τ_h, ~40 ms; Nguyen *et al.* 1996) than the WT channel, were both less sensitive to the removal of K_o^+ than their WT counterpart. Lastly, the H404R mutant, which inactivated with a slightly slower rate than the WT (τ_h, ~340 ms; Nguyen *et al.* 1996), was extremely sensitive to the removal of K_o^+. Taken together our results suggest that the sensitivity of Kv channels to removal of K_o^+ is not coupled to their rate of C-type inactivation.

Mutation of a potassium-binding site in the pore enhances $[K^+]_o$ dependence

The P-region of all potassium-selective channels that have been isolated thus far, from bacteria to humans, contains the G(Y/F)GD motif, which is thought to form the selectivity filter. This sequence forms a shallow ~0·6 nm trough at the centre of the external vestibule (Ranganathan, Lewis & MacKinnon, 1996; Aiyar *et al.* 1996). Since the tyrosine in this signature sequence has been reported to be a potassium-binding site (Ranganathan *et al.* 1996; Aiyar *et al.* 1996), perturbations at this position might destabilize the channel and increase its sensitivity to removal of K_o^+. As shown in Fig. 6, a WT–Y400V dimer construct greatly enhanced the sensitivity of the channel to 0 mM K_o^+. Current through the WT–Y400V dimer was almost completely abolished by removal of K_o^+ at pH_o 7·6 (Fig. 6), a behaviour resembling that of the H404N and H404R mutants. Very similar results were observed for a WT–D402N construct (data not shown). Thus, the loss of conductance upon removal of K_o^+ is not restricted to residues at position 404 alone. Further studies will ascertain whether substitutions at neighbouring positions have a similar destabilizing effect.

Nature of the non-conducting state: mechanism of current loss in zero $[K^+]_o$

There are several hypotheses that could explain the loss of outward current upon removal of K_o^+. One possibility is that the channel is forced into an inactivated conformation in 0 mM K_o^+, either through an increase in the rate of C-type inactivation or via a slowing of the recovery from inactivation (Levy & Deutsch, 1996), which would reduce the number of functional channels available for opening upon depolarization. This possibility does not seem likely for the following reason. Kv1.3 exhibits cumulative inactivation

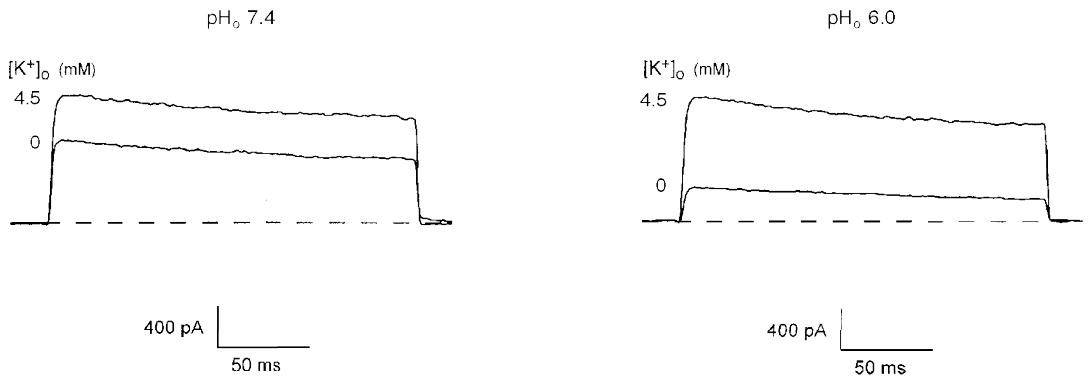


Figure 5. Effect of reducing $[K^+]_o$ and pH_o on current through the slowly inactivating G380F mutant Kv1.3 channel

Current was elicited as described in the legend to Fig. 1 and in identical solutions.

and typically requires 30 s to recover from C-type inactivation following a standard 200 ms depolarizing pulse to 40 mV from a holding potential of −80 mV. If the absence of K_o^+ abolished current by slowing recovery from inactivation, re-addition of 4·5 mM K_o^+ would elicit current only after a short delay – the time required to transition from the inactivated to the closed conformation. For example, a 200 ms exposure to 0 mM K_o^+ should result in a delay similar to a 200 ms depolarization, i.e. ~30 s (compare Fukushima, Hagiwara & Henkart, 1984; Cahalan, Chandy, DeCoursey & Gupta, 1985). The data presented in Fig. 7*A* belay this prediction. Following a ~1 min exposure to 0 mM K_o^+, current was restored almost instantaneously upon re-exposure to 4·5 mM K_o^+. Although these results suggest that this first mechanism is unlikely to be the cause of $[K^+]_o$ dependence, it remains to be experimentally disproven. A second mechanism to explain the loss of current in 0 mM K_o^+ predicts that the Kv1.3 channel, in the absence of K_o^+, undergoes a conformational change into a non-conducting 'closed' state. To distinguish between these two possibilities, we exploited a novel dihydroquinoline compound that preferentially and almost irreversibly binds to and blocks the inactivated conformation of Kv1.3 (Nguyen *et al.* 1996). If the channel adopts an inactivated state in 0 mM K_o^+, CP-339,818 would be expected to interact with this channel conformation and prevent it from reopening upon the re-addition of 4·5 mM K_o^+. In contrast, if the channel transitions into a non-conducting closed state in the absence of K_o^+, CP-339,818 would not bind to or block this conformation of the channel, and upon re-exposure to 4·5 mM K_o^+, current would remain unchanged compared with the control current.

