

K⁺ Channels in Cardiomyocytes of the Pulmonate Snail *Helix*

S.A. Kodirov^{1,2,3}, V.L. Zhuravlev², V.K. Pavlenko³, T.A. Safonova², J. Brachmann¹

¹Department of Cardiology, Medical University Hospital Heidelberg, 69120 Heidelberg, Germany

²Department of General Physiology, Saint Petersburg University, 199034 Saint Petersburg, Russia

³Department of Biophysics, Saint Petersburg University, 199034 Saint Petersburg, Russia

Received: 14 April 2003/Revised: 25 November 2003

Abstract. We used the patch-clamp technique to identify and characterize the electrophysiological, biophysical, and pharmacological properties of K⁺ channels in enzymatically dissociated ventricular cells of the land pulmonate snail *Helix*. The family of outward K⁺ currents started to activate at –30 mV and the activation was faster at more depolarized potentials (time constants: at 0 mV 17.4 ± 1.2 ms vs. 2.5 ± 0.1 ms at + 60 mV). The current waveforms were similar to those of the A-type family of voltage-dependent K⁺ currents encoded by Kv4.2 in mammals. Inactivation of the current was relatively fast, i.e., $50.2 \pm 1.8\%$ of current was inactivated within 250 ms at + 40 mV. The recovery of K⁺ channels from inactivation was relatively slow with a mean time constant of 1.7 ± 0.2 s. Closer examination of steady-state inactivation kinetics revealed that the voltage dependency of inactivation was U-shaped, exhibiting less inactivation at more depolarized membrane potentials. On the basis of this phenomenon, we suggest that a channel encoded by Kv2.1 similar to that in mammals does exist in land pulmonates of the *Helix* genus. Outward currents were sensitive to 4-aminopyridine and tetraethylammonium chloride. The last compound was most effective, with an IC_{50} of 336 ± 142 $\mu\text{mol l}^{-1}$. Thus, using distinct pharmacological and biophysical tools we identified different types of voltage-gated K⁺ channels.

Key words: Mollusc — A-type K⁺ currents — Delayed rectifier — Ventricular cells

Present address for S.A.K.: Brigham and Women's Hospital, Cardiovascular Division, Harvard Medical School, 75 Francis St., Thorn 1216, Boston, MA 02115.

Correspondence to: S.A. Kodirov; email: skodirov@hms.harvard.edu

Introduction

A heart is a group of muscular cells that are electrically coupled so that the cells beat as if they were a syncytium. Like the mammalian heart, the mollusc heart exhibits regular action potentials (Huddart & Hill, 1996; Zhuravlev et al., 2001). Spontaneous action potentials with a spike-like waveform have also been recorded from the single cardiac cells of the snail *Lymnaea* (Yeoman & Benjamin, 1999). In these molluscs the depolarization phase of cardiac action potentials is due to the influx of Ca²⁺ ions; the K⁺ channel accounts for repolarization. Although the voltage-dependent outwardly rectifying K⁺ channels were first characterized by Hodgkin and Huxley (1952) in invertebrates more than 50 years ago and the ionic currents responsible for the resting membrane potential in the heart of the snail *Lymnaea* were described by Brezden, Gardner & Morris (1986) about 15 years ago, there have been very few studies of ion channels in invertebrate cardiac myocytes. Since the mollusc heart is regulated by cholinergic, serotonergic, and peptidergic neurotransmitters (Buckett et al., 1990; Brezden, Benjamin & Gardner, 1991; Kuwasawa & Hill, 1997), we were interested in understanding their role in the modulation of ion channels in this organ in general and in the generation of noncholinergic junction potentials (Zhuravlev et al., 2001) in particular. To determine the role of these junction potentials we first characterized the ionic channels present in cardiomyocytes of the gastropod snail *Helix*. Although logically the knowledge about ion channels in the heart of pond snail *Lymnaea* (Brezden et al., 1986, 1999; Yeoman & Benjamin, 1999; Yeoman, Brezden & Benjamin, 1999) could be extended to land pulmonates, to date nothing is known about the ion channels and their role in the latter molluscs. Thus, our data describe the voltage-gated K⁺ channels in the heart of *Helix*.

The properties of these currents closely resemble those of mammals and contribute to the repolarization phase of action potentials. Some of our data appeared in a preliminary communication (Kodirov, Zhuravlev & Kreye, 1995).

Materials and Methods

CELL ISOLATION

Ventricular myocytes were dissociated from hearts of snail *Helix*. The animals were kept at room temperature (20–24 °C), and fed ad libitum on vegetable. The procedure of cell isolation was modified from that described previously (Maruyama et al., 1987; Pfrunder & Kreye, 1991). In brief, the heart was dissected from the animal, and the auricle and the remains of the aorta were removed. The ventricle was cut exactly into two pieces in order to protect it from an excessive exposure to enzymes. The cells were then dissociated by digestion for 20–30 min in KB solution (Isenberg & Klockner, 1982) containing papain (20 units ml⁻¹) and collagenase (1 mg ml⁻¹). After digestion, the cells were centrifuged, the supernatant was removed, and the pellet was resuspended in a fresh KB solution.

ELECTROPHYSIOLOGY

The surfaces of dissociated *Lymnaea* ventricle cells have been reported to have numerous invaginations, which makes the whole-cell patch-clamp recordings difficult. Therefore, in some previous studies, sharp microelectrodes were used in order to perform voltage-clamp measurements in these cells (Yeoman & Benjamin, 1999; Yeoman et al., 1999). Although we faced the same difficulties with the majority of cardiomyocytes of *Helix* we were able to conduct whole-cell recordings (Hamill et al., 1981) in these cells. Data were acquired with an RK-400 patch- and cell-clamp amplifier (Bio-Logic) and the pClamp 6.0.3 software package (Axon Instruments). The current signals were filtered at 1 kHz and stored for later off-line computer analyses; the sampling interval was 2 kHz. Microelectrodes were fabricated from capillary tubes (WPI) with the P87 puller (Sutler Instruments). Electrodes had resistances of between 1.5 and 4 MΩ when filled with an intracellular solution. The calculated junction potential between external and internal standard solutions was 7.3 mV at 24°C. Series resistance was electrically compensated to minimize the duration of the capacitive current. Electrophysiological experiments were performed at room temperature (20–24°C).

