Molecular cloning and functional expression of a potassium channel cDNA isolated from a rat cardiac library

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A full-length K^+ channel cDNA (RHK1) was isolated from a rat cardiac library using the polymerase chain reaction (PCR) method and degenerate oligonucleotide primers derived from K^+ channel sequences conserved between *Drosophila Shaker* H4 and mouse brain MBK1. Although RHK1 was isolated from heart, its expression was found in both heart and brain. The RHK1-encoded protein, when expressed in *Xenopus* oocytes, gated a 4-aminopyridine (4-AP)-sensitive transient outward current. This current is similar to the transient outward current measured in rat ventricular myocytes with respect to voltage-dependence of activation and inactivation, time course of activation and inactivation, and pharmacology.

Cardiac tissue expression; Transient outward K^+ current; PCR cloning; *Xenopus* oocyte expression

1. INTRODUCTION

Electrophysiological studies suggest that there are at least 10 distinct K^+ conductances in the heart [1–10]. These conductances appear to be critical for the determination of various phases during the cardiac excitation cycle, especially phase 1 repolarization, phase 3 repolarization, and resting membrane potential. A molecular understanding of cardiac K^+ channels should give fundamental insight into mechanisms underlying the normal cardiac action potential, and how this excitability may be altered by pathology or by drugs [11]. Of particular interest is the contribution that ischemia-induced alterations in K^+ conductance or K^+ accumulation may play in reentry arrhythmias. A related issue of therapeutic interest is the differential expression of K^+ channels in heart versus other excitable tissues. Some antiarrhythmic drugs appear to exert their action by modifying cardiac K^+ channels, but their usefulness has been limited by an apparent lack of selectivity [12]. If so, molecular analyses of cardiac channels could provide a basis for the design of more specific agents [13]. Cardiac K^+ channels have generally been viewed as being distinct from their brain counterparts, however, it is possible that all major cardiac channels show substantial expression in other excitable tissues. To address some of these issues, we have initiated a molecular analysis of cardiac K^+ channels by cloning a cDNA expressed in rat heart.

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2. MATERIALS AND METHODS

2.1. *PCR cloning and sequencing of cDNAs*

Degenerate oligonucleotides (20 bases) to prime a PCR amplification were synthesized according to the *Drosophila Shaker* H4 cDNA sequence: a sense sequence corresponded to amino acid residues 413–419 and an antisense sequence corresponded to residues 480–486 [14]. The amino acid sequences are identical between *Shaker* and mouse MBK1 [15]. Template was recombinant lambda phage DNA from a rat cardiac cDNA library prepared by Clontech from adult rat heart poly(A)$^+$ RNA. 1.6×10^6 independent clones were divided into 7 pools and amplified in separate runs: 94°C, 1 min; 45°C, 1 min; and 55°C, 2 min for 40 cycles using Taq DNA polymerase. Amplified products from the PCR were size-fractionated, isolated, cloned into pBluescript vector (Stratagene), and sequenced using a Sequenase kit (US Biochemical Co.). Sequence data were analyzed on an IBM-XT with software developed by Dr A. Goldin, Caltech. A small PCR clone corresponding to RHK1 was used as a hybridization probe to screen the rat heart cDNA library. Twenty-eight cDNAs were isolated, and we chose 2 with large inserts, RHK1a and RHK1b, to examine in detail. All of the cDNA sequencing was performed using both dGTP and 7-deazo-dGTP reactions. In the consensus RHK1 sequence (3201 bp), RHK1a extends from nucleotides 1 to 3122 and contains the entire open reading frame; RHK1b is an incomplete cDNA, and extends from 1342 to 3201. An initiation sequence used by eukaryotic ribosomes (ACCATGG) is present at the putative RHK1 initiation codon [16].

