Differential Effects of Elevating $[K]_o$ on Three Transient Outward Potassium Channels

Dependence on Channel Inactivation Mechanisms

Gea-Ny Tseng and Julie Tseng-Crank

We carried out a systematic study on the effects of elevating $[K]_o$ on the properties of a transient outward potassium channel encoded by a cardiac cDNA (RHK1) and compared them with those on two Shaker potassium channels (H-4 and H-37). The amino acid sequences of all three channels are known, and their structure–function relations have been partially characterized. All three channels were expressed in Xenopus oocytes and studied under double-microelectrode voltage-clamp conditions. For all three channels, elevating $[K]_o$ caused an increase in the channels' chord conductances and a negative shift in the calculated activation curves. However, in other aspects of channel properties that are related to the channels' inactivation processes, there were differences in the changes induced by increasing $[K]_o$:

1. Elevating $[K]_o$ caused a positive shift in the steady-state inactivation curves of RHK1 and H-4 but did not cause any shift in H-37.
2. Elevating $[K]_o$ slowed the time course of inactivation of H-37 but did not cause any significant changes in the time course of RHK1 or H-4.
3. Elevating $[K]_o$ accelerated the rate of recovery from inactivation of RHK1 and H-4 but slowed the recovery time course of H-37. Our experiments show that elevating $[K]_o$ can cause a wide range of effects on the transient outward potassium channels. Furthermore, raising $[K]_o$ induced similar changes in RHK1 and H-4 (inactivation mediated by an "N-type" mechanism) that were different from the changes in H-37 (inactivation mediated by a "C-type" mechanism). Therefore, our data suggest that part of the effects of elevating $[K]_o$ on channel properties may depend on the channel's inactivation mechanism. This hypothesis is supported by results from experiments studying the effects of elevating $[K]_o$ on a mutant RHK1 channel (RHK1A3-25), which apparently lacks the N-type and C-type inactivation mechanisms. (Circulation Research 1992;71:657-672)

KEY WORDS  •  Xenopus oocytes  •  A-type potassium channel  •  extracellular potassium accumulation
  •  molecular cloning  •  cDNA expression  •  site-directed mutagenesis

Changing $[K]_o$ can alter currents through potassium channels by varying the reversal potentials and open-channel current–voltage (I-V) relations. Moreover, elevating $[K]_o$ has been shown to cause an increase in the conductances of potassium channels. A shift in the voltage dependences of potassium channel gating, and alterations in the kinetics of potassium channel deactivation, inactivation, or recovery from inactivation. These actions of $K_+ ions on K channels have been explained by hypothetical interactions between potassium ions and channel-gating apparatus or between potassium ions and channel-blocking ions. For example, it has been postulated that increasing $[K]_o$ can delay channel closing (deactivation) by an interaction between potassium ions inside the channel pore and the channel gate (occupancy hypothesis). Increasing $[K]_o$ may accelerate the recovery of the channel from inactivation by repelling the inactivation gate from the pore. For the inward rectifier potassium channel, the high $K_+$-induced increase in channel conductance and a positive shift in the voltage dependence of channel activation have been accounted for by an ionic repulsion of blocking ions that come from the cytoplasm by $K_+$ ions. Similarly, increasing $[K]_o$ can delay the time course of inactivation of the potassium current in human T lymphocytes possibly by repelling blocking ions from the channel pore. It is conceivable that various potassium channels with different structures may respond differently to an elevation of $[K]_o$. A comparative study of the effects of changing $[K]_o$ on potassium channels with known amino acid sequences and structure–function relations will provide insights into the molecular mechanisms of actions of potassium ions and allow tests for the above hypotheses.

We carried out a systematic study of effects of elevating $[K]_o$ on the function of three potassium channels: RHK1, H-4, and H-37. RHK1 is a mammalian transient outward potassium channel isolated from a rat heart library. H-4 and H-37 are two transient outward potassium channels isolated from the Shaker locus of Drosophila. The amino acid sequences of all three
channels are available.\textsuperscript{17,19} All three channels have six putative transmembrane domains (termed S1–S6), with both N- and C-termini in the cytoplasm.\textsuperscript{17,19} S4 is believed to be the voltage sensor of voltage-gated ion channels.\textsuperscript{20} H-4 and H-37 are identical in the S4 domain, having seven positively charged residues.\textsuperscript{19} There is only one uncharged residue in the S4 domain of RHK1 (isoleucine 446) that is different from that in H-4 or H-37 (valine).\textsuperscript{17} The segment between S5 and S6 (H-5 linker) is believed to form the channel pore-lining region.\textsuperscript{21} This region is highly conserved among the three. The sequence is identical between H-4 and H-37.\textsuperscript{19} There is only one amino acid in RHK1 (lysine 532) that is different from that in H-4 or H-37 (threonine). This residue is located at the outer mouth of the pore.\textsuperscript{17,21} The molecular mechanism of inactivation, on the other hand, is different in these three channels. For H-4, it has been well established that the inactivation is mainly mediated by the cytoplasmic N-terminal structure of the channel\textsuperscript{22} via a “ball-and-chain” model\textsuperscript{23} (N-type inactivation).\textsuperscript{24} Although RHK1 is longer than H-4 in the N-terminal region by 80 amino acids, the inactivation of RHK1 may be mediated by a mechanism similar to that in H-4 (see “Results”). In both channels, the N-termini contain a hydrophobic region followed by a cluster of positively charged residues that are important for the inactivation process. On the other hand, the N-terminus of H-37 does not contain a structural pattern resembling that of RHK1 or H-4.\textsuperscript{19} Therefore, N-type inactivation probably is weak or negligible in H-37.\textsuperscript{25} Recently, another type of inactivation mechanism, the C-type inactivation, has been described for a \textit{Shaker} K channel (\textit{Sh} A).\textsuperscript{24} For this type of inactivation, the sequence close to the C-terminus is important. This mode of inactivation does not seem to involve a cytoplasmic domain. Instead, a residue in the S6 transmembrane domain close to the extracellular surface has been shown to play a crucial role in the C-type inactivation.\textsuperscript{24} A valine in this position (as in \textit{Sh} A) makes the C-type inactivation develop faster and the recovery from it slower than if an alanine occupies this position.\textsuperscript{24} H-37 and \textit{Sh} A share the same sequence in the transmembrane domains and the C-terminal region.\textsuperscript{19} Therefore, inactivation in H-37 may be mediated mainly by the C-type mechanism. H-4 and RHK1 both have alanines in the S6 position.\textsuperscript{17,19} Consequently, C-type inactivation may not be important for these two channels. The goal of the present study was to compare the effects of elevating \(K_0\) on these three K channels and, based on this comparison and our knowledge of the structure–function relations of these channels, to infer the mechanism of action of \(K_0\) ions.

In the heart, the transient outward potassium current plays several roles in influencing the cardiac electrical activity.\textsuperscript{26} First, in many regions of the heart, this current is important for phase 1 repolarization of the action potential.\textsuperscript{26,27} Second, by setting the voltage of early plateau phase, this current may influence action potential duration\textsuperscript{26,28} and thus cardiac contractility\textsuperscript{29} by affecting the activation and inactivation of other plateau currents. Under abnormal conditions when the cell membrane is partially depolarized, the magnitude and time course of the transient outward current may affect the rate of impulse initiation\textsuperscript{29} or conduction.\textsuperscript{31} Therefore, it is important to understand the modulation of the cardiac transient outward potassium channel by pathological conditions, such as \(K_0\) accumulation.\textsuperscript{32} RHK1 is almost identical in sequence to a potassium channel cDNA isolated from a human heart library.\textsuperscript{33} Therefore, studies on RHK1 may bear relevance to an understanding of the physiology and pathology of the human heart. Our observations of the effects of elevating \(K_0\), on the properties of RHK1 suggest that under abnormal conditions when there is \(K_0\) accumulation, the cardiac transient outward current may play an important role in determining the cardiac electrical activity due to the acceleration of recovery of this current from inactivation. This, in conjunction with a diminished sodium current, may further impair impulse conduction, which may be antiarrhythmic or arrhythmogenic, depending on the balance of the conditions.