We chose the H404N mutant for these experiments because of its sensitivity to 0 mM K_o^+ at pH_o 7·4 (Fig. 3), and also because it is potently blocked by CP-339,818 (Fig. 7*A* and Nguyen *et al.* 1996). Figure 7*A* shows first the control current in 4·5 mM K_o^+ that is reversibly suppressed by removing K_o^+. After that procedure, again in the presence of 4·5 mM K_o^+, externally applied CP-339,818 (1 μM) could only block current through the H404N channel when depolarizing pulses were applied. No current reduction could be observed after a 3 min exposure to the drug in the absence of depolarizing pulses. This is consistent with the idea that channel block occurs only if the channel is in the inactivated state, confirming earlier results (Nguyen *et al.* 1996). After starting the depolarizing pulses, the drug blocked the current in a use-dependent manner with a time constant for the reduction of peak current of ~100 s (Fig. 7*A*). This indicated to us that the channel had changed its conformation to one that was recognized by the drug: presumably the inactivated state. In a parallel experiment we wanted to find out whether the drug could act on the channel in the absence of K_o^+. Figure 7*B* shows first the control current at 4·5 mM K_o^+ that is suppressed by removing K_o^+. The channels were then exposed to extracellular CP-339,818 (1 μM) in 0 mM K_o^+ for 1 min, and 4·5 mM K_o^+ in combination with fresh drug were then re-applied to the cell for 30 s, no depolarizing pulses being administered during this 90 s period. Current amplitude at the end of the 1 min exposure to CP-339,818 and 0 mM K_o^+ remained unchanged (Fig. 7*B*) indicating that the drug did not bind to or block the H404N channel in the absence of K_o^+. In the presence of 4·5 mM K_o^+, CP-339,818 blocked the channel in a use-dependent fashion with the temporal behaviour seen in Fig. 7*A*. This set of experiments suggests that 0 mM K_o^+ induces the channel to adopt a non-conducting closed state which is unable to bind CP-339,818; this conformation is distinct from the inactivated state which can bind the drug.

Can the non-conducting state undergo inactivation?

To determine whether the non-conducting closed state could transition into the inactivated conformation, we repeated the protocol used in the previous experiment with one difference. During the 1 min exposure to the drug in 0 mM K_o^+, we subjected the cell to two depolarizing pulses to enable the channels, if they were capable, to transition from the non-conducting closed conformation to a non-conducting inactivated state. The fraction of channels that made this transition would bind CP-339,818 and be unavailable for reopening when exposed to 4·5 mM K_o^+. Consistent with this expectation, re-application of 4·5 mM K_o^+ only partially

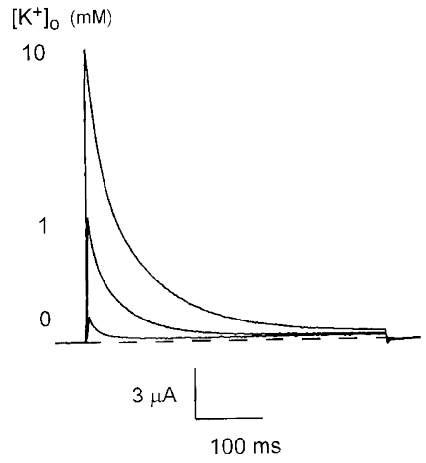


Figure 6. Effect of reducing $[K^+]_o$ on current through a dimer of WT–Y400V mutant Kv1.3 channels expressed in *Xenopus* oocytes at pH_o 7·6

Current was elicited using 450 ms depolarizing pulses to 40 mV (holding potential, −100 mV) every 30 s in ND96 solution in the absence and presence of 1 or 10 mM K_o^+.

restored the current amplitude (Fig. 7*C*), and the drug then blocked the channel with almost the same time course as for Fig. 7*A* and *B*. These results argue that the non-conducting closed state induced by 0 mM K_o^+ can undergo C-type inactivation upon depolarization with kinetics similar to the transition from the 'normal' closed to the 'normal' inactivated conformation. The resulting non-conducting inactivated state is recognized by CP-339,818 in much the same manner as the normal inactivated conformation.