2.2. *Synthesis of cRNA and* Xenopus *oocyte expression*

To prepare transcripts, RHK1a was subcloned into the *Eco*RI site of the pBluescript vector under control of the T7 promoter. Template DNA was prepared by digestion with *Hin*dIII followed by proteinase K treatment. A Pharmacia TransProbe T kit was used for the

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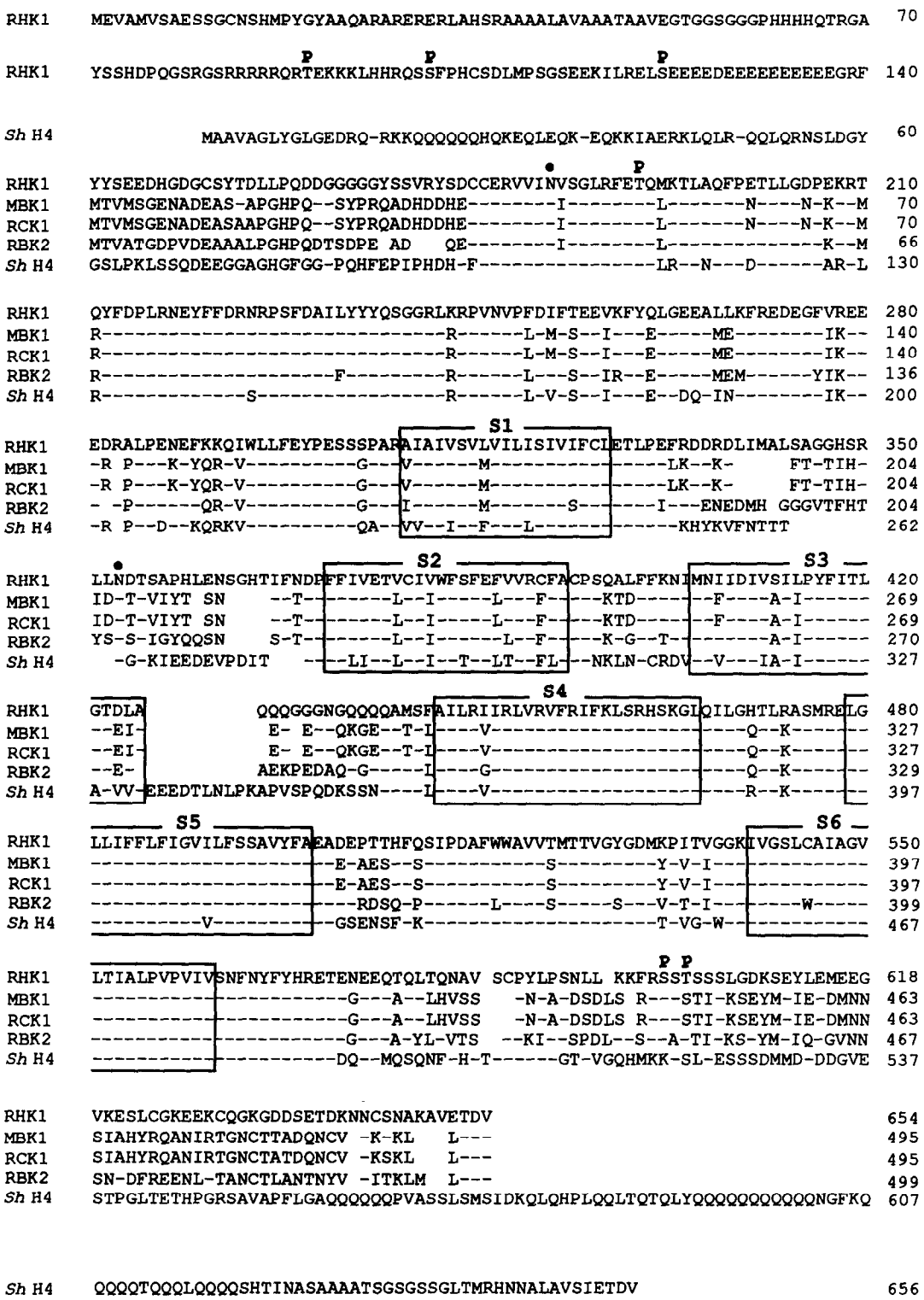