SOLUTIONS AND CHEMICALS

In all experiments a physiological solution of the following composition was used (in mmol l⁻¹): 135 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 1 MgCl₂, 1 CaCl₂, 10 HEPES, and 10 glucose. The pH of this solution was adjusted with NaOH to 7.4. The cells in the chamber were superserved with the appropriate external solution by means of a peristaltic pump. The pipette solution contained (in mmol l⁻¹): 140 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, 5 Mg₂ATP, 0.1 GTP (pH of 7.2 was adjusted with KOH). KB solution contained (in mmol l⁻¹): 85 KCl, 30 K₂HPO₄, 5 Na₂ATP, 5 MgSO₄, 0.2 EGTA, 5 creatine, 20 taurine, 5 pyruvate, 5 sodium 3-hydroxy-β-butyrate, 20 glucose, and 10 HEPES; pH of 7.2 was adjusted with KOH. To this solution albumin (1 mg ml⁻¹) was also added. Stock solutions of 4-aminopyridine (4-AP) and tetraethylammonium (TEA) in 10-mmol l⁻¹ concentration were prepared. All chemicals were purchased from Sigma.

DATA ANALYSIS

All data were initially recorded without leak subtraction, which was performed later in some cells with a pClamp protocol (Axon Instruments). Origin (Microcal) and Microsoft Excel software were used for data analyses.

The TEA-induced inhibition of current amplitude within 250 ms was calculated as $I_{TEA}/I_{control}$. The mean relative current was plotted as a function of TEA concentration, which was fitted with the sigmoidal curve of logistic function:

$$y = A_2 + (A_1 - A_2)/(1 + (x/x_0)^p),$$

where A_1 and A_2 are the initial and final values, respectively; x_0 is the concentration that produces the half-maximal inhibition (IC_{50}); p is a slope value.

The voltage dependency of activation was determined by fitting the time constants with the sigmoidal curve of the Boltzmann function:

$$y = A_2 + (A_1 - A_2)/(1 + \exp((x - x_0)/dx)),$$

where A_1 and A_2 are the initial and final values, respectively; x_0 is the half-maximal ($V_{0.5}$) value; and d_x is a slope factor. This function was also used for the determination of half-maximal activation and inactivation voltages.

The dissociation constant (K_D) was estimated (Denton & Leiter, 2002) using the equation:

$$K_D = ([TEA] \times I_{TEA})/(I_{control} - I_{TEA}),$$

where [TEA] is the tested concentration of TEA; I_{TEA} and $I_{control}$ are the amplitudes of current in the presence and the absence of TEA, respectively.

The time constants for activation and inactivation of current were calculated using the exponential function of the Chebyshev method (Clampfit 6.0.3) according to equation:

$$f(t) = A_i \times \exp(-t/\tau_i) + C,$$

where A_i and C are the amplitude of activating (or inactivating) and steady-state components, respectively; τ_i is time constant for current activation and inactivation, respectively. In order to obtain the activation time constants, data were fitted from the start of current activation to its peak value; inactivation τ values were defined by the data points ranging between the peak current value and those at the end of pulse duration.

The data are presented as the means \pm SEM and n corresponds to the number of experiments. Student's t -test was used to examine the difference between data groups; $P < 0.05$ was considered significant.

Results

HOLDING-POTENTIAL DEPENDENCE OF OUTWARD CURRENT

Healthy cells contracted in response to high K⁺ leaking from the pipettes before the whole-cell configuration was established. This was a reversible response, i.e., if the membrane of the cell was really undamaged, the observed contraction was always followed by immediate relaxation.

The currents were elicited with voltage steps ranging between -50 and +80 mV from holding

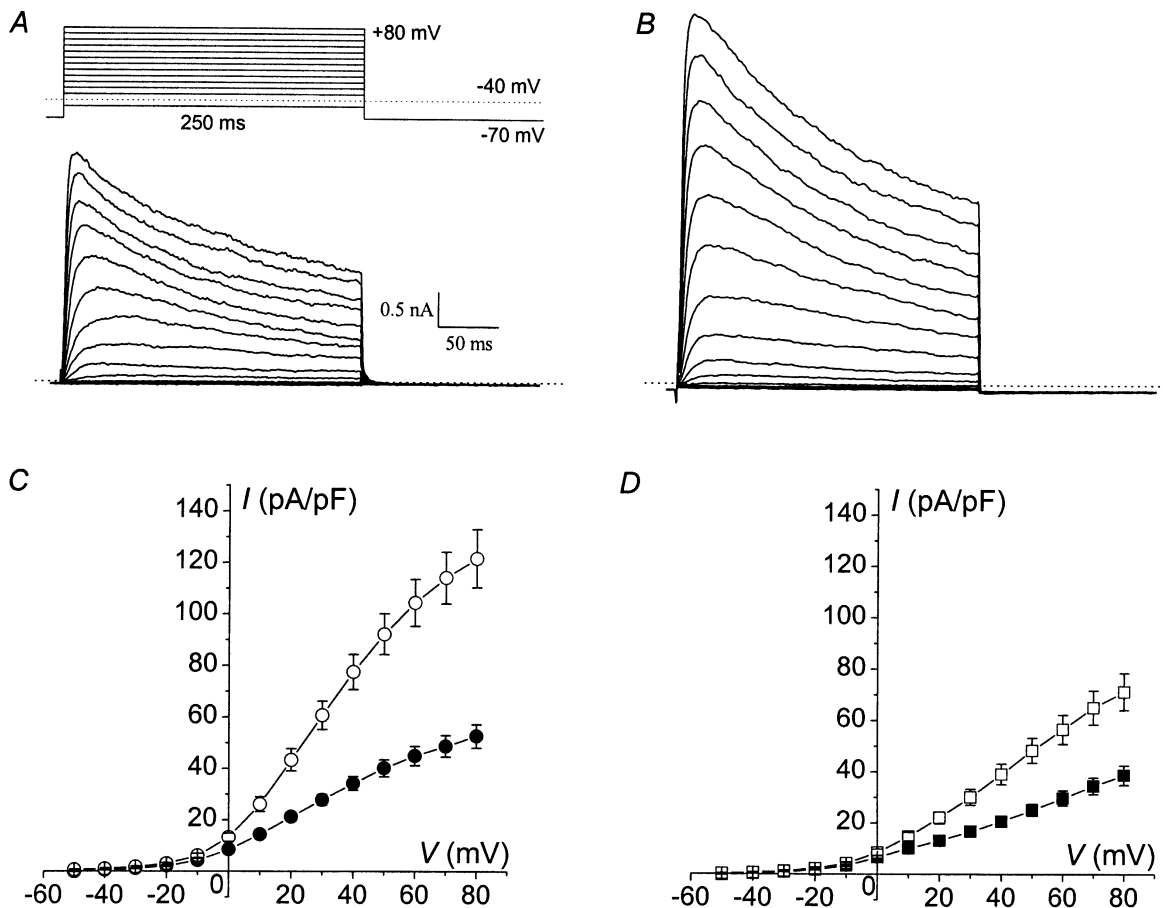


Fig. 1. Identification of K^+ currents. (A) A family of potassium currents was evoked in standard physiological solution in response to voltage pulses from -50 to $+80$ mV from a holding potential of -40 mV. The pulse protocol is shown in the upper part of Fig. 1A. (B) The current obtained from the same cell under similar conditions, but at a holding potential of -70 mV. Under these

conditions the amplitude of outward currents was increased. *I-V* relationships for peak current densities (C) and those measured at the end of 250-ms pulses (D) are shown. Empty and filled symbols are current densities at holding potentials of either -70 mV or -40 mV, respectively. Scale bars shown in A refer to B as well.

