Two Components of Cardiac Delayed Rectifier K^+ Current

Differential Sensitivity to Block by Class III Antiarrhythmic Agents

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ABSTRACT An envelope of tails test was used to show that the delayed rectifier K^+ current (I_K) of guinea pig ventricular myocytes results from the activation of two outward K^+ currents. One current was specifically blocked by the benzenesulfonamide antiarrhythmic agent, E-4031 (IC_{50} = 397 nM). The drug-sensitive current, "I_{Kr}" exhibits prominent rectification and activates very rapidly relative to the slowly activating drug-insensitive current, "I_{Ks}." I_{Ks} was characterized by a delayed onset of activation that occurs over a voltage range typical of the classically described cardiac I_K. Fully activated I_{Ks}, measured as tail current after 7.5-s test pulses, was 11.4 times larger than the fully activated I_{Kr}. I_{Kr} was also blocked by d-sotalol (100 μM), a less potent benzenesulfonamide Class III antiarrhythmic agent. The activation curve of I_{Kr} had a steep slope (+7.5 mV) and a negative half-point (−21.5 mV) relative to the activation curve of I_{Ks} (slope = +12.7 mV, half-point = +15.7 mV). The reversal potential (E_{rev}) of I_{Kr} (−93 mV) was similar to E_K (−94 mV for $[K^+]_o$ = 4 mM), whereas E_{rev} of I_{Ks} was −77 mV. The time constants for activation and deactivation of I_{Kr} made up a bell-shaped function of membrane potential, peaking between −30 and −40 mV (170 ms). The slope conductance of the linear portion of the fully activated I_{Kr}-V relation was 22.5 S/F. Inward rectification of this relation occurred at potentials > −50 mV, resulting in a voltage-dependent decrease in peak I_{Kr} at test potentials > 0 mV. Peak I_{Kr} at 0 mV averaged 0.8 pA/pF (n = 21). Although the magnitude of I_{Kr} was small relative to fully activated I_{Ks}, the two currents were of similar magnitude when measured during a relatively short pulse protocol (225 ms) at membrane potentials (−20 to +20 mV) typical of the plateau phase of cardiac action potentials.

INTRODUCTION

Depending upon species and cell type, a number of different K^+ currents may be responsible for limiting cardiac action potential duration (APD). In guinea pig

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ventricular myocytes, the currents responsible for termination of the action potential plateau are the slowly activating, delayed rectifier K^+ current (Matsuura et al., 1987), I_K, and perhaps the recently described plateau K^+ current, I_{Kp} (Yue and Marban, 1988). I_K has been well characterized in vertebrate cardiac cells (Bennett et al., 1985; Giles and Shibata, 1985; Hume et al., 1986; Simmons et al., 1986; Matsuura et al., 1987; Shibasaki, 1987). In other cardiac cell types, different outward K^+ currents are responsible for terminating the plateau phase of the action potential. For example, the major outward K^+ current responsible for repolarization in adult rat ventricular myocytes is a transient (inactivating) outward K^+ current (Josephson et al., 1984). Repolarization of action potentials in rabbit sinoatrial and atrioventricular node cells is accomplished by activation of a transient outward (Nakayama and Irisawa, 1985), and more importantly by a "delayed rectifier" K^+ current (DiFrancesco et al., 1979; Nakayama et al., 1984; Shibasaki, 1987) that activates much faster than the typical I_K of guinea pig ventricular cells.

One category of antiarrhythmic drugs (so-called Class III antiarrhythmic agents) are characterized by their ability to prolong cardiac APD without having significant effects on either Na^+ or Ca^{2+} currents. Sotalol, a benzenesulfonamide β-blocker and Class III antiarrhythmic agent, has been reported to block I_K in rabbit Purkinje fibers (Carmeliet, 1985). Several structural analogues of sotalol have recently been developed which retain Class III antiarrhythmic activity, but lack β-blocker activity. One example is E-4031 (1-[2-(6-methyl-2-pyridyl)ethyl]-4-(4-methylsulfonylaminobenzoyl)piperidine; Fig. 8). E-4031 is a potent Class III agent, prolonging APD at concentrations as low as 0.1 μM (Sawada et al., 1988; Sanguinetti et al., 1989), and has been reported to block I_K (Sawada, 1989). In contrast to this report, we have not observed significant block of time-dependent I_K during large depolarizing pulses in isolated guinea pig ventricular myocytes with either d-sotalol or E-4031 (Sanguinetti et al., 1989). Instead, we have found that both of these benzenesulfonamides block a component of I_K not previously described in guinea pig ventricular myocytes (Sanguinetti and Jurkiewicz, 1989).

METHODS

Cell Preparation

Guinea pig ventricular myocytes were isolated using a modification of the procedure described by Mitra and Morad (1985) as detailed previously (Kamp et al., 1989). The isolated cells were stored in a solution of the following composition, in millimolar: 132 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.2. The cells were kept at room temperature until used, within 10 h after isolation.

Voltage-Clamp Technique and Microelectrode Fabrication

A List EPC-7 was used to clamp either voltage or current of the isolated cells. Series resistance was compensated 40–70%, and current was low-pass filtered with an eight-pole Bessel filter at a cut-off frequency of 2 kHz.