Fig. 1 The deduced RHK1 amino acid sequence compared with sequences of MBK1, RCK_1, RBK2, and *Drosophila Shaker* H4 cDNA. Dashes represent amino acid identities; gaps are introduced to improve overall alignment; and presumptive transmembrane helices (S1 to S6) are boxed. For RHK1, potential phosphorylation sites are marked with a P: cAMP-dependent protein kinase sites 90 and 600, and type II Ca^{2+}/CaM-dependent protein kinase sites 90, 101, 122, 190, and 602. Residues 596–602 (KKFRSST) encode an interesting structure in RHK1 containing both PKA and PKC sites. This structure is conserved in MBK1, RCK1, and RBK2, although the sequences encoding it and the region surrounding it are divergent. *Shaker* H4 only contains the PKA site. Putative N-linked glycosylation sites in RHK1 are marked with a dot (residues 182, 353 and 643). Among them, residue 182 is conserved among other K^+ channels. Residues 353 and 643 are in two areas of extensive diversity; however, glycosylation sites are found in analogous positions in RCK1, RBK2, MBK1, and *Shaker*. The RHK1 nucleotide sequence is available from the Genebank database (M30867).

transcription reaction following the manufacturer's protocol, except for: (i) 1 μCi of [α-^{32}P]UTP was used instead of 10 μCi, (ii) ATP, UTP, and CTP were 0.5 mM, GTP was 0.1 mM, and m^{7}GpppG was 0.5 mM. Transcribed RNAs were checked with formaldehyde/agarose gel electrophoresis and autoradiography. RNA was dissolved in water to 0.2 μg/ml and 50 nl of RNA solution was injected into each oocyte. Oocytes were incubated at 20°C in ND96 (in mM: NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, and Hepes 5, pH 7.5), supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml) and Na-pyruvate (2.5 mM). For electrophysiology, oocytes were bathed in Ca^{2+}-free ND96 solution, supplemented with 1.8 mM $MgCl_2$, to minimize Ca^{2+}-activated Cl^- currents. No time-dependent outward currents were observed in uninjected oocytes. Standard 2-microelectrode voltage clamp was used with 3 M KCl-filled electrodes (resistance 1–2 MΩ). The gain of the voltage clamp amplifier (Dagan 8500) was increased until the capacitative transient was over by 2–4 ms and the ratio of membrane voltage step to command voltage step was ≥0.9 at a step size of 100 mV. Pulse protocol generation, data acquisition and analysis were performed on an IBM/AT using pClamp software (Axon Instruments). Experiments were carried out at room temperature (21–23°C). All average values are mean ± SD.

3. RESULTS AND DISCUSSION

A full-length cDNA, RHK1, was isolated from a rat heart library using the PCR cloning method as described. The deduced amino acid sequence contains one long open reading frame of 654 amino acid residues (corresponding to nucleotides 81–2042). The sequence reveals an overall structure similar to that described for other voltage-gated K^+ channels with 6 putative membrane-spanning segments labeled S1 to S6 in Fig. 1 [14–15,17–21]. RHK1 also contains two interesting sequence motifs described previously: the segment S4, which may be involved in voltage sensing; and a leucine zipper, which may be involved in gating, located between S4 and S5 [22]. As in many other K^+ channels, the carboxyl-terminus of RHK1 ends with the sequence TDV. Compared with RCK1, RBK2, MBK1, and *Shaker* H4 channels, RHK1 is most highly conserved in the presumptive transmembrane segments and the 135 residues preceding S1, showing 69–80% amino acid identity [14–15,17–19]. RHK1 shows low homology to a recently isolated delayed rectifier (drk1), showing 38% amino acid identity over the conserved regions [23]. The most unusual feature of RHK1 is in the amino-terminus which is longer than channels RCK1, RBK2, and MBK1 by some 140 residues and which contains unusual strings of A, G, R, and E. RHK1 is virtually identical to a recently described rat brain cDNA, RCK4 [24]. The two cDNAs appear to be derived from the same gene although there are over 30 differences in DNA sequences, which may be derived from natural variations among rat strains. The most notable differences in the coding region are 2 frameshifts in the amino-terminal region (residues 84–88) which reads RRRRQ (RHK1) vs EEEAT (RCK4). Other coding differences are at residues 42 (L vs A), 94 (K insertion in RCK4), and 309 (A vs G).