potentials of -40 mV (Fig. 1A) and -70 mV (Fig. 1B) in 10-mV increments. The pulse protocol is drawn in Fig. 1A. Duration of the tested pulses was 250 ms. The outward currents evoked during depolarization up to $+20$ mV were sustained and did not show significant inactivation, but at a voltage range between $+30$ and $+80$ mV the currents were significantly inactivated. At a holding potential of -40 mV, the net outward current was relatively small (Fig. 1A), but significantly increased at more negative holding potentials, for example, at -70 mV (Fig. 1B). Note that the waveforms of currents under both conditions were similar, revealing the activation of the same channels. In *Lymnaea* the holding-potential dependence of A-type K^+ current that exhibited steady-state inactivation at more positive voltages was determined in the presence of 5 mM TEA (Yeoman & Benjamin, 1999). Here we tested this phenomenon under control conditions, i.e., experiments were conducted in standard physiological

solution (Fig. 1A, B). Thus, outward K^+ currents in *Helix*, unlike those in the blue mussel (Curtis, Delpy & Williamson, 1999), cannot be separated into two components, i.e., A-type (I_A) and delayed rectifier (I_K), by different holding potentials. However, inactivation of the current was significantly faster at a holding potential of -70 mV, i.e., $50.2 \pm 1.8\%$ ($n = 18$, $P < 0.01$) of current was inactivated within 250 ms at $+40$ mV step potential. This value under identical conditions at holding potential of -40 mV was $39.8 \pm 1.7\%$ ($n = 31$). Starting at -30 mV, the *I-V* relationships of peak current (Fig. 1C) and those measured at the end of 250-ms pulses (Fig. 1D) show activation of outward current. The *I-V* relationship of peak current shows prominent outward rectification (Fig. 1C), and the average values of current densities at holding potentials of -40 and -70 mV at $+60$ mV were 44.9 ± 3.2 pA pF $^{-1}$ ($n = 31$) and 104.5 ± 9.1 pA pF $^{-1}$ ($n = 18$, $P < 0.001$), respectively. Thus, the properties of outward currents were similar to those

of voltage-dependent outwardly rectifying potassium channels.

DOSE-DEPENDENT EFFECTS OF TEA

Outward K^+ current was also characterized by applying relatively low concentrations of TEA. Figures 2A and B show the currents evoked from a holding potential of -40 mV to either $+20$ or $+80$ mV (these two membrane potentials were chosen randomly for the clarity of presentation). Application of $100 \mu\text{mol l}^{-1}$ (Fig. 2A, B; \circ) and 10 mmol l^{-1} TEA (Fig. 2A, B; \square) significantly decreased the current magnitude. Moreover, currents evoked at $+20$ mV (Fig. 2A) in the presence of $100 \mu\text{mol l}^{-1}$ TEA showed crossover. The activation under these conditions was slow, and no significant inactivation during the 250-ms voltage steps was observed. Under control conditions the current started to activate at -30 mV ($n = 31$) and gradually increased in amplitude at more positive potentials (Fig. 2C; \bullet). The I - V relationship of peak currents demonstrates a pronounced outward current despite the presence of $100 \mu\text{mol l}^{-1}$ TEA (Fig. 2C; \circ). Cumulative application of TEA up to 10 mmol l^{-1} significantly blocked the current (Fig. 2C; \square). Thus, a dose-response curve for the TEA effect at tested concentrations ranging from 1 nmol l^{-1} to 30 mmol l^{-1} ($n = 3$ – 10) was obtained (Fig. 2D). The TEA-induced inhibition of current amplitude within 250 ms at $+40$ mV was calculated as $I_{\text{TEA}}/I_{\text{control}}$. The relative current was plotted as a function of TEA concentration to estimate both an IC_{50} of $336 \pm 142 \mu\text{mol l}^{-1}$ and a slope value of 0.8 ± 0.2 . Note that 30 mmol l^{-1} TEA did not further decrease the amplitude of current (Fig. 2D). The effect of TEA was not significantly voltage-dependent at membrane potentials ranging between $+20$ and $+80$ mV, as shown in Fig. 2E, where \blacktriangle and \triangle are $100 \mu\text{mol l}^{-1}$ and 10 mmol l^{-1} TEA, respectively. The estimation of TEA dissociation constant (see Materials and Methods) did not reveal voltage dependence (Fig. 2F; \diamond and \blacklozenge are 1 and 10 mmol l^{-1} TEA, respectively). The effect of TEA was reversible upon washout (*data not shown*; however, see Fig. 3).

COMPARISON OF CURRENT BLOCKADE BY 4-AP AND TEA

In the next sets of experiments, first the effects of 4-AP on outward currents in *Helix* ventricular cells were evaluated (Fig. 3). Figure 3A shows the currents evoked using the pulse protocol shown in Fig. 3E. One of characteristics of A-type currents is their sensitivity to the K^+ channel blocker 4-AP. To test this phenomenon, the cells were exposed to increasing concentrations of 4-AP up to 1 mmol l^{-1} . Figure 3B shows the currents evoked in the presence of 1 mmol

l^{-1} 4-AP, which significantly affects the amplitude of outward K^+ current. The average values of current densities at $+50$ mV (holding potential: -70 mV) under control conditions and in the presence of 1 mmol l^{-1} 4-AP were $106.8 \pm 22.9 \text{ pA pF}^{-1}$ and $79.8 \pm 25.8 \text{ pA pF}^{-1}$ ($n = 3$, $P < 0.05$), respectively. The significant portion of remaining outward current after the application of 1 mmol l^{-1} 4-AP (Fig. 3B) was blocked by TEA. Sensitivity to TEA is one of the properties of delayed rectifier K^+ currents, including those in molluscs (Yeoman & Benjamin, 1999). The outward current traces in the presence of both 1 mmol l^{-1} 4-AP and 1 mmol l^{-1} TEA are illustrated in Fig. 3C. The outward current in *Helix* ventricular cells was more sensitive to TEA (Fig. 3C) than to 4-AP (Fig. 3B) at 1 mmol l^{-1} concentration. The block caused by 4-AP and TEA was almost entirely reversible with washout (Fig. 3D). The 4-AP- and TEA-sensitive currents for this particular cell are shown in Fig. 3E and F, respectively. Note that the currents sensitive to TEA (Fig. 3F) and those under control conditions (Fig. 3A; see also Fig. 1B and Fig. 4A) have identical waveforms. Thus, the peak and pseudo steady-state outward K^+ currents were similarly sensitive to TEA (see Discussion).