**Materials and Methods**

**Potassium Channel cDNA In Vitro Transcription and Oocyte Expression**

The cDNA sequences of RHK1,\textsuperscript{17} H-4, and H-37\textsuperscript{19} in pBluescript vector (Stratagene Inc., La Jolla, Calif.) were used to prepare DNA templates for in vitro cRNA synthesis. The plasmid vector contains polycistronic sites surrounded at two ends by T7 and T3 RNA polymerase promoters and initiation sites. For each of the cDNAs, the T7 promoter precedes the 5' end; therefore, T7 RNA polymerase (Pharmacia) was used to generate the full-length cRNA. Supercil plasmid DNA was digested with \textit{Hindlll} (a restriction enzyme site in the polycistronic region 3' downstream from the cDNA insert) and further treated with 200 μg/ml proteinase K (Boehringer Mannheim Corp., Indianapolis, Ind.) for over 3 hours at 37°C to remove any RNase contamination. The DNA was then extracted with phenol/chloroform and precipitated with 0.3 M sodium acetate and ethanol. The transcription reaction was carried out in a mixture of the following composition: 40 mM Tris HCl (pH 7.5), 6 mM MgCl\(_2\), 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 30 units RNase inhibitor, 1 μCi [α-\textit{32P}]-UTP, 100 μM GTP, 500 μM each of ATP, UTP, and CTP, and cap analogue m^7GpppG (Promega Corp., Madison, Wis.), 2 μg linear template DNA, and 5 units/μl T7 RNA polymerase. After incubation at 37°C for 120 minutes, 2 units Dnase I (Promega) was added, and incubation was continued at 37°C for another 15 minutes to destroy the template. The cRNA product was extracted with phenol/chloroform and precipitated with 0.3 M sodium acetate and ethanol. For each transcription reaction, the quality of the cRNA product was checked by denaturing agarose gel electrophoresis and autoradiography. The cRNA was resuspended in 10 μl RNase-free water for microinjection.

\textit{Xenopus} oocytes were released from follicular cell layers by collagenase treatment.\textsuperscript{34} Four to 6 hours after isolation, oocytes were microinjected with a cRNA solution at 40–50 nl each. The oocytes were incubated at 18°C in an ND96 solution containing (mM) NaCl 96, KCl 2, CaCl\(_2\) 1.8, MgCl\(_2\) 1.0, HEPES 5, and sodium pyruvate 2.5 (pH 7.5 with NaOH), which was supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Membrane currents were studied 2–6 days after injection.
FIGURE 1. Graphs showing the effects of elevating \([K]_o\) on the peak current–voltage relations (top panels) and the apparent activation curves (bottom panels) of three potassium channels: RHK1 (left), H-4 (middle), and H-37 (right). To construct the peak current–voltage relation, depolarization steps from a holding voltage of −80 mV (RHK1 and H-4) or −100 mV (H-37) to different test voltages \((V_t)\) for 400 msec (RHK1 and H-37) or 80 msec (H-4) were applied at an interpulse interval of 15 seconds (RHK1 and H-37) or 5 seconds (H-4). Current tracings were leak-subtracted using scaled current tracings induced by a step from −80 or −100 mV to −60 mV (below the channels’ activation thresholds) as templates. The amplitude of the peak current \((I_{peak})\) was measured as the difference between the outward peak and the holding current level and plotted against \(V_t\). The peak chord conductance at each \(V_t\) (g) was calculated by dividing \(I_{peak}\) by the driving force: \(g=I_{peak}/(V_t-EK)\), where \(E_K\) is the potassium equilibrium potential. The method of estimating \(E_K\) for each of the oocytes is described in “Materials and Methods.” The activation curve was constructed by dividing g at each \(V_t\) by the peak conductance at +70 mV (gmax) and plotted against \(V_t\). Shown in the bottom panels are data points superimposed on curves calculated from single-sigmoidal (H-4 and H-37) or double-sigmoidal (RHK1) functions with the best-fit values of half-maximum activation voltages \((V_{0.5})\) and slope factors \((k)\); for the single-sigmoidal function, \(g/g_{max}=1/[1+\exp(V_{0.5}-V_t)/k]\); for the double-sigmoidal function, \(g/g_{max}=A_1/[1+\exp(V_{0.5,1}-V_t)/k_1]+A_2/[1+\exp(V_{0.5,2}-V_t)/k_2]\), where \(A\) denotes the fraction of channels activated in the negative \((1)\) or positive \((2)\) voltage range. For H-4, the \(V_{0.5}\) and \(k\) are −5.8 and 13.1 mV at 2 mM \([K]_o\) and −16.6 and 10.1 mV at 20 mM \([K]_o\). For H-37, the \(V_{0.5}\) and \(k\) are −15.6 and 9.4 mV at 2 mM \([K]_o\) and −20.4 and 8.2 mV at 20 mM \([K]_o\). For RHK1, the following parameter values fit the data best: at 2 mM \([K]_o\), \(A_1=0.48, V_{0.5,1}=-26.6\) mV, \(k_1=8.6\) mV, \(A_2=0.52, V_{0.5,2}=+24.6\) mV, and \(k_2=19.7\) mV; at 20 mM \([K]_o\), \(A_1=0.38, V_{0.5,1}=-3.1\) mV, \(k_1=5.5\) mV, \(A_2=0.42, V_{0.5,2}=+9.8\) mV, and \(k_2=16.9\) mV.

In Vitro Mutagenesis

Site-directed in vitro mutagenesis was carried out after a modified procedure of Kunkel.35 RHK1 cDNA cloned into pBluescript M13 phagemid vector (Stratagene) was transformed into BW313 host E. coli cell line (dut· ung·) and used for the preparation of uridine-incorporated single-stranded template DNA.36 Oligonucleotide designed with RHK1Δ3-25 mutation was synthesized with a DNA synthesizer (model 380B, Applied Biosystems, Inc., Foster City, Calif.) and purified with high-performance liquid chromatography (series II 1090 liquid chromatograph, Hewlett-Packard Co., Palo Alto, Calif.). The oligonucleotide sequence, 5’ CCCAAAACCTACCCACCAGGAGGAGCCTGAGAGAAGG3’, harbored a deletion mutation that removed amino acid residues 3-25 from the N-terminus of RHK1. The circular single-stranded template DNA (0.1 pmol) and kinased oligonucleotide (2 pmol) were mixed in 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, and 50 mM NaCl, denatured by heating to 95°C, and then renatured with a slow equilibration back to the room temperature. The reaction mixture was adjusted to 400 µM each of dATP, dGTP, dCTP, and dTTP, 25 mM Tris-HCl (pH 7.9), 15 mM MgCl₂, 25 mM NaCl, 10 mM dithiothreitol, and 0.25 mM ATP, in a total volume of 20 µl. DNA synthesis reaction was started by adding 3 units T4 DNA polymerase and 2 units T4 DNA ligase. The tube was kept on ice for 5 minutes, then at room temperature for 10 minutes, and finally incubated in 37°C overnight. An aliquot of the reaction mixture was used to transform the dut· ung· DH5α host strain. Miniplasmid DNAs were prepared from six transformed colonies. The mutation site and surrounding sequences were checked with a restriction band shift and double-stranded DNA sequencing. Four of the six transformants contained the correct deletion, and among the four, two independent mutants were used for cRNA synthesis. The results were the same from these two cRNAs, thus minimizing the likelihood of interference from random mutations derived from errors by T4 DNA polymerase.