Southern blot analysis under high stringency conditions suggests that RHK1 is encoded by a single gene, i.e. one or two bands are observed in each restriction digest of rat genomic DNA (Fig. 2A) [24]. RHK1 is expressed in heart and brain, but not other tissues (Fig. 2C). Northern analysis showed that the RHK1 probe hybridizes to heart and brain poly(A)$^+$ RNA of about 6.0 and 4.5 kb. The 6.0 kb band was present in about equal amounts; the 4.5 kb band was relatively more abundant in the brain (note, that comparisons are not corrected for the proportionately larger amount of membrane channel proteins present in brain vs heart) [25–26]. No hybridization was detected when liver, skeletal muscle, spleen, kidney, stomach, and aorta RNA was probed with RHK1. The absence of transcripts in these tissues, particularly smooth muscle and skeletal muscle, is consistent with the absence of the transient outward current in these tissues.

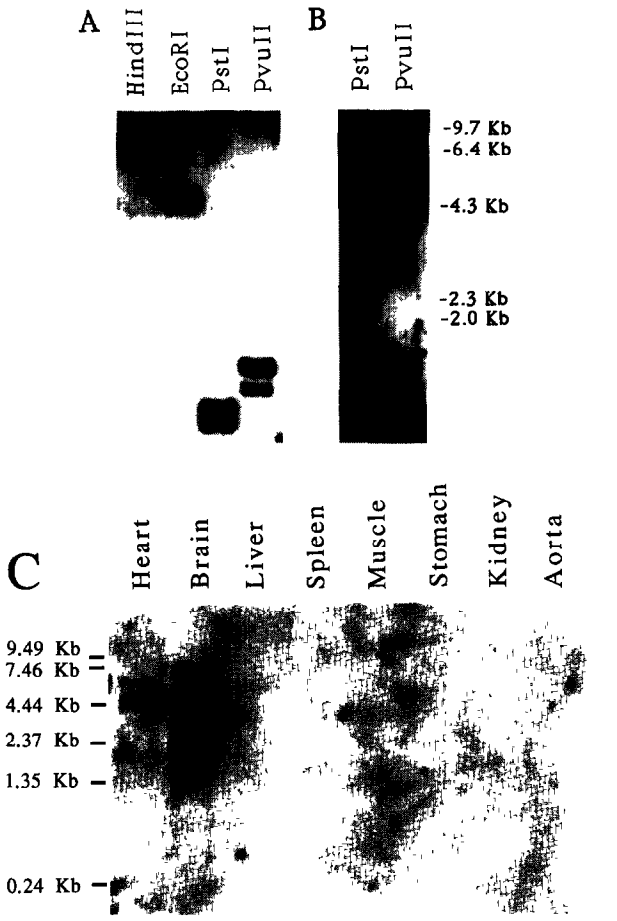

Fig. 2. Southern and Northern blot analysis. (A) RHK1 and RCK4 are probably encoded by the same gene since Southern blot analysis under high stringency conditions shows bands of similar sizes recognized by the RHK1 and RCK4 [24]. (B) Under lowered stringencies, some additional bands are observed, particularly in digestions with *PstI* (1.8 kb fragment) and *PvuII* (1.6 kb fragment). The 1.8 kb *PstI* band resembles one that hybridizes to RCK3 [14]. (C) Expression of RHK1 is detected in the heart and in the brain by Northern blot hybridization under high stringency conditions.