KINETIC PROPERTIES

In further experiments the effects of longer depolarizing pulses were examined to find the true steady-state, i.e., noninactivating, current. Membrane currents were evoked at voltages ranging between -90 and $+60$ mV for 400 ms from a holding potential of -50 mV; after each step the membrane potential was changed to a constant $+40$ mV for 400 ms. However, even during these relatively long pulses, outward currents continued to decay and reached only a pseudo steady state within 400 ms (Fig. 4A). Hyperpolarizing the cell to potentials between -90 and -60 mV produced only small linear leakage currents (Fig. 4A), confirming the absence of an inward rectifier K^+ current (Yeoman & Benjamin, 1999). The effect of TEA on the current was tested under conditions similar to those shown in Fig. 2. Application of TEA ($n = 5$) dose-dependently reduced the peak and steady-state currents (*data not shown*).

To determine the potential dependence of K^+ current activation, the peak currents that could be recorded during < 50 -ms pulses were analysed (Fig. 4A, B). Note that the current traces in Fig. 4B are shown in an expanded time and amplitude scale (scale bars are shown as an inset). The outward current elicited by depolarization to potentials ranging from -10 to $+80$ mV from a holding potential of -50 mV was analyzed. Unlike the A-type currents described in *Lymnaea*, the activation of current was relatively fast, ranging from 11.9 ± 0.7 ms at $+20$ mV to 1.7 ± 0.1 ms at $+80$ mV (Fig. 4B, F; $n = 20$, $P < 0.001$).

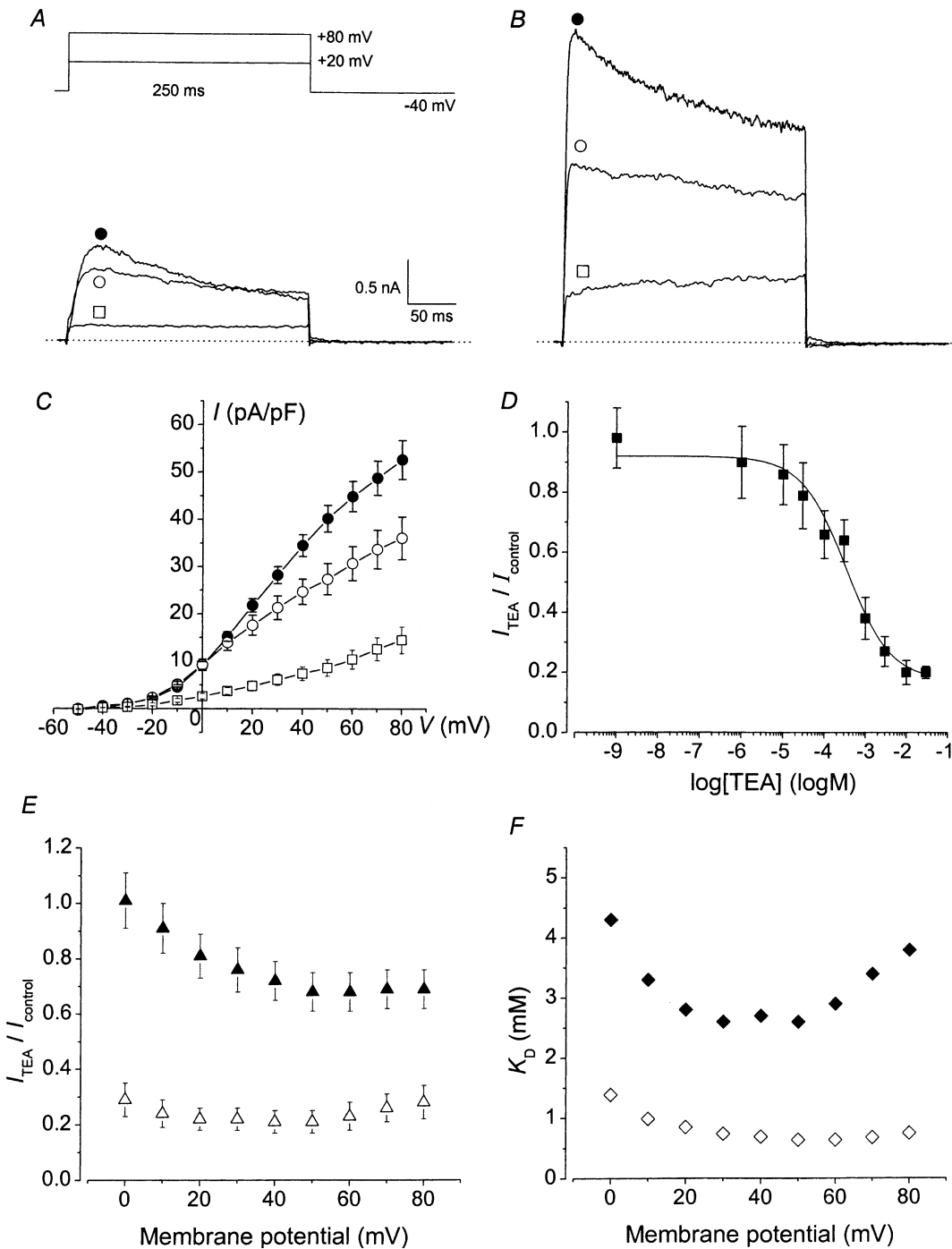


Fig. 2. Dose-dependent inhibition of current by TEA. (A) Membrane currents were recorded with constant 250-ms test pulses to potentials ranging between -50 and $+80$ mV from a -40 mV holding potential. For the illustration, only current traces at $+20$ (A) and $+80$ mV (B) are chosen. Currents were evoked before (\bullet) and after the application of $100 \mu\text{mol l}^{-1}$ (\circ) and 10mmol l^{-1} (\square) TEA; corresponding $I-V$ relationships are superimposed in C. (D) Dose-response relationship of TEA effect ($n = 3-10$). The tested

concentration ranged between 1nmol l^{-1} and 30mmol l^{-1} and peak outward currents were normalized as $I_{\text{TEA}}/I_{\text{control}}$. Data were fitted with the sigmoidal curve of logistic function and the IC_{50} of $336 \pm 142 \mu\text{mol l}^{-1}$ was estimated. (E) The effect of TEA at membrane potentials ranging between ± 0 and $+80$ mV, where \blacktriangle and \triangle are $100 \mu\text{mol l}^{-1}$ and 10mmol l^{-1} TEA, respectively. (F) TEA dissociation constant (\diamond and \blacklozenge are 1 and 10mmol l^{-1} TEA, respectively).