Electrophysiological Experiments

The oocytes were placed in a tissue chamber and superfused at room temperature (21–23°C) with ND96 solution at a rate of approximately 3 ml/min. Membrane currents were studied using the conventional two-microelectrode voltage-clamp technique with an AxoClamp-2A.
amplifier (Axon Instruments, Foster City, Calif.). Both voltage and current electrodes were filled with 3 M KCl solution and had tip resistances of 1–2 MΩ. A grounded shield was placed between the two electrodes to decrease capacitance coupling. During the experiments, the external solution was changed to a modified calcium-free ND96 solution (CaCl₂ was replaced by MgCl₂) to minimize the interference from an endogenous calcium-activated chloride current.37 In solutions containing 20 and 40 mM [K]₀, the concentration of NaCl was reduced to 78 and 58 mM, respectively, to maintain the osmolarity. In solutions containing 2 mM [K]₀ and 78 mM [Na]₀, 18 mM choline chloride or 32 mM sucrose was added to substitute for NaCl.

Voltage-clamp protocol generation and data acquisition were controlled by an IBM/AT-compatible computer through PCLAMP software (Axon Instruments) via a 12-bit digital-to-analog and analog-to-digital converter (Axon Instruments). Data were filtered at 2 kHz with an eighth-pole Bessel filter (Frequency Devices Inc., Haverhill, Mass.), digitized on-line at a sampling interval of 0.08 msec (H-4) or 0.5 msec (RHK1 and H-37), and stored on disk for off-line analysis. The voltage-clamp protocols and data analyses will be described in the corresponding figure legends. Three methods were used for curve fitting. The first method used the program PEAKFIT (Jandel Scientific, Corte Madera, Calif.) to fit the activation and inactivation curves with a simple Boltzmann (single-sigmoidal) or double-sigmoidal function. The second method used the program CLAMPFIT (part of PCLAMP) to fit the time courses of current decay during depolarizations with single- or double-exponential functions. The time course of channel recovery from inactivation (restoration) was fit with a single- or double-exponential function using a simplex algorithm.38 Wherever applicable, the data were averaged and presented as mean±SD. Statistical significance was tested by paired or unpaired Student’s t test.

In the calculation of chord conductances (see Figure 1 legend), we needed to estimate the reversal potential of the channels. The reversal potentials were approximated by the potassium equilibrium potential (Eₖ) for all three channels, based on their high selectivities for potassium ions.17,18 Since variations in [K]₀ among oocytes might occur even in the same batch of oocytes because of differences in cellular conditions and Na⁺-K⁺ pump activity, it was of importance to determine the Eₖ for each of the oocytes studied. We made an indirect estimation of Eₖ in the following way. For each oocyte, we first estimated the Eₖ at 40 mM [K]₀, by the resting membrane potential at this level of [K]₀, taking advantage of the fact that at such a high [K]₀, the resting membrane conductance was dominated by potassium conductance through endogenous potassium channels.39,40 This was tested in eight oocytes expressing RHK1. Eₖ was directly determined and compared with the resting membrane potential at 40 mM [K]₀. The two differed by 3±2 mV (measured Eₖ was more negative than the resting membrane potential). Therefore, at 40 mM [K]₀, Eₖ was estimated to be 3 ± 2 mV negative to

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**Table 1. Effects of Elevating [K]₀ on the Properties of Potassium Channels**

<table>
<thead>
<tr>
<th>Channel</th>
<th>Peak chord conductance</th>
<th>Voltage dependence of activation</th>
<th>Voltage dependence of steady-state inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 mM [K]₀</td>
<td>40 mM [K]₀</td>
<td>20 mM [K]₀</td>
</tr>
<tr>
<td></td>
<td>g20/g2</td>
<td>g40/g2</td>
<td>g20/g2</td>
</tr>
<tr>
<td>RHK1</td>
<td>1.85±0.42*</td>
<td>2.16±0.57*</td>
<td>10</td>
</tr>
<tr>
<td>H-4</td>
<td>1.37±0.19*</td>
<td>1.41±0.35*</td>
<td>11</td>
</tr>
<tr>
<td>H-37</td>
<td>1.16±0.16*</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Voltage dependence of activation

<table>
<thead>
<tr>
<th>2 mM [K]₀</th>
<th>20 mM [K]₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>V₀.5 (mV)</td>
<td>k (mV)</td>
</tr>
<tr>
<td>RHK1</td>
<td>33.7±6.1</td>
</tr>
<tr>
<td>H-4</td>
<td>-6.0±4.5</td>
</tr>
<tr>
<td>H-37</td>
<td>-9.9±6.0</td>
</tr>
</tbody>
</table>

Voltage dependence of steady-state inactivation

<table>
<thead>
<tr>
<th>2 mM [K]₀</th>
<th>20 mM [K]₀</th>
<th>40 mM [K]₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>V₀.5 (mV)</td>
<td>k (mV)</td>
<td>V₀.5 (mV)</td>
</tr>
<tr>
<td>RHK1</td>
<td>-55.0±4.4</td>
<td>3.5±1.0</td>
</tr>
<tr>
<td>H-4</td>
<td>-35.1±1.3</td>
<td>4.1±0.5</td>
</tr>
<tr>
<td>H-37</td>
<td>-35.2±4.7</td>
<td>3.9±3.6</td>
</tr>
</tbody>
</table>

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**Notes:**

- g20/g2 and g40/g2, peak chord conductance (g) measured at 20 and 40 mM [K]₀, respectively, and normalized to g at 2 mM [K]₀; n, number of oocytes studied. For activation properties, V₀.5 is half-maximum activation voltage; k is slope factor (subscript 1 and 2 for RHK1 denote the components activated in negative and positive ranges, respectively); and A₁ is fraction of the former component. Values are mean±SD. g was calculated at +70 mV (see Figure 1 legend).

*tp<0.01 vs. ratio of 1 by one-sided paired t test; tp<0.05 vs. corresponding value at 2 mM [K]₀; and tp<0.02 vs. corresponding value at 2 mM [K]₀.
FIGURE 2. Graphs showing the effects of elevating $[K]_o$ on the voltage dependence of steady-state inactivation of three potassium channels: RHK1 (top panel), H-4 (middle panel), and H-37 (bottom panel). The steady-state inactivation curve was constructed using data obtained from a double-pulse protocol: From a holding voltage of $-80 \text{ mV}$ (RHK1 and H-4) or $-100 \text{ mV}$ (H-37), a conditioning step to different voltages ($V_c$) was applied for 2 seconds, which was followed by a test pulse to $+20 \text{ mV}$ for 500 msec (RHK1 and H-37) or 100 msec (H-4). These double pulses were applied at an interval of 15 seconds (RHK1 and H-37) or 5 seconds (H-4). The current tracing during each of the test pulses was leak-subtracted using a current tracing induced by a test pulse that followed $V_c$ that had totally inactivated the channel. The peak current amplitude was then measured as the difference between the outward peak current and current level at the end of the test pulse ($I_t$). The peak current amplitude was normalized by the maximal peak current amplitude ($I_{\text{max}}$) recorded after a negative $V_c$ that had totally removed inactivation ($I_t/I_{\text{max}}$). The relation between $I_t/I_{\text{max}}$ and $V_c$ was fit with a Boltzmann function to obtain the half-maximum inactivation voltage ($V_{1/2}$) and slope factor ($k$): $I_t/I_{\text{max}} = 1/[1 + \exp((V_c - V_{1/2})/k)]$. Shown in each panel are data points superimposed on curves calculated from the Boltzmann equation using the best-fit values of $V_{1/2}$ and $k$. The values of $V_{1/2}$ and $k$ are the following: for RHK1, $-56.5$ and $4.0 \text{ mV}$ at $2 \text{ mM } [K]_o$, $-52.7$ and $4.0 \text{ mV}$ at $20 \text{ mM } [K]_o$, $-53.2$ and $4.2 \text{ mV}$ at $40 \text{ mM } [K]_o$; for H-4, $-35.1$ and $4.8 \text{ mV}$ at $2 \text{ mM } [K]_o$, $-30.5$ and $5.0 \text{ mV}$ at $20 \text{ mM } [K]_o$, $-29.8$ and $5.7 \text{ mV}$ at $40 \text{ mM } [K]_o$; for H-37, $-36.3$ and $3.1 \text{ mV}$ at $2 \text{ mM } [K]_o$, $-35.9$ and $3.4 \text{ mV}$ at $20 \text{ mM } [K]_o$, $-34.9$ and $3.2 \text{ mV}$ at $40 \text{ mM } [K]_o$.