DISCUSSION

We have characterized currents through different *Shaker*-related K^+ channels and through WT and mutant Kv1.3 channels in the presence and absence of K_o^+ and at different pH_o. Among the *Shaker*-related channels investigated, only Kv1.3 and Kv1.4 show a reduction of the outward current amplitude upon removal of K_o^+. We confirmed and extended the results of Pardo *et al.* (1992) who reported that positively charged residues at the external TEA-binding site are responsible for this K^+ sensitivity. Hydrophobic residues at the corresponding position render the channel insensitive to removal of K_o^+. Several other observations, however, indicate that it is not merely the presence of charge, but the presence of a nitrogen-containing group at this position that appears to be critical for the observed phenomenon. For instance, the neutral, polar residue asparagine rendered the channel sensitive to removal of K_o^+. On the other hand, other non-nitrogen-containing polar substitutions (threonine and serine) did not alter the sensitivity of the channel to the absence of K_o^+. In addition, we present evidence that residues outside the H404 site (Y400 and D402) also contribute to the sensitivity of the Kv1.3 channel to $[K^+]_o$. Mutations in the K^+ channel signature sequence render the channel extremely sensitive to the absence of K_o^+.

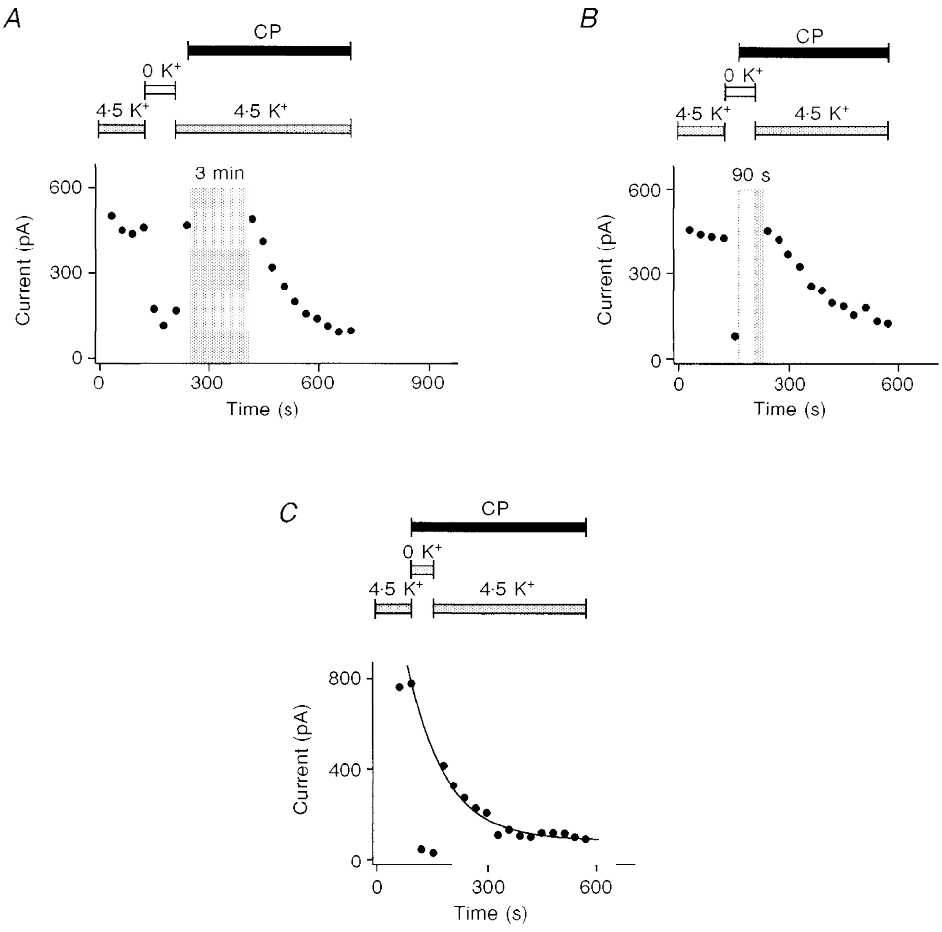
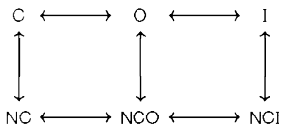


Figure 7. Effect of CP-339,818 on current through the H404N mutant Kv1.3 channel in the presence and absence of K_o^+, with or without application of depolarizing pulses

Currents were elicited with 200 ms depolarizing voltage steps to 40 mV (holding potential, −80 mV). *A*, peak currents are plotted against time during the experiment in the indicated solutions. No block of the peak current by 1 μM CP-339,818 in 4·5 mM K^+ occurred if the membrane was not depolarized (grey area). *B*, similar record to that shown in *A*. Again, no block of the peak current by 1 μM CP-339,818 occurred, this time in 0 mM K_o^+, if the membrane was not depolarized (area outlined in white and grey). *C*, 1 μM CP-339,818 was applied firstly in the absence of K_o^+. After 60 s $[K^+]_o$ was increased to 4·5 mM in the presence of the drug.