Plotting of the obtained time constants (τ) against the voltage commands yielded the activation curve (Fig. 4F; Δ). These data were best fitted with the sigmoidal

curve of Boltzmann function, and the half-maximal activation voltage of $+18.7 \pm 3.8$ mV and the slope factor of 16.3 ± 3.2 mV ($n = 20$) were found. Thus, a

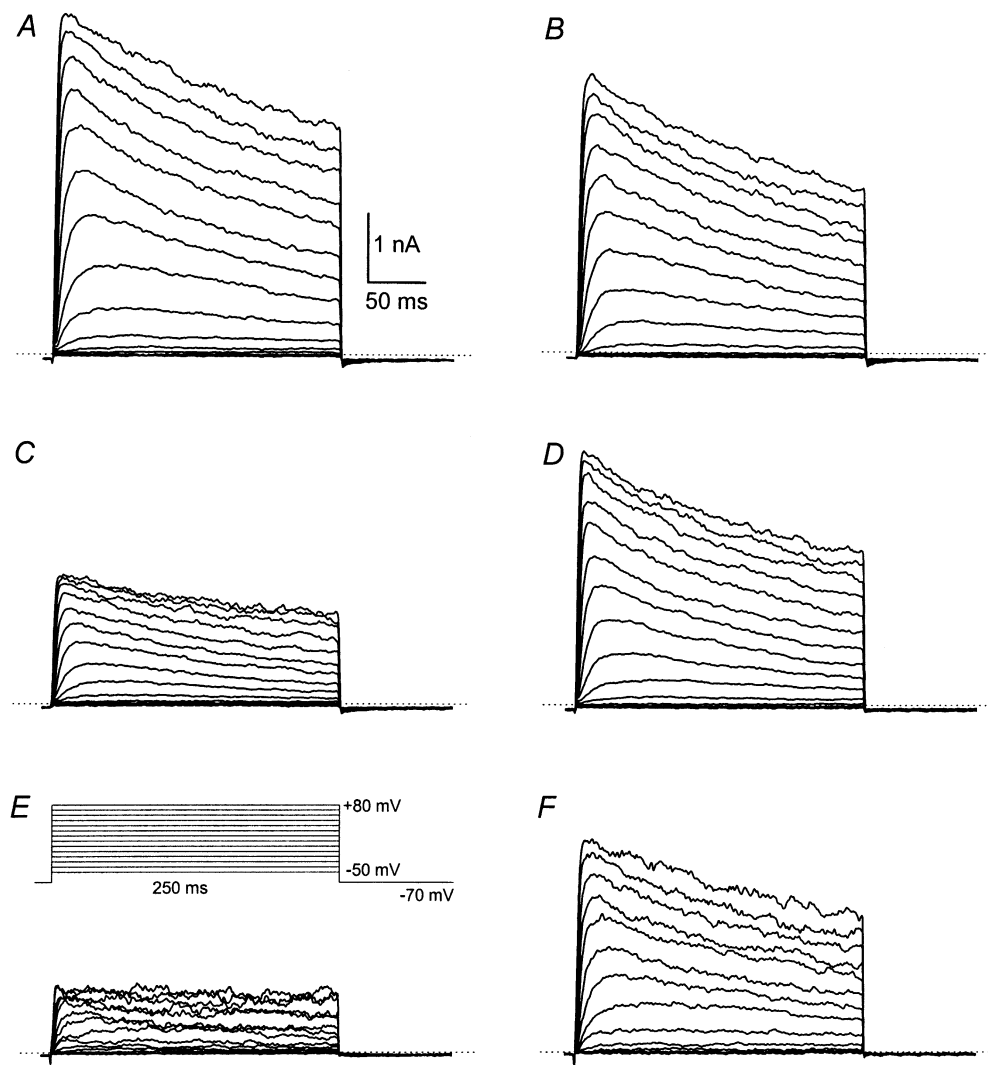


Fig. 3. Comparison between the effects of 4-AP and TEA. (A) Currents elicited under control conditions from a holding potential of -70 mV. The pulse protocol is shown in E. (B) Effects of 1 mmol l^{-1} 4-AP. (C) Currents recorded after administration of

1 mmol l^{-1} TEA in the presence of 1 mmol l^{-1} 4-AP. (D) The effect of agents was reversed by perfusion with standard saline. (E and F) Subtracted 4-AP- and TEA-sensitive currents, respectively.

comparison of currents distinguished by depolarizing voltages demonstrated a significant difference in the activation time constants, i.e., this property of the channel was voltage dependent (Fig. 4F). On the basis of both fast activation rates and relatively depolarized thresholds of these K^+ currents, they could contribute to the repolarization phase of the action potentials, as also suggested by their TEA sensitivity (Yeoman & Benjamin, 1999).

Next, the peak outward currents were normalized to the current with the maximal amplitude (at $+60$ mV) as I/I_{max} and plotted as a function of command voltages (Fig. 4D; ■). The half-maximal chord conductance occurred at $+25.3 \pm 1$ mV with a slope factor of 15.5 ± 0.7 mV. For the analysis of steady-state inactivation, the currents were evoked at constant $+40$ mV (shown in Fig. 4A and expanded in C),

normalized to the maximal value, and plotted versus prepulse voltages. Half-maximal inactivation occurred at -29.9 ± 4.4 mV ($n = 21$) with slope factor of 17.3 ± 4.2 mV (Fig. 4D; □). To determine whether or not the steady-state inactivation kinetics of K^+ current in *Helix* ventricular cells were voltage dependent, the current magnitude in response to a depolarizing pulse was fitted with an exponential function (Fig. 4C). Here, the same experiment shown in Fig. 4A is demonstrated in an expanded time and amplitude scale. The steady-state inactivation of current at $+40$ mV (Fig. 4C) was best fitted to a single exponential with a mean time constant of 200.3 ± 13.9 ms ($n = 20$) at a prepulse of -90 mV (Fig. 4F; ▲). Figure 4E shows the $I-V$ relationship for peak (●) and pseudo steady-state (○) currents evoked at potentials ranging between -90 mV and $+60$ mV