FIGURE 3. Effects of elevating $[K]_o$ on the time course of current decay during depolarization for three potassium channels: RHK1 (top panel), H-4 (middle panel), and H-37 (bottom panel). Shown are current tracings induced by steps from $-80$ to $+40 \text{ mV}$ (RHK1), from $-80$ to $+60 \text{ mV}$ (H-4) or from $-100$ to $+70 \text{ mV}$ (H-37) and recorded from the same oocytes at various $[K]_o$ ($2, 20,$ and $40 \text{ mM}$, as marked). To facilitate the comparison of time courses of current decay, for each channel the time-dependent components of the current have been scaled to the same amplitude.

The resting membrane potential. The $[K]_o$ was then calculated from the estimated $E_K$ at 40 mM $[K]_o$, using the Nernst equation:

$$E_K = (RT/F) \ln ([K]_o/[K]_i)$$

where $R$ is gas constant, $T$ is temperature in °K, and $F$ is Faraday constant. The values of $E_K$ at 20 and 2 mM $[K]_o$ were then calculated using the same equation and the calculated value of $[K]_o$, assuming that $[K]_i$ did not change significantly during the period of one experiment (30–40 minutes). The difference between calculated $E_K$ and directly measured $E_K$ at 2 and 20 mM $[K]_o$ for RHK1 was small (4±3 and $-1±2 \text{ mV}$, respectively, $n=8$; calculated $E_K$ relative to measured $E_K$). Similar estimation was applied to H-4 and H-37, because it was expected that the endogenous potassium channels were similar among oocytes injected with different cRNAs. The calculated $[K]_o$ values were 127.8±8.8, 106.8±24.1, and 110.3±17.7 for RHK1 ($n=10$), H-4 ($n=11$), and H-37 ($n=11$).

Results

We characterized the effects of elevating $[K]_o$ on the following properties of RHK1, H-4, and H-37: 1) chord
conductance, 2) voltage dependence of activation and inactivation, and 3) kinetics of inactivation and recovery from inactivation. As stated in the introduction, these three channels may share a common mechanism of voltage-dependent activation.\textsuperscript{17,19,20,21} However, inactivation in RHK1 and H-4 may be mediated by an N-type mechanism,\textsuperscript{22} whereas that in H-37 may be mediated by a C-type mechanism.\textsuperscript{24} These may form the basis for similar as well as differential responses of these channels to an elevation in [K\textsubscript{o}].

\textbf{Effects of Elevating [K\textsubscript{o}] on the Chord Conductances and Voltage Dependences of Activation of Potassium Channels}

The peak I-V relations of RHK1, H-4, and H-37 recorded at levels of [K\textsubscript{o}], of 2, 20, and 40 mM are shown in the top panels of Figure 1. For both H-4 and H-37, increasing [K\textsubscript{o}], led to a concentration-dependent reduction of the peak current amplitudes at all test voltages that was readily reversible on restoring [K\textsubscript{o}], to 2 mM. The decrease in outward current on elevating [K\textsubscript{o}], was mainly due to a reduction in the driving force for potassium efflux through the channels. For RHK1, the peak current amplitudes at 20 and 40 mM [K\textsubscript{o}], exceeded that at 2 mM [K\textsubscript{o}], at voltages positive to −20 and 0 mV, respectively, thus causing a “crossover” of the peak I-V relations. The most likely explanation for such a crossover phenomenon is that the conductance of the potassium channel is increased by raising [K\textsubscript{o}], and the increase in channel conductance outweighed the reduction in driving force at positive voltages, leading to a net increase in current amplitude. Elevating [K\textsubscript{o}], also increased the channel conductances of H-4 and H-37 (Table 1). The difference in the high K\textsubscript{o}-induced changes in the peak I-V relations seen in the top panels of Figure 1 (a crossover in RHK1 but not in H-4 or H-37) suggested that the degree of increase in channel conductance was greater in RHK1 than in H-4 or H-37 or that RHK1 had a lower dissociation constant (K\textsubscript{d}) for the effect of K\textsubscript{o} ions on the single-channel conductance than the K\textsubscript{d} for the effect of K\textsubscript{o} ions on H-4 or H-37. The effect of increasing [K\textsubscript{o}], on the channel conductance was examined by determining the peak chord conductances at different levels of [K\textsubscript{o}]. Elevating [K\textsubscript{o}], caused a concentration-dependent increase in channel conductance (Table 1). The degree of increase was higher for RHK1 than for H-4 or H-37, consistent with the data shown in the top panels of Figure 1. However, inference of changes in single-channel conductance from calculated chord conductance based on whole-cell current measurement may be subject to errors (see “Discussion”).

We investigated the effects of elevating [K\textsubscript{o}], on the peak I-V relations of the channels. The results showed an apparent increase in channel conductance; however, Pardo et al\textsuperscript{42} have suggested an effect of [K\textsubscript{o}], on the number of channels. Shown in the bottom panels of Figure 1 are the activation curves of these three channels at 2 and 20 mM [K\textsubscript{o}], (for construction of the activation curve, see the Figure 1 legend). The functions needed to fit the activation curves of these potassium channels differ: the activation curves of H-4 and H-37 could be fit well with a simple Boltzmann (single-sigmoidal) function,\textsuperscript{18,43} whereas the activation curves of RHK1 required a double-sigmoidal function (see Figure 1 legend for equations used for curve fitting). Some possible explanations for this behavior of RHK1 will be presented in “Discussion.” Elevating [K\textsubscript{o}], from 2 to 20 mM caused a negative shift in the activation curves of all three channels, with the amount of shift larger in RHK1 and H-4 than in H-37. Data are summarized in Table 1. For H-4 and H-37, elevating [K\textsubscript{o}], caused a negative shift in the half-maximum activation voltages and a reduction in the slope factors of the simple Boltzmann fits. For RHK1, elevating [K\textsubscript{o}], increased the fraction of the component in the double-sigmoidal function whose activation occurred in the negative voltage range. There was also a negative shift in the half-maximum activation voltages of both components, although these changes were not statistically significant.
From these observations, we conclude that elevating \([K]\)\textsubscript{o} induced qualitatively similar changes in channel conductances and activation curves of all three potassium channels. However, in the following channel properties that are related to the inactivation process, elevating \([K]\)\textsubscript{o} induced different effects on these three channels that could be divided into two patterns: RHK1 and H-4 were similar, but the two were different from H-37.

Effects of Elevating \([K]\)\textsubscript{o} on the Voltage Dependences of Steady-State Inactivation of Potassium Channels

The effects of increasing \([K]\)\textsubscript{o} on the channels' voltage dependences of inactivation were evaluated by constructing the steady-state inactivation curves at different levels of \([K]\)\textsubscript{o}, using a double-pulse protocol. The voltage-clamp protocol and data analysis are described in the legend of Figure 2. For both RHK1 and H-4, raising \([K]\)\textsubscript{o} from 2 to 20 mM caused a positive shift in the half-maximum inactivation voltage without a significant change in the slope factor. Elevating \([K]\)\textsubscript{o} to 40 mM induced very little further changes (Figure 2). These changes in RHK1 and H-4, although small, were consistent among oocytes and were statistically significant (Table 1). The steady-state inactivation curves of H-37 recorded at different levels of \([K]\)\textsubscript{o}, on the other hand, were almost superimposable (Figure 2, bottom panel). Data are summarized in Table 1.