Figure 8. Simplified scheme of Kv1.3 channel transitions by depolarizations and removal of K_o^+

Not all possible transitions are shown. C, closed; O, open; I, inactivated; NC, non-conducting closed; NCO, non-conducting open; NCI, non-conducting inactivated.



Comparison of the K^+ sensitivity of Kv1.3 with other channels

Pardo *et al.* (1992) and Lopez-Barneo, Hoshi, Heinemann & Aldrich (1993) studied the effects of removal of K_o^+ on the fly *Shaker* channel and its mammalian homologues. Their studies were conducted on WT and on deletion mutants lacking the N-terminal ball responsible for fast inactivation (N-type). Both groups discuss the possibility that the non-conducting state induced by 0 mM K_o^+ might be a consequence of a change in the inactivation properties of the channel, since they observe a faster time course of C-type inactivation during depolarizations and a slower recovery from inactivation upon removal of K_o^+. The dependence of recovery from inactivation on $[K^+]_o$ was also reported for current through Kv1.3 (Levy & Deutsch, 1996). Pardo *et al.* (1992) and Lopez-Barneo *et al.* (1993), however, exclude this apparently simple possibility for two reasons. First, recovery from C-type inactivation in low and high $[K^+]_o$ was significantly faster than the interval between pulses used in their experimental protocol, and could therefore not account for the decrease of current amplitude in 0 mM K_o^+. Second, changing the holding potential from −90 to −160 mV, a manoeuvre that should accelerate the recovery from inactivation and make more channels available for opening, did not appreciably increase current amplitude. They conclude that closed channels can go directly into the inactivated state and that this transition is influenced by $[K^+]_o$. Raising $[K^+]_o$ would prevent this closed state inactivation and would therefore result in more channels going through the open state, leading to larger currents and vice versa (Lopez-Barneo *et al.* 1993). Their postulation of a closed inactivation transition being dependent on $[K^+]_o$ is equivalent to our model implying a $[K^+]_o$-dependent transition from the closed to a non-conducting state that can still undergo the transition to a non-conducting inactivated state (see Fig. 8).

More recently other investigators have also observed current loss in *Shaker* B channels upon simultaneous removal of potassium from both the extracellular and cytoplasmic sides of the channel, but not upon removal of K_o^+ alone (Heinemann, Starkus & Rayner, 1996; Gomez-Lagunas, 1997). The phenomenon they are studying is clearly different from the non-conducting state we observe in Kv1.3 channels in 0 mM K_o^+.

The non-conducting state

Several hypotheses can explain the loss of outward current upon removal of K_o^+. One hypothesis is that the removal of K_o^+ enhances the proportion of channels in the inactivated state (see Fig. 8), either through an increase in the rate of C-type inactivation or through a slowing of the recovery from inactivation. In either case the proportion of channels that occupy the inactivated state (see Fig. 8) is increased. A second hypothesis suggests that the channels undergo a conformational change upon removal of K_o^+, resulting in a new non-conducting conformation which is distinct from the inactivated state of the channels (see Fig. 8). Several observations lead us to favour the latter hypothesis. (1) Recovery from C-type inactivation is much slower than recovery from the K^+-induced conformation. For example, recovery from inactivation following a 200 ms pulse takes at least 30 s, whilst the inactivated state induced by a substantially longer depolarizing pulse (e.g. 30 s) would require minutes for complete recovery. In contrast, following a 30 s application of 0 mM K_o^+, re-application of 4·5 mM K_o^+ elicits a current well within 30 s (see Fig. 7*C*). (2) A drug (CP-339,818) that preferentially blocks the C-type inactivated state of the channel (Nguyen *et al.* 1996) does not bind to or block the conformation induced by 0 mM K_o^+ (see Fig. 7). This result strongly argues that the Kv1.3 channel in the absence of K_o^+ assumes a conformation distinct from the inactivated state elicited by a depolarizing pulse. (3) Administration of repeated depolarizing pulses to the channels bathed in 0 mM K_o^+ causes this novel 'non-conducting closed' state to transition to a 'non-conducting inactivated' conformation that is blocked by CP-339,818. The simple model proposed in Fig. 8 explains our results.

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