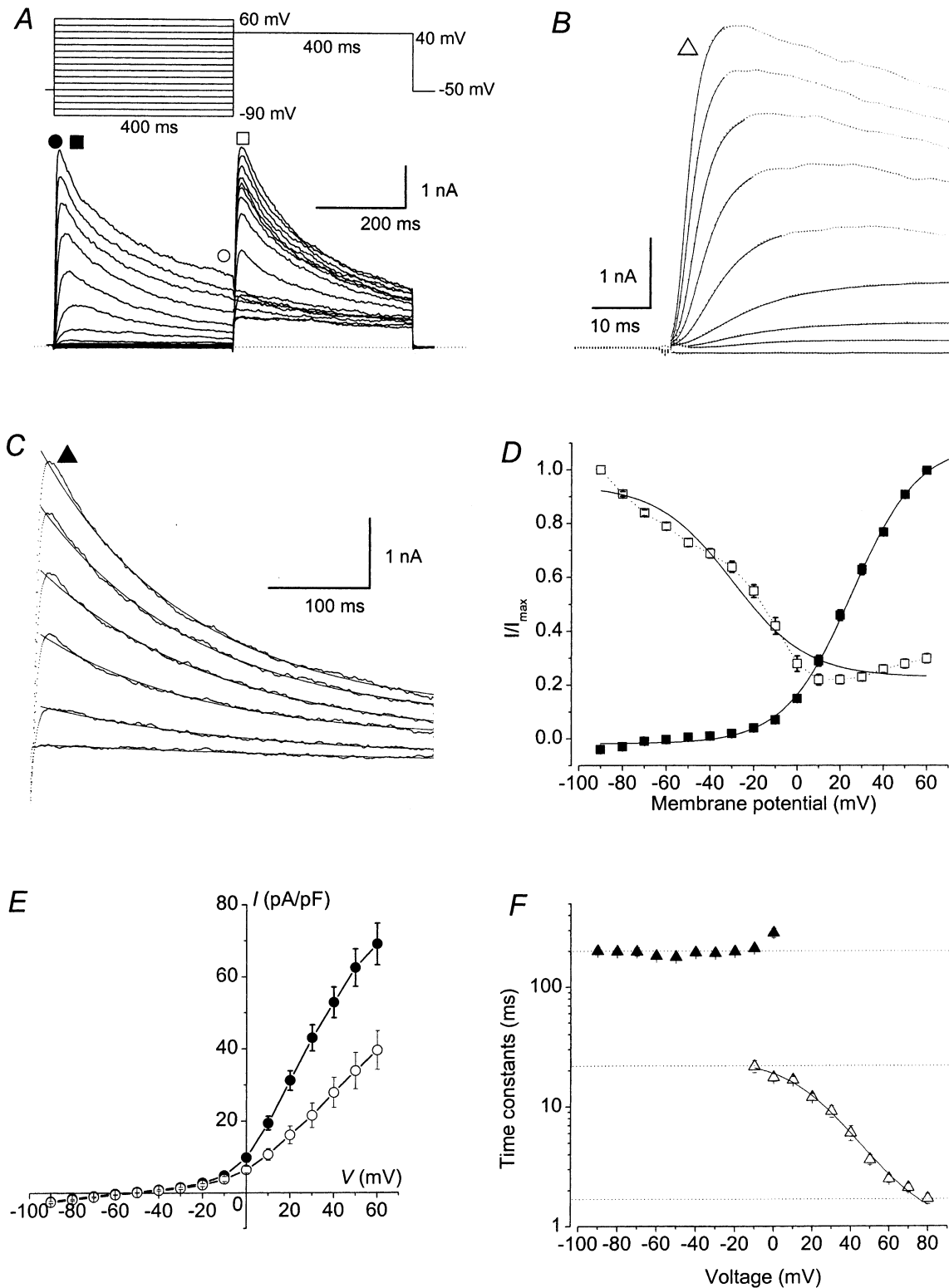


Fig. 4. Biophysical properties of K⁺ currents. (A) Steady-state inactivation of outward current. To measure the activation and inactivation kinetics, the channels were activated by command steps to between -90 and +60 mV from a holding potential of -50 mV. (B and C) The same current traces shown in A are shown in an expanded time and amplitude scale. These figures demonstrate the analysis of activation and inactivation properties of current, respectively. Note that single-exponential curves and current traces

superimposed. (D) Half-maximal voltage for K⁺ current activation and inactivation. (E) The values of both peak outward (●) and pseudo steady-state current densities (at the end of 400 ms, ○) are plotted versus membrane potentials ($n = 21$). (F) Time constants for both the activation (△, B) and inactivation (▲, C) were estimated by fitting either the current rise or decay with the single-exponential function of the Chebyshev method.

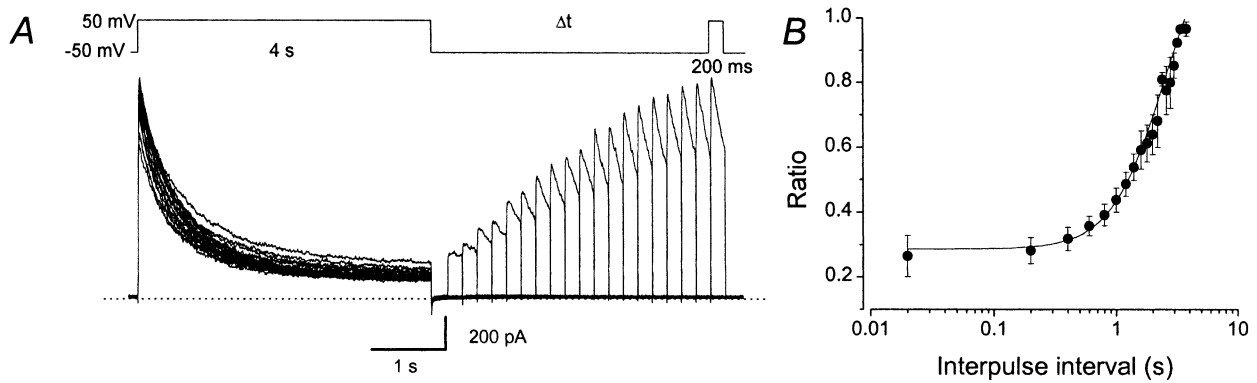


Fig. 5. Recovery of K^+ channels from inactivation. (A) Outward K^+ currents were recorded using a combination of two pulses to constant $+50$ mV from a holding potential of -50 mV. Tested interpulse intervals ranged between $0.02 - 3.8$ s. (B) The peak

outward currents were normalized as I_{PULSE2}/I_{PULSE1} . The time constant ($\tau = 1.7 \pm 0.2$ s, $n = 4$) for recovery from inactivation was defined by fitting data points with the single-exponential function.

under control conditions. The examples of raw data from which this $I-V$ plot was calculated are shown in Fig. 4A. At more depolarized potentials, i.e., starting at -20 mV, the cells show a pronounced outward current that increased as the membrane potential became more depolarized (Fig. 4A, E). K^+ current displayed outward rectification at depolarized voltages positive to -20 mV and a tendency to saturate between $+50$ and $+60$ mV (Fig. 4E). The average values of peak current densities and those measured at the end of 400-ms pulses at $+60$ mV (holding potential: -50 mV) were 71.1 ± 5.9 pA pF $^{-1}$ and 40.2 ± 5.2 pA pF $^{-1}$ ($n = 22$), respectively. The latter values further confirm holding potential-dependent activation of channel (see above, Fig. 1 C, D).