RHK1 channel activity was examined in the *Xenopus* oocyte expression system. A transient outward current

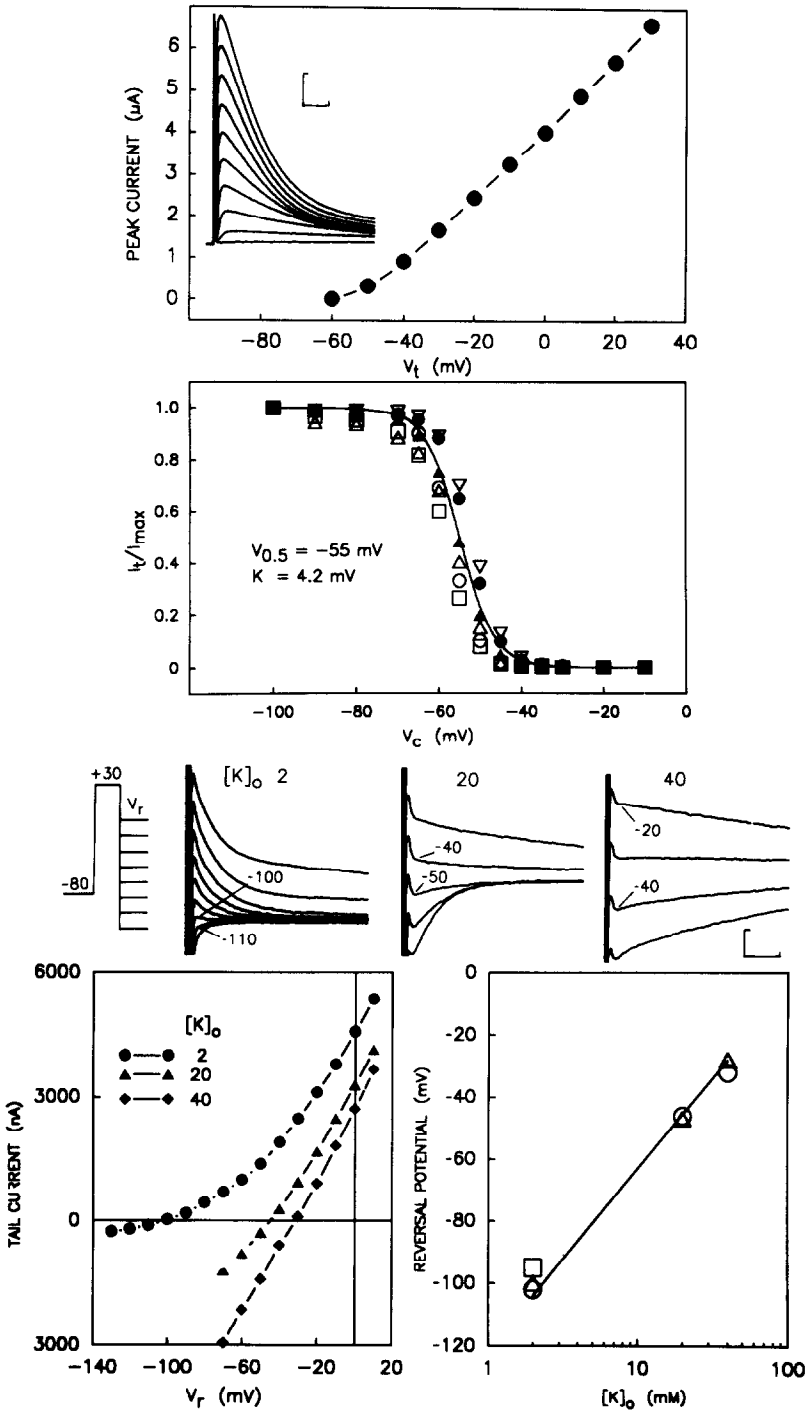

Fig. 3. Functional features of RHK1 channels expressed in *Xenopus* oocytes. (A) Voltage dependence of activation (upper panel). The insert shows current traces induced by 500 ms-depolarizing steps from a holding voltage (V_h) of −80 mV to test voltages (V_t) ranging from −60 to +30 mV, in 10-mV increments. Calibration: 25 ms and 1 μA. The plot shows peak current-voltage relationship. Peak current (I_t) was the difference between the outward peak and the current level 500 ms after the start of depolarization. (Lower panel) Steady state inactivation. Membrane potential was stepped from a V_h of −80 mV, to a conditioning voltage (V_c) ranging from −100 to −10 mV for 2 s, and then to a test pule of +20 mV. I_{max} is taken as I_t at V_c = −100 mV (n = 6, symbols represent data from separate experiments). For each experiment, voltage of half-maximum inactivation ($V_{0.5}$) and slope factor (k) were determined from the relationship between I_t/I_{max} and V_c which was best fit by the Boltzmann function ($I_t/I_{max} = 1 + \exp[(V_c - V_{0.5})/k]\} - 1$). The smooth curve was calculated from the Boltzmann function using the average of experimental values. (B) K^+ selectivity of the RHK1 channel. (Upper panels) The voltage clamp protocol is depicted: membrane potential was stepped from a V_h of −80 mV, to +30 mV for 10 ms, and then to different repolarization voltages (V_r) ranging from +20 to −120 mV for 500 ms. Also depicted are superimposed tail currents recorded at different $[K^+]_o$; the marked voltages are those which bracket the reversal potential. At $[K^+]_o$ of 20 and 40 mM, upward deflections caused by overcompensation of the capacitative transient are superimposed