Effects of Elevating \([K]\)\textsubscript{o} on the Rate of Channel Inactivation During Depolarization

For RHK1 and H-4, increasing \([K]\)\textsubscript{o} did not alter the time course of current inactivation (decay) during depolarization (Figure 3, top and middle panels; see also Figure 5). Similar observations were obtained by Demo and Yellen\textsuperscript{15} in their study on the effects of raising \([K]\)\textsubscript{o} on H-4. On the other hand, increasing \([K]\)\textsubscript{o} slowed the time to reach peak current and time course of decay of H-37 in a concentration-dependent manner (Figure 3, bottom panel; see also Figure 5).

To quantify the prolonging effect of high \([K]\)\textsubscript{o} on H-37, the time constants of decay of H-37 at different levels of \([K]\)\textsubscript{o} over a wide voltage range were measured. Data from a representative experiment are shown in the top panel of Figure 4. The time course of decay of H-37 could be adequately fit with a single-exponential function (inset in the top panel of Figure 4). Elevating \([K]\)\textsubscript{o} caused a consistent increase in the time constant of decay at all voltages (from −20 to +70 mV). This

![Figure 5](http://circres.ahajournals.org/)

**FIGURE 5.** Effects of elevating \([K]\)\textsubscript{o} on the rate of recovery from inactivation (restitution) of three potassium channels: RHK1 (panel A), H-4 (panel B), and H-37 (panel C). A double-pulse voltage-clamp protocol was used to measure the restitution time course: From a holding voltage of −80 mV (RHK1 and H-4) or −100 mV (H-37), double pulses each to +20 mV for 50 msec (H-4) or 500 msec (RHK1 and H-37) with a varying interpulse interval were applied every 15 seconds. The currents during the first pulse and the second pulse were leak-subtracted using a template created by the same series of voltage steps except that the depolarization pulses were to −60 mV (below the channels' activation thresholds). The amplitudes of the time-dependent components were measured. The difference in the amplitude between the first pulse and the second pulse was normalized by the amplitude of the first pulse (percent inactivated), and its relation to the interpulse interval (restitution time course) was studied. In the left part of each panel, superimposed original current tracings during the first and the second pulses at 2 mM (top tracing), 20 mM (middle tracing), and 40 mM (bottom tracing) \([K]\)\textsubscript{o} are illustrated. To facilitate comparison of the restitution time courses, the current tracings during the first pulses were scaled to the same amplitude. Note also that the time scale in panel B is different from those in panels A and C: double pulses lasted 50 msec for H-4 but 300 msec for RHK1 and H-37. The right part of each panel shows a semilogarithmic plot of the restitution time courses. Data points are superimposed on lines calculated from a single-exponential function with the best-fit values of time constants. The time constants of restitution at 2, 20, and 40 mM \([K]\)\textsubscript{o} are 1,949, 438, and 313 msec for RHK1; 53.7, 33.3, and 28.8 msec for H-4; and 999, 1,492, and 2,291 msec for H-37, respectively.
The prolonging effect was completely reversible after restoring [K], to 2 mM. The time constant of decay of H-37 was increased by 50–80% and 100–140% on elevating [K], from 2 to 20 and 40 mM, respectively (n=7, bottom panel of Figure 4). The plot in the lower panel of Figure 4 also shows that the high K,-induced prolongation of H-37 displayed very little voltage dependence: a voltage shift of 355 mV is required for an e-fold change in the degree of prolongation. Assuming that one potassium ion acts on each channel, this voltage dependence indicates that the site of potassium ion action in the H-37 channel is located at an electrical distance of only 0.07 from the extracellular surface.

**Effects of Elevating [K]o on the Time Course of Recovery From Inactivation (Restitution) of Potassium Channels**

The time course of restitution was evaluated by a double-pulse protocol (see Figure 5 legend). Using this voltage-clamp protocol, the restitution time course of RHK1 at 2, 20, and 40 mM [K], could be adequately fit with a single-exponential function, as exemplified on the right side of Figure 5A. The restitution was accelerated on elevating [K],. The average time constants of restitution of RHK1 at 2, 20, and 40 mM [K], were 2.2±0.6, 0.7±0.4, and 0.4±0.2 seconds (n=6). For H-4, the time course of restitution at all three levels of [K], could also be adequately fit with a single-exponential function (right side of Figure 5B). The time constants of restitution became shorter as [K], was increased. The average restitution time constants at 2, 20, and 40 mM [K], were 51±17, 30±3, and 25±4 msec (n=8). For H-37, there was a delay in the onset of restitution during the initial 400 msec after the first depolarization pulse. Therefore, the time course of restitution was characterized by fitting data points obtained at interpulse intervals of ≥400 msec with a single-exponential function (right side of Figure 5C). In contrast to RHK1 and H-4, increasing [K], from 2 to 20 and 40 mM caused a concentration-dependent slowing of the restitution of H-37. The average restitution time constants were 796±181, 1,029±327, and 1,508±708 msec at 2, 20, and 40 mM [K],, respectively. The plot in the top panel of Figure 6 compares the responses of these three potassium channels to an elevation of [K], in their restitution time courses. H-4 and RHK1 are similar in that their restitution time courses were accelerated when [K], became higher. However, the degree of acceleration induced by the same amount of [K], elevation was higher for RHK1 than for H-4. The restitution of H-37 was slowed by increasing [K],, a response opposite those of the other two.

The accelerating effect of high K, on the restitution of RHK1 and H-4 displayed a profound voltage dependence (bottom panel of Figure 6). This was similar between RHK1 and H-4: high K,-induced acceleration was greater at a holding potential (Vh) of −100 mV and less at Vh of −60 mV, relative to that at Vh of −80 mV. This voltage dependence corresponded to a relation of 58.8-mV voltage shift per e-fold change in the degree of acceleration. Assuming that potassium ions worked on the channels on a one-to-one basis, this observation suggests that the sites of potassium ion action in RHK1 and H-4 are at an electrical distance of 0.43 from the extracellular surface, i.e., about halfway in the transmembrane electrical field. There was no significant voltage dependence in the slowing effect of high K, on the restitution of H-37. Elevating [K], from 2 to 20 mM decreased the rate of restitution of H-37 by a factor of

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Panel A: Graph summarizing the effects of elevating [K], on the restitution time courses of three potassium channels: RHK1, H-4, and H-37. Data are taken from six, eight, and five experiments for RHK1, H-4, and H-37, respectively. The voltage-clamp protocol and data analysis used to obtain the restitution time constants were as described in Figure 5. To facilitate the comparison of changes in the restitution time constants among potassium channels, the time constants obtained at 20 and 40 mM [K], were normalized to that at 2 mM [K], (normalized restitution time constant) and averaged. Shown are means with standard deviation bars (for the data point of RHK1 at 40 mM [K],, the standard deviation bar is smaller than the symbol size). The lines and errors are added for the sake of clarity. Panel B: Graph showing the voltage dependence of the accelerating effect of elevating [K], on the restitution of RHK1 and H-4. For each of the experiments, the oocyte was first superfused with the 2 mM [K], solution, and the time courses of restitution at a control holding voltage (Vh) of −80 and a test Vh of −100 or −60 mV were measured. The [K], was then raised to 20 or 40 mM, and the time courses of restitution at the same levels of Vh were measured again. The ratio of restitution time constants at 2 mM [K], to that at 20 or 40 mM [K], was calculated for each Vh. The ratio at the test Vh was then normalized to the ratio obtained at the control Vh (normalized ratio of restitution time constants), averaged, and plotted against Vh on a semilogarithmic scale. Data are taken from four measurements of RHK1 at Vh of −100 mV (○), 1.33±0.14, two measurements of RHK1 at Vh of −60 mV (△, mean with range), four measurements of H-4 at Vh of −60 mV (□, 0.72±0.22), and one measurement of H-4 at Vh of −100 mV (×). The solid line represents the relation of a 58.8-mV shift per e-fold change in the high K,-induced acceleration of restitution.
hydrophobic region\(^2\) (Figure 7A). Furthermore, this mutant channel is similar to the wild-type channel in having very weak or negligible C-type inactivation because of the alanine in the S6 domain.\(^2\) If the responses of RHK1 and H-4 were due to an interaction between \(K_o\) ions and the N-type inactivation gating apparatus, these responses would be diminished in RHK1\(^\Delta3\)-25. If the responses of H-37 to changing \(K_o\) were due to a lack of the N-type inactivation, the responses of RHK1\(^\Delta3\)-25 would resemble those of H-37. On the other hand, if the responses of H-37 to raising \(K_o\) were due to an interaction between \(K_o\) ions and the C-type inactivation gating apparatus, then RHK1\(^\Delta3\)-25 would be different from H-37 in its responses to changing \(K_o\). Moreover, we evaluated the effects of increasing \(K_o\) on the chord conductance and voltage dependence of activation of RHK1\(^\Delta3\)-25. If the channels' responses in these aspects were independent of the N- or C-type inactivation (as suggested by the similarities in the effects of raising \(K_o\)) on these two properties of RHK1, H-4, and H-37; Figure 1 and Table 1), the responses of RHK1\(^\Delta3\)-25 should be similar to those of the other channels.