The recovery of K^+ channels from inactivation was relatively slow. In order to study this property of the channel, the outward K^+ currents were recorded using a double-pulse protocol. Membrane potential was depolarized to constant $+50$ mV from a holding potential of -50 mV and the interval between two pulses was varied from 0.02 to 3.8 s (Fig. 5A). Note that recovery from inactivation was almost complete only after 3 s. For the analysis of recovery from inactivation, the currents evoked by the second pulse were normalized to those activated by the first one (as I_{PULSE2}/I_{PULSE1}), and the obtained ratio was plotted versus interpulse intervals (Fig. 5B). The latter value at $+50$ mV was best fitted to a single-exponential function with a mean time constant of 1.7 ± 0.2 s ($n = 4$). These data support the notion that in *Helix* heart the total outward K^+ currents may consist of multiple components (see Discussion).

Discussion

Delayed rectifier (I_K) and A-type K^+ currents (I_A) were first characterized in neurons of molluscs

(Connor & Stevens, 1971a, 1971b; Neher, 1971; Thompson, 1977). In cardiac cells of molluscs, I_K and I_A are also present and they have been shown to be differently sensitive to holding potentials (Curtis et al., 1999; Yeoman & Benjamin, 1999). In *Lymnaea*, I_A channels are completely inactivated at holding potentials of -40 mV, but not the I_K channels. Moreover, I_K could be activated even from a holding potential of -10 mV. I_K in *Lymnaea* has been shown to be sensitive to TEA; A-type K^+ current was blocked by low concentrations of 4-AP ($1-5$ mmol l $^{-1}$) and was relatively insensitive to 10 mmol l $^{-1}$ TEA (Yeoman & Benjamin, 1999). I_A currents in this preparation are masked under delayed rectifier currents and could be unmasked by application of 10 mmol l $^{-1}$ TEA. In *Helix*, already under control conditions (Fig. 1), the current waveforms were different from those in *Lymnaea*. These currents, unlike most A-type K^+ currents, including those in *Lymnaea* heart, are highly sensitive to external TEA with an IC_{50} of 336 ± 142 μ mol l $^{-1}$ ($n = 3-10$; Fig. 2D). Similar sensitivity to TEA (IC_{50} of 88 ± 12 μ mol l $^{-1}$) is known only for the atypical cloned A-type K^+ currents encoded by HKShIIIc (human K^+ channel ShIII cDNA) gene (Rudy et al., 1991). Since an external TEA exhibits high affinity to A-type K^+ currents in *Helix* heart, one might suggest that its effects should not be voltage-dependent. This was, indeed, the case (Fig. 2E). Thus, our data revealed that neither IC_{50} (data not shown) nor K_D for TEA block (Fig. 2F) of I_A currents in *Helix* heart, unlike those in neurons (Denton & Leiter, 2002) of the same species, were significantly voltage-dependent. Extracellular TEA (100 μ mol l $^{-1}$) reduced the amplitude of peak outward K^+ current and decreased the decay of current. The latter effects resulted in a crossover of current with those recorded under control conditions. It is interesting to note that, although the current waveforms under control conditions are not identical,

the observed activation threshold of -20 mV for the delayed rectifier current in *Lymnaea* ventricular cells is comparable to that recorded in *Helix*. The differences in outward-current waveforms probably underlie the relatively slow in vivo heart rate in *Lymnaea* (0.3 Hz) compared with *Helix* (~ 1 Hz).

Thus, in *Helix* heart, similar to mammals, probably several or at least two voltage-dependent K^+ channels contribute to the total outward currents. One of the components may be similar to the A-type K^+ current encoded by *Kv4.2* (Bähring et al., 2001; Nadal et al., 2001). We came to this conclusion after detailed analysis of the activation kinetics of currents evoked by a stepwise change in membrane potential (Fig. 4A). The activation of current was voltage dependent and relatively fast (Fig. 4B). Approximation of activation time constants between -10 and $+80$ mV yielded a half-maximal value ($V_{0.5}$) of $+18.7 \pm 3.8$ mV, and the τ value at $+20$ mV (a value close to $V_{0.5}$) was 11.9 ± 0.7 ms (Fig. 4F; $n = 20$). Unlike A-currents described in *Lymnaea*, the activation of currents in *Helix* was relatively fast, ranging from 21.5 ± 2.4 ms at -10 mV to 6 ± 0.8 ms at $+40$ mV (Fig. 4B, F; $n = 20$, $P < 0.001$). The values for the same voltages are about half those described for the cardiac cells of the pond snail, i.e., 50.4 ± 5.2 and 12 ± 1.4 ms, respectively (Yeoman & Benjamin, 1999). This discrepancy probably resulted from differences in experimental conditions, but it may also indicate variation in the properties of identical channels between different species of animals. Moreover, since the majority of native channels represent a combination of both $Kv \alpha$ and β subunits (Trimmer, 1998), possible effects of auxiliary subunits may be suggested, as evidenced by comparison of activation time constants with those in previous studies of mammalian A-type K^+ currents either encoded by *Kv4.2* alone or co-encoded by *Kv4.2* and *KChIP*, i.e., 7.3 ± 0.6 and 5 ± 0.4 ms, respectively (Nadal et al., 2001). Interestingly, these τ values and those obtained in our studies (see above, Fig. 4B, F) were comparable within the same membrane potentials. Inactivation of the current in *Lymnaea* was fairly rapid, and its amplitude decreased by $53.3 \pm 4.9\%$ during the 200-ms voltage step. This phenomenon was also shown under similar but not identical conditions (for example, pulse duration and the absence of TEA differed) in *Helix*, where $50.2 \pm 1.8\%$ ($n = 18$) of current was inactivated within 250 ms at $+40$ mV (Fig. 1B). Although in *Helix* the cardiac current waveforms were clearly distinct from those of neurons, the previous organ, similarly to the latter one, may possess two or three kinds of A-type K^+ currents (Bal et al., 2001). This suggestion is evidenced by experiments (Fig. 1A) that were carried out, similar to those in neurons (Furukawa, Kandel & Pfaffinger, 1992; Alekseev & Ziskin, 1995; Bal et al., 2001), at a holding potential of -40 mV, a

membrane potential at which a traditional A-type K^+ currents are inactivated. The recovery of K^+ channels from inactivation in *Helix* cardiac cells, unlike those of the A-type K^+ currents encoded by *Kv4.2* (Bähring et al., 2001), was relatively slow with a mean time constant of 1.7 ± 0.2 s ($n = 4$). Therefore, we suggest that the total outward currents in *Helix* may have multiple components.