on the initial tail currents. Calibration: 7.5 ms and 500 nA. (Lower left) The plot shows the instantaneous current-voltage relationship for 3 values of $[K^+]_o$. Tail current amplitude was determined by fitting a single exponential function to the current between 5 and 500 ms after repolarization, and then extrapolating back to the start of the repolarization step. Reversal potentials (E_{rev}) were determined by the intersections of the curves with the voltage aixs. (Lower right) Plot shows E_{rev} at different $[K^+]_o$. Symbols represent data from different experiments (n = 3). The straight line is calculated from the Nernst equation, assuming intracellular $[K^+]_i$ = 120 mM. For all experiments in this figure, the time between trials was 10 s.

was recorded 2–4 days after microinjection of RHK1 cRNA into oocytes (Fig. 3A). The voltage threshold for activation of the RHK1 current was −50 mV; the peak current amplitude increased as the voltage became more positive up to +80 mV. The voltage dependence of inactivation for RHK1 is shown in the steady state inactivation curve of Fig. 3A. The data from individual oocytes were fitted with a Boltzmann function; the average half-maximum inactivation voltage ($V_{0,5}$) was −55.0 ± 3.8 mV and the slope factor (k) was 4.2 ± 0.8 mV ($n=9$). Fig. 3B shows the instantaneous current-voltage relationship and reversal potential (E_{rev}) of RHK1: E_{rev} was −105, −45, and −28 mV at 2, 20, and 40 mM $[K^+]_o$, respectively. Assuming that the channel is selective for K^+ at 40 mM $[K^+]_o$, $[K^+]_i$ is estimated to be 120 mM using the Nernst equation and E_{rev}. Based on this estimate of $[K^+]_i$, E_{rev} should be −104 and −45.5 mV in 2 and 20 mM $[K^+]_o$, respectively, close to the observed values. Therefore, we suggest that RHK1 is a purely K^+-selective channel.