**Effects of Elevating \(K_o\) on the Properties of an RHK1 Mutant Channel (RHK1\(^\Delta3\)-25) That Lacks N-Type and C-Type Inactivation**

Figure 7 shows that elevating \(K_o\), from 2 to 20 mM induced changes in the activation curve of RHK1\(^\Delta3\)-25 similar to those seen in the wild-type channel. Moreover, such a change in \(K_o\), caused an increase in the peak chord conductance at +70 mV by a factor of 1.7, similar to that seen in the wild-type channel. Similar observations were obtained in four other experiments. Elevating \(K_o\) did not cause any shift in the voltage dependence of inactivation of RHK1\(^\Delta3\)-25 (n=3, Figure 8), a response different from that seen in the wild-type channel. Although RHK1\(^\Delta3\)-25 did not show an appreciable decay during depolarization for a short duration (comparable to that used in the experiments on wild-type channel, Figure 7A), its current did decline with a complicated time course when the depolarization was prolonged (Figure 9). Elevating \(K_o\) had minimal effects on the initial decay phase of RHK1\(^\Delta3\)-25 (<5 seconds) but accelerated the late phase of decay (n=4, Figure 9). This response of RHK1\(^\Delta3\)-25, therefore, was different from those of the other three channels. Finally, elevating \(K_o\) did not induce any appreciable changes in the restitution time course of RHK1\(^\Delta3\)-25 (n=6; one example is shown in Figure 10). This response of RHK1\(^\Delta3\)-25 again was different from those of the other three channels.

Our observations therefore suggest that the high \(K_o\)-induced positive shift in the steady-state inactivation curve and acceleration of the restitution in RHK1 depend on an intact N-type inactivation mechanism in this channel. Destroying the N-type inactivation in RHK1 by specific mutagenesis abolished the effects of high \(K_o\) on these properties of the channel. The same may apply to H-4. The effects of increasing \(K_o\) on H-37, i.e., a slowing of the development of and recovery from inactivation, were not mimicked by RHK1\(^\Delta3\)-25, which lacks the C-type inactivation. These results suggest that these responses of H-37 to changing \(K_o\), are due to an efficient C-type inactivation mechanism in this...
channel. On the other hand, the effects of changing [K]o on the channels' chord conductances and voltage dependences of activation do not seem to depend on either N- or C-type inactivation.

Observed Effects of Elevating [K]o on RHK1 Were Not Induced by the Simultaneous Reduction in [Na]o

It has been reported that reducing [Na]o can alter the transient outward current in rat ventricular myocytes.\(^45\) Moreover, in neurons\(^46\) and guinea pig ventricular myocytes,\(^47\) an intracellular sodium-activated potassium channel has been described that may display a time course similar to a transient outward current.\(^48\) Since there was a simultaneous reduction in [Na]o in the high [K]o solutions used in the experiments, this change in [Na]o, or possibly a subsequent decrease in [Na]o might partially account for the changes we observed in high [K]o solutions. We tested this possibility by examining the effects on RHK1 of lowering [Na]o, without a simultaneous elevation in [K]o (NaCl substituted by choline chloride or sucrose) and comparing them with those

![Figure 8](image)

**Figure 8.** Lack of effects of elevating [K]o on the voltage dependence of inactivation of the mutant RHK1 channel (RHK1Δ3-25). Panel A: Original current tracings at 2 and 20 mM [K]o during both the conditioning pulses (Vc) and the test pulses. The holding voltage was −80 mV. The Vc lasted 5 seconds and ranged from −70 to −30 mV (marked next to the current tracings during the test pulses). The test pulse was to +20 mV for 2.5 seconds. Note that the current tracings during Vc and the test pulses are not shown on the same time scale and that channel inactivation was not complete during either Vc or the test pulses. The cycle length was 15 seconds. Current tracings during the test pulses were leak subtracted, and the peak current amplitudes were determined as the differences between the outward peaks and the zero current level. Panel B: Inactivation curves of RHK1Δ3-25 at the two levels of [K]o. The data are superimposed on curves calculated from the modified Boltzmann equation: \(I/I_{\text{max}} = A_0/[1+\exp(V_c-V_{0.5})/k] + (1-A_0)\), where \(I\) is the current at the test pulse, \(I_{\text{max}}\) is the maximal \(I\), \(A_0\) is the fraction of inactivated channels, \(V_{0.5}\) is half-maximum inactivation voltage, and \(k\) is the slope factor. The values of parameters are the following: for 2 mM [K]o, \(A_0=0.64\), \(V_{0.5}=-48.7\) mV, and \(k=3.6\) mV; for 20 mM [K]o, \(A_0=0.67\), \(V_{0.5}=-48.0\) mV, and \(k=3.9\) mV.

![Figure 9](image)

**Figure 9.** Comparison of the decay time courses of the mutant RHK1 channel (RHK1Δ3-25) at 2 and 20 mM [K]o. The currents were induced by test pulses from a holding voltage of −80 mV to 0 mV for 50 seconds. To facilitate comparison, the time-dependent components of the current tracings have been scaled to matched amplitudes.

![Figure 10](image)

**Figure 10.** Lack of effects of elevating [K]o on the restitution of the mutant RHK1 channel (RHK1Δ3-25). Panel A: Original current tracings during the first and second pulses at 2 and 20 mM [K]o. The holding voltage was −80 mV. Both the first and the second pulses were to 0 mV. The first pulse lasted 5 seconds, and the second pulse lasted 2.5 seconds. The interpulse interval was varied from 250 to 4,000 msec. The interval between double pulses was 30 seconds. Current amplitude was measured as the difference between the holding current and the peak outward current. Panel B: Restitution time course of RHK1Δ3-25 plotted on a semilogarithmic scale. The format of the plot is the same as those shown in Figure 5.
seen when \([Na]\), was lowered along with an elevation in \([K]\), (as in our experimental conditions). Figure 11 illustrates the results from this experiment. It is clear that only elevating \([K]\) could increase the peak chord conductance and that it caused a negative shift in the activation curve of RHK1 (Figure 11A). Reducing \([Na]\), without an elevation of \([K]\), caused a small but discernable and reversible negative shift in the steady-state inactivation curve of RHK1, opposite to the positive shift seen when \([K]\), was increased along with a reduction of \([Na]\), (Figure 11B). Elevating \([K]\), but not reducing \([Na]\), could accelerate the restitution of RHK1 (Figure 11C). These observations suggest that the effects on the properties of RHK1 we observed were mediated by \(K\) ions.