Closer examination of steady-state inactivation kinetics led us to an interesting observation. Namely, the voltage dependency of inactivation was not classical, but like *Kv2.1* U-shaped (Klemic et al., 1998), exhibiting less inactivation at more depolarized membrane potentials. After taking into account this phenomenon and the above-mentioned outcome of analysis, we suggest that channels encoded by *Kv2.1* and *Kv4.2*, similarly to those in mammals, do exist in land pulmonates of the *Helix* genus.

The support of the Deutsche Forschungsgemeinschaft within SFB320 "Herzfunktion und ihre Regulation" is gratefully acknowledged. The authors greatly appreciate the excellent technical support of Isolde Villhauer and Klara Güth.

References

- Alekseev, S.I., Ziskin, M.C. 1995. Two types of A-channels in *Lymnaea* neurons. *J. Membrane Biol.* **146**:327–341
- Bähring, R., Boland, L.M., Varghese, A., Gebauer, M., Pongs, O. 2001. Kinetic analysis of open- and closed-state inactivation transitions in human *Kv4.2* A-type potassium channels. *J. Physiol* **535**:65–81
- Bal, R., Janahmadi, M., Green, G.G., Sanders, D.J. 2001. Two kinds of transient outward currents, I_A and I_{Adepol} , in F76 and D1 soma membranes of the subesophageal ganglia of *Helix aspersa*. *J. Membrane Biol.* **179**:71–78
- Brezden, B.L., Benjamin, P.R., Gardner, D.R. 1991. The peptide FMRFamide activates a divalent cation-conducting channel in heart muscle cells of the snail *Lymnaea stagnalis*. *J. Physiol.* **443**:727–738
- Brezden, B.L., Gardner, D.R., Morris, C.E. 1986. A potassium-selective channel in isolated *Lymnaea stagnalis* heart muscle cells. *J. Exp. Biol.* **123**:175–189
- Brezden, B.L., Yeoman, M.S., Gardner, D.R., Benjamin, P.R. 1999. FMRFamide-activated Ca^{2+} channels in *Lymnaea* heart cells are modulated by "SEELY," a neuropeptide encoded on the same gene. *J. Neurophysiol.* **81**:1818–1826
- Buckett, K.J., Dockray, G.J., Osborne, N.N., Benjamin, P.R. 1990. Pharmacology of the myogenic heart of the pond snail *Lymnaea stagnalis*. *J. Neurophysiol.* **63**:1413–1425
- Connor, J.A., Stevens, C.F. 1971a. Inward and delayed outward membrane currents in isolated neural somata under voltage clamp. *J. Physiol.* **213**:1–19
- Connor, J.A., Stevens, C.F. 1971b. Voltage clamp studies of a transient outward membrane current in gastropod neural somata. *J. Physiol.* **213**:21–30
- Curtis, T.M., Depledge, M.H., Williamson, R. 1999. Voltage-activated currents in cardiac myocytes of the blue mussel, *Mytilus edulis*. *Comp. Biochem. Physiol A* **124**:231–241
- Denton, J.S., Leiter, J.C. 2002. Anomalous effects of external TEA on permeation and gating of the A-type potassium current in *H. aspersa* neuronal somata. *J. Membrane Biol.* **190**:17–28

- Furukawa, Y., Kandel, E.R., Pfaffinger, P. 1992. Three types of early transient potassium currents in *Aplysia* neurons. *J. Neurosci.* **12**:989–1000
- Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100
- Hodgkin, A.L., Huxley, A.F. 1952. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**:449–472
- Huddart, H., Hill, R.B. 1996. Modulatory mechanisms in the isolated internally perfused ventricle of the whelk *Busycon canaliculatum*. *Gen. Pharmacol* **27**:809–818
- Isenberg, G., Klockner, U. 1982. Calcium tolerant ventricular myocytes prepared by preincubation in a “KB medium”. *Pfluegers Arch.* **395**:6–18
- Klemic, K.G., Shieh, C.C., Kirsch, G.E., Jones, S.W. 1998. Inactivation of Kv2.1 potassium channels. *Biophys. J.* **74**:1779–1789
- Kodirov, S., Zhuravlev, V., Kreye, V. 1995. The membrane conductance of cardiac single cells obtained from the edible snail, *Helix pomatia*, is carried mainly by K⁺ ions. In: The 8th Symposium on Invertebrate Neurobiology, Tihany, Hungary
- Kuwasawa, K., Hill, R. 1997. Evidence for cholinergic inhibitory and serotonergic excitatory neuromuscular transmission in the heart of the bivalve *Mercuria mercenaria*. *J. Exp. Biol* **200**:2123–2135
- Maruyama, I., Yoshida, C., Kobayashi, M., Oyamada, H., Momose, K. 1987. Preparation of single smooth muscle cells from guinea pig taenia coli by combinations of purified collagenase and papain. *J. Pharmacol Methods* **18**:151–161
- Nadal, M.S., Amarillo, Y., Vega-Saenz de Miera, E., Rudy, B. 2001. Evidence for the presence of a novel Kv4-mediated A-type K⁺ channel-modifying factor. *J. Physiol.* **537**:801–809
- Neher, E. 1971. Two fast transient current components during voltage clamp on snail neurons. *J. Gen. Physiol* **58**:36–53
- Pfrunder, D., Kreye, V.A. 1991. Tedisamil blocks single large-conductance Ca²⁺-activated K⁺ channels in membrane patches from smooth muscle cells of the guinea-pig portal vein. *Pfluegers Arch.* **418**:308–312
- Rudy, B., Sen, K., Vega-Saenz de Miera, E., Lau, D., Ried, T., Ward, D.C. 1991. Cloning of a human cDNA expressing a high voltage-activating, TEA-sensitive, type-A K⁺ channel, which maps to chromosome 1 band p21. *J. Neurosci. Res.* **29**:401–412
- Thompson, S.H. 1977. Three pharmacologically distinct potassium channels in molluscan neurones. *J. Physiol* **265**:465–488
- Trimmer, J.S. 1998. Regulation of ion channel expression by cytoplasmic subunits. *Curr. Opin. Neurobiol* **8**:370–374
- Yeoman, M.S., Benjamin, P.R. 1999. Two types of voltage-gated K⁺ currents in dissociated heart ventricular muscle cells of the snail *Lymnaea stagnalis*. *J. Neurophysiol* **82**:2415–2427
- Yeoman, M.S., Brezden, B.L., Benjamin, P.R. 1999. LVA and HVA Ca²⁺ currents in ventricular muscle cells of the *Lymnaea* heart. *J. Neurophysiol* **82**:2428–2440
- Zhuravlev, V., Bugaj, V., Kodirov, S., Safonova, T., Staruschenko, A. 2001. Giant multimodal heart motoneurons of *Achatina fulica*: a new cardio regulatory input in pulmonates. *Comp. Biochem. Physiol A* **130**:183–196