The kinetics of activation for RHK1 became faster at more positive test voltages (Fig. 4), i.e. time to peak was 14.8 ± 4.6 ms at −20 mV and 11.0 ± 3.0 ms at +30 mV ($n=6$). The time course of current decay was described by a single exponential, that was accelerated at more positive voltages (Fig. 4). The decay time constant was 65.4 ± 17.4 and 45.2 ± 16.7 ms at −20 and +30 mV, respectively ($n=6$). The recovery from inactivation followed a single exponential time course in a voltage-dependent manner with a time constant of 2.2 ± 0.6 s ($n=6$) at −80 mV (Fig. 4).

4-AP and Ba^{2+} blocked the RHK1 current with a half-maximum inhibition concentration of 1.2 mM and 0.75 mM, respectively. Cs^+ (10 mM) decreased the current by only 10% while tetraethylammonium (20 mM) did not affect the current.

For comparison with the present study, the transient outward current in rat ventricular myocytes was studied under the same voltage clamp protocols. Properties of the transient outward current in rat ventricular myocytes are: voltage threshold for activation (−40 mV); steady-state inactivation ($V_{0,5}=-57$ mV, $k=4$ mV); time constant of inactivation (30–40 ms at +60 mV); time constant of recovery from inactivation (30 ms at −80 mV). Also, 4-AP (5 mM) totally abolishes the current; Ba^{2+} (1 mM) decreases the current by 60%; and Cs^{2+} (10 mM) decreases the current by about 10%.

The functional properties of RHK1 are similar to the transient outward current in rat ventricular myocytes, which is believed to be responsible for phase 1 repolarization [2]. Indeed, the two currents are virtually identical in voltage dependence of activation and inactivation, kinetics of activation and inactivation, and pharmacological sensitivity. The two currents, however, differ in recovery from inactivation with the ventricular myocyte current recovering more rapidly [2]. An interesting possibility is that RHK1 is missing an additional subunit that affects recovery from inactivation [27]. Although RHK1 may account for a major outward current in the heart, it also shows substantial brain expression. An implication is that major channels, such as RHK1, represent functions critical for several types of excitable membranes. We are presently investigating the possibility that other brain channels could also show heart expression, with the anticipation that a more complete molecular picture of cardiac excitability may soon be forthcoming.

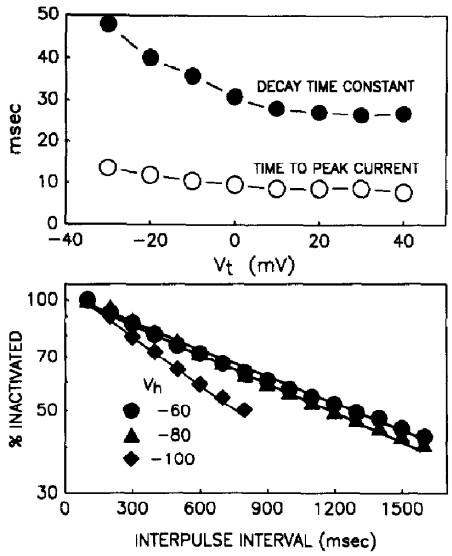

Fig. 4. Kinetic properties of RHK1. (Upper panel) Plot shows kinetics of activation and inactivation of RHK1 at various test voltages (V_t). Voltage clamp protocol is described in the upper panel of Fig. 3A. The time to peak current was measured from the start of the depolarizing pulse to the peak of I_t. The time constant of current decay was obtained from a single exponential function fit to the difference in current between I_t and 200 ms after the start of depolarizing pulse. (Lower panel) Plot shows the time course of recovery from inactivation at 3 different holding potentials. Voltage clamp protocol was a variable interpulse interval (0.01–4.6 s) separating two test pulses (+20 mV, 100 ms). The time between successive trials was 10 s. Percent inactivated was the decrease in the peak current amplitude between the first and second test pulses. The time course of recovery from inactivation was described by a single exponential function. The lines were calculated using best-fit time constants: 1.8, 1.6, and 1.3 sec for V_h = −60, −80, and −100 mV, respectively.

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