**Discussion**

**Consideration of the Experimental Design and Data Analysis**

*Xenopus* oocytes have endogenous ion channels that need to be suppressed for an unambiguous observation of implanted exogenous channels. A nominally calcium-free external solution, supplemented with magnesium, was used in the experiments to minimize the interference from the endogenous calcium-activated chloride current.37 Indeed, with \([Ca]\), of 1.8 mM, there was a prominent transient outward current in the voltage range positive to \(-10\) mV that was sensitive to external chloride removal (substituted by methanesulfonate, data not shown). After removing \(Ca\),, this current component was abolished. Under these conditions, depolarization pulses from a holding voltage of \(-80\) or \(-100\) mV to test voltages up to \(+70\) mV induced only small currents (\(\leq 100\) nA at \(+70\) mV) that were largely time independent. The test voltage in these experiments was limited to \(+70\) mV to avoid interference from an endogenous delayed rectifier-like current, which is activated by depolarizations to \(+80\) mV or more positive voltages.

The activation curve of RHK1 is fit better with a double-sigmoidal function than with a single-sigmoidal (simple Boltzmann) function (Figure 1). This was a consistent observation of RHK1 and unusual compared with other voltage-gated ion channels (however, see Reference 48). We considered several possibilities of experimental error that might account for such a behav-

The values for the half-maximum inactivation voltage (\(V_{1/2}\)) are as follows: control \(ND96\), \(-60.6\) mV; low sodium (choline chloride) with \(2\) mM \([K]\), \(-63.6\) mV; control \(ND96\), \(-56.2\) mV; low sodium (sucrose) with \(2\) mM \([K]\), \(-59.9\) mV; control \(ND96\), \(-53.6\) mV; low sodium with \(20\) mM \([K]\), \(-50.7\) mV. The slope factors were similar (3.1–3.4). For the sake of clarity, the inactivation curves have been shifted along the voltage axis so that the values of \(V_{1/2}\) of curves recorded in the control solution (\(ND96\)) coincide with the averaged control \(V_{1/2}\) of \(-56.8\) mV (abscissa was thus marked as “normalized \(V_{1/2}\”). Since the three control curves are superimposable, only the one with open circles is shown. Panel C: Elevating \([K]\), but not reducing \([Na]\), could enhance the restitution of RHK1. The voltage-clamp protocol and data analysis were the same as those described for Figure 5.
ior of RHK1. First, it might arise from a poor voltage control that was due to large current amplitudes of RHK1. This was not likely because similar activation curves were obtained in RHK1-expressing oocytes with maximal peak current amplitudes ranging from 1 to 6 μA. Furthermore, the current amplitude in the experiments shown in Figure 1 was smaller in RHK1 than in H-4 and H-37. Second, it could be due to interference from channel inactivation. This was unlikely because H-4 and H-37 are also inactivating currents. More importantly, a mutant RHK1 channel that lost the N-type inactivation, RHK1Δ3-25, still required a double-sigmoidal function to fit its activation curve (n=6, Figure 7). Other possibilities included errors introduced by an inaccurate determination of E_k or a nonlinearity in the open-channel I-V relation of RHK1. At 2 mM $[K]_o$, the open-channel I-V relation of RHK1 showed an outward rectification: the I-V relation is linear in the voltage range positive to −40 mV but has a reduced slope conductance between −40 mV and the reversal potential. Therefore, the activation curve of RHK1 that was calculated based on the chord conductance at 2 mM $[K]_o$ will give rise to an underestimation of the true level of channel activation in the voltage range between the activation threshold (−50 mV) and −40 mV. However, this type of error introduced by using the chord conductance should not occur in the calculation of the activation curve of RHK1 at 20 mM $[K]_o$, since the open-channel I-V relation at this level of $[K]_o$ is linear in the voltage range around and positive to the reversal potential. Furthermore, the possibility that the complicated shape of the activation curve of RHK1 was entirely due to errors introduced by using chord conductance and estimating E_k was made less likely by the results from experiments that compared the activation curve of RHK1 constructed from the peak I-V relation with that from a tail I-V relation (Figure 12). The tail I-V method avoided the assumption of a linearity in the open-channel I-V relation and the determination of E_k. Moreover, since the test pulse in this protocol lasted only 10 msec in the positive voltage range, its voltage could be extended to more positive levels without a serious interference from the endogenous outward current (see above), which developed with a time constant of 100–150 msec at +90 mV. Activation curves generated by these two methods from the same cell were very similar and needed a double-sigmoidal function for a good fit (n=4, Figure 12). Therefore, we conclude that this unusual behavior of RHK1 was not likely to be totally due to experimental errors, and we need to seek further explanations. One straightforward explanation will be that in these RHK1-expressing oocytes there are two populations of channels (or two modes of channel gating) that display widely separated voltage dependences of activation. There have been precedents for the notion that oocytes injected with one species of RNA can generate ion channels with two distinct gating kinetics.49,50 However, in these two studies the difference in channel gating is related to the kinetics of inactivation but not the voltage dependence of activation, as is the possible mechanism proposed here for RHK1. The second possibility involves membrane voltage-dependent changes in charge-carrying capability of intracellular cations through the channel pore. For the delayed rectifier potassium channels in squid axons,51 it

**FIGURE 12.** Comparison of the activation curves of the potassium channel RHK1 constructed using two protocols: the peak current–voltage (I-V) relation and the tail I-V relation. $[K]_o$ was 2 mM. The voltage-clamp protocol used to generate the peak I-V relation and the calculation and normalization of the chord conductance were as described in the Figure 1 legend. To generate the tail I-V relation, a test pulse from a holding voltage of −80 mV to various voltages (from −50 to +90 mV) for a duration during which the current reached a peak without appreciable decay (10–20 msec) was followed by repolarization to −60 mV. The tail currents were fit with a single-exponential function. The instantaneous tail current amplitude was determined by extrapolating the current to time zero and normalized by the maximum tail current amplitude induced by the +90-mV pulse. The relation between the normalized chord conductance or the normalized tail current amplitude (fraction activated) and the test voltage ($V_t$) was fit with the single- and double-sigmoidal functions given in the Figure 1 legend. Panel A: Families of original current tracings induced by the peak I-V protocol (for the left tracings, the lowest tracing was −60 mV, and the highest tracing was +70 mV; in 10-mV increments) and the tail I-V protocol (for the right tracings, the lowest tail current tracing followed a test pulse to −50 mV, and the highest tail current tracing followed a test pulse to +90 mV, in 10-mV increments; the current tracings during the test pulses positive to −40 mV are off scale). Panels B and C show data points superimposed on curves calculated from a single-sigmoidal function (1-sigmoidal, panel B) or a double-sigmoidal function (2-sigmoidal, panel C). Open circles represent data points generated from the peak I-V protocol, and filled circles are data from the tail I-V protocol.
has been shown that Na ions are pushed through the channel pore, thus carrying charges, only at strong depolarizations. It is possible that for RHK1 channels some intracellular cations (probably sodium ions) may become more efficient charge carriers as the voltage becomes more positive. Therefore, because of an increase in the charge-carrying capacity of intracellular cations, the peak outward current amplitude continues to increase as the depolarization exceeds the plateau voltage for channel activation. These two possibilities await tests by single-channel recordings. Finally, it is possible that a complicated shape of the activation curve may reflect an intrinsic voltage-dependent activation process of RHK1. The double-sigmoidal function fitted the activation curve better than the single-sigmoidal function because the former allowed more free parameters for fitting, but not because there were two separate voltage-dependent components.

The error introduced by constructing the activation curves using the chord conductances based on the driving force, as discussed above for RHK1, may contribute to the observed negative shift in the activation curves of these potassium channels on elevating [K]. More experimentation is required to rigorously test the possibility of this error in our observations. Furthermore, single-channel analysis of conductance at different levels of [K] will provide an unequivocal quantification of the effects of elevating [K] on the single-channel conductances of these potassium channels.

The high K-induced acceleration of restitution of RHK1 was abolished in the deletion mutant channel, RHK1Δ3-25 (Figure 10). However, the design of the restitution experiments on RHK1Δ3-25 needs some discussion. Since the decay of the mutant channel was extremely slow in these experiments, the duration of the first pulse (T) was prolonged to 5 seconds, which caused an inactivation of the mutant channel by only approximately 50% (e.g., Figure 10A). Therefore, this protocol was different from that used in the experiments on the wild-type channel, in which T was 10 times shorter (500 msec) and the inactivation was largely complete during the first pulse (Figure 5A). Either or both of these two factors might contribute to the observed difference in the response of the mutant channel in its restitution to elevating [K]. Therefore, we tested in the wild-type channel whether prolonging T to 5 seconds (the same as T in the experiments on RHK1Δ3-25) or shortening T to 50 msec (that caused an inactivation of the wild-type channel by only 50%) would change the effects of elevating [K], on the restitution process. For the wild-type channel, with T values of 50, 500, and 5,000 msec, the restitution time constants were 2,241, 1,956, and 1,936 msec at 2 mM [K], respectively. These time constants were shortened to 674, 621, and 656 msec, respectively, on elevating [K] to 20 mM at the same levels of T. Therefore, in the wild-type channel, increasing [K] led to an acceleration of restitution by a factor of 3.0–3.3 in a T range of 50–5,000 msec. These observations suggested that the lack of effects of elevating [K] on the restitution of RHK1Δ3-25 was not due to the design of the voltage-clamp protocol but resulted from the destruction of the N-type inactivation.

**Speculation on the Molecular Mechanism of Actions of K, Ions on the Potassium Channels**

The effects of elevating [K], on the properties of RHK1, H-4, and H-37 and the deletion mutant of RHK1 (RHK1Δ3-25) are summarized in Table 2. Increasing [K], can increase the chord conductance and cause a negative shift in the activation curve in all four channels we studied, with either the N-type inactivation (RHK1, H-4), C-type inactivation (H-37), or an apparent lack of both (RHK1Δ3-25). This indicates that these effects of K, are mediated by structural components independent of the inactivation mechanism. In the other aspects, elevating [K], induced similar changes in RHK1 and H-4 that were different from the effects of increasing [K], on H-37. For RHK1Δ3-25, the responses to an elevation of [K], were different from those of the other three channels. A comparison between RHK1 and RHK1Δ3-25 suggests that, for the former, the positive shift in the steady-state inactivation curve and the acceleration of the restitution depend on an intact N-type inactivation mechanism. The same may be true for H-4. A comparison between H-37 and RHK1Δ3-25 suggests that for H-37, the slowing in the development of and recovery from inactivation induced by elevating [K], relies on an efficient C-type inactivation mechanism.

Table 2. Summary of Effects of Elevating [K], on the Properties of Potassium Channels

<table>
<thead>
<tr>
<th>Properties</th>
<th>RHK1</th>
<th>RHK1Δ3-25</th>
<th>H-4</th>
<th>H-37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage dependence of activation</td>
<td>Increase in the proportion of the negative component</td>
<td>Similar to RHK1</td>
<td>Negative shift</td>
<td>Negative shift</td>
</tr>
<tr>
<td>Peak chord conductance</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Voltage dependence of inactivation</td>
<td>Positive shift</td>
<td>No change</td>
<td>Positive shift</td>
<td>No change</td>
</tr>
<tr>
<td>Rate of inactivation during depolarization</td>
<td>No change</td>
<td>Accelerated</td>
<td>No change</td>
<td>Slowed</td>
</tr>
<tr>
<td>Rate of recovery from inactivation</td>
<td>Accelerated</td>
<td>No change</td>
<td>Accelerated</td>
<td>Slowed</td>
</tr>
<tr>
<td>Voltage dependence of K,'s effects</td>
<td>Significant</td>
<td>.</td>
<td>Significant</td>
<td>Small</td>
</tr>
</tbody>
</table>

RHK1, H-4, and H-37 are potassium channels. RHK1Δ3-25 is a mutant RHK1 channel in which the N-terminal amino acids numbered three to 25 have been deleted.
the channel returns to the resting state. The middle part of each panel illustrates cartoons of different channel conformations. The orientation of the channel is such that the extracellular compartment is on the top and the cytoplasmic compartment is on the bottom. The voltage-sensor (S4) domains are denoted by hatched areas, which can be displaced upwardly (toward the extracellular surface) on membrane depolarization.\textsuperscript{20,23,54} The S4 domains control the positions of "activation gates" (filled arrowheads), which determine whether potassium ions (K\textsuperscript{+}) can flow through the channel pore or not.

Although RHK1 is longer than H-4 by 80 amino acids in the N-terminal region, the two share the same pattern in the N-terminus of having a stretch of hydrophobic residues followed by a cluster of positive charges.\textsuperscript{17,18} For H-4, it has been shown that this forms the "inactivation gate."\textsuperscript{22} The same may be true for RHK1. The inactivation gate is represented by a tethered ball with a positive charge sign in the cartoon shown in the middle part of the top panel of Figure 13.\textsuperscript{22,55} The inactivation gate can bind to a receptor site in the channel and plug the channel pore only after activation gates are fully open (state O)\textsuperscript{22,55} or are almost fully open (state C').\textsuperscript{56} Therefore, inactivation is coupled to activation.\textsuperscript{22,54,55} The channel can return to the resting state (C) only after the inactivation gate has left the channel. This process may traverse an open state (O*), as seen in a single-channel study on H-4 that shows that channel reactivation is most often preceded by channel reopening during the recovery interval.\textsuperscript{15} K\textsubscript{o} ions can interact with the inactivation gate at negative membrane voltages (state O*). Elevating [K\textsubscript{o}], will increase the local [K] inside the pore. This may speed up the exit of the inactivation gate from the channel, possibly by an electrostatic repulsion, leading to an acceleration of the return of the channel to the resting state (Figure 5).\textsuperscript{15} This effect of increasing [K\textsubscript{o}] can be accentuated by membrane hyperpolarization, because the membrane electrical field will draw K\textsubscript{o} ions deeper into the pore, making them more efficient in repelling the inactivation gate. This is consistent with the experimental observations that making the \textit{V\textsubscript{i}} more negative enhances the high K\textsubscript{o}-induced acceleration of restitution in RHK1 and H-4 (Figure 6). K\textsubscript{o} ions may also interact with the inactivation gate at moderate depolarizations before the
channel becomes fully open (state C'). Elevating [K+]o may increase the [K] in the site of potassium ion action, retarding the binding of the inactivation gate. This hindrance of channel inactivation from closed state(s) may cause a positive shift in the steady-state inactivation curve of RHK1 and H-4 (Figure 2). However, increasing [K+]o did not affect the rate of channel inactivation (decay) during a maintained depolarization in either RHK1 or H-4 (Figure 3). This could be because in the presence of an outward potassium current the local [K] in the vicinity of the potassium action site, which is deep inside the pore according to the strong voltage dependence of K+ action in RHK1 and H-4 (Figure 6B), may be mainly determined by [K] in the cytoplasm but not by [K+]o. Therefore, inactivation proceeds at the same rate despite an increase in [K+]o. Similar observations and interpretation concerning a lack of effect of elevating [K+]o on the decay time course of H-4 have been presented by Demo and Yellen.15 However, based on our data, other possibilities cannot be ruled out. The quantitative differences between RHK1 and H-4 may be explained by the difference in the amount of positive charges in their N-termini. RHK1 has more positive charges than H-4; this may cause a slower rate of recovery from inactivation and a greater acceleration of recovery by the same amount of [K+]o, elevation in RHK1 than in H-4. For H-37, the N-terminus does not contain a structural pattern of a hydrophobic region followed by positive charges similar to that seen in RHK1 and H-4.19 The channel has the C-type inactivation that is mediated by a hydrophobic residue in the S6 domain close to the extracellular surface.24 The kinetics of C-type inactivation are voltage independent,24 indicating that, similar to the N-type inactivation, the C-type inactivation may not be accompanied by a charge movement across the cell membrane and that the C-type inactivation may be coupled to channel activation. The molecular mechanism for C-type inactivation has not been elucidated (however, see the legend of Figure 13 for a proposed mechanism of C-type activation).

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