The suction microelectrode technique described by Giles and Shibata (1985) was used to voltage clamp the cells. Microelectrodes were made by using a two-stage puller (model PP-83; Narashige, Tokyo, Japan) with square bore (1.0 mm o.d.) borosilicate capillary tubing (Glass

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Company of America, Millville, NJ). Pipettes were filled with 1 M K gluconate and 50 mM KCl. The electrodes had resistances of 4–8 MΩ when filled with this solution. After establishing a gigaseal upon the cell surface, suction was applied to the pipette using a 1.0-ml gas-tight syringe attached to the suction port of the microelectrode holder via air-tight tubing. As noted by Giles and Shibata (1985), K^+ leakage from the pipette into the cell was negligible when short-shanked electrodes were used and when negative pressure (0.5–1.0 ml on syringe) was maintained. Lack of significant K^+ leakage was assured for each cell by repeatedly measuring the resting potential in current-clamp mode as the experiment progressed. Assuming a $[K^+]_i$ of 140 mM, a 2-mV hyperpolarization in resting membrane potential would correspond to a 10-mM rise in $[K^+]_i$. If the membrane potential hyperpolarized >2 mV the experiment was discarded. Such experiments were rare and only occurred when negative pressure on the pipette was not adequately maintained. The advantage of this technique is that dialysis of the cell is minimized, preventing washout of potential intracellular regulators of channel activity. The disadvantage also relates to the lack of dialysis, since control of internal ion concentrations is not possible. As the experiment progressed, in some cells during large depolarizing pulses an outward shift in the instantaneous current occurred. To correct for this shift, drug-sensitive current was defined as changes in time-dependent current relative to the control condition. This procedure was justified since time-dependent current in untreated cells did not change during the time (<5 min) required for these experiments. Currents measured in this manner were indistinguishable with respect to kinetics and voltage dependence compared with currents from the many cells in which there was no detectable shift in instantaneous current.

The cells were bathed in the same solution used to store the cells, with the addition of 200 nM nisoldipine to block L-type Ca^{2+} currents. Nisoldipine is a specific Ca^{2+} channel blocker, without effects on K^+ currents (Kass, 1982). Na^+ current was inactivated by a holding potential of −40 mV. The cell chamber was superfused with solutions at a rate of 1.0 ml/min and the temperature was maintained at 35°C.

Current-Voltage Relationship of Drug-sensitive Currents

The holding potential was −40 mV for these experiments and the cell was clamped for 225 ms to increasingly positive potentials in 10-mV steps at a rate of 12 pulses/min. Tail currents were measured upon repolarization to −40 mV for 750 ms. A split digital clock rate was used to digitize these currents. Thus, in Figs. 1, 7, 11, and 15, the tail currents are plotted on a reduced time scale, such that they appear as long as the currents recorded during the depolarizing steps. Drug-sensitive currents were obtained by digital subtraction of currents measured in the presence of 5 μM E-4031 or 100 μM d-sotalol from control currents measured before exposure to the drug.

Data Analysis

Data acquisition and analysis was performed using pClamp software (Axon Instruments, Inc., Foster City, CA). Curve fitting of current traces and Boltzmann distributions (activation and inactivation curves) were performed using the Marquardt least-squares method of nonlinear regression analysis. All data are expressed as mean ± SEM.

Chemicals

E-4031 was synthesized by Dr. H. Selnick of the Department of Medicinal Chemistry at Merck, Sharp and Dohme Research Laboratories (West Point, PA). d-Sotalol was kindly provided by Bristol Meyers Company (Evansville, IN). Each drug was dissolved in distilled water to make 10-mM stock solutions.

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RESULTS

E-4031 Blocks a Component of I_K

We and others have reported that E-4031 and sotalol prolong APD of guinea pig papillary muscles at concentrations between 0.1–3 μM for E-4031 (Sawada et al., 1988; Sanguinetti et al., 1989), and 1–300 μM for sotalol (Carmeliet, 1985). The maximum increase in APD was 30–40% with either drug. We confirmed this effect with E-4031 in isolated guinea pig ventricular myocytes. Exposure to 3 μM E-4031 for 1 min, sufficient time for the drug to reach steady-state effects, lengthened APD measured at 90% repolarization from an average of 339 ± 44 ms to 420 ± 64 ms (n = 4), an increase of 24%. Note that only the rate of phase 2 repolarization was slowed by E-4031 (Fig. 1 *A*), an effect observed in all the cells studied. The initial and

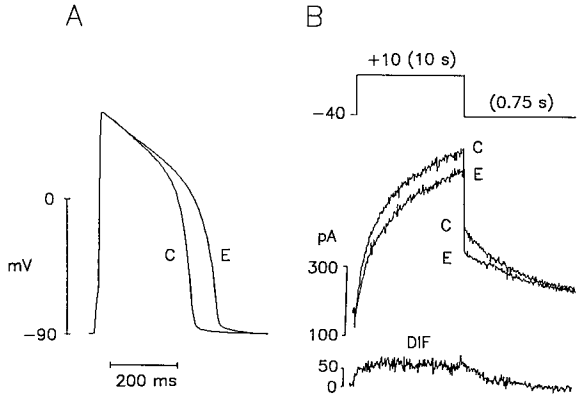

FIGURE 1. Effect of E-4031 on action potentials and outward currents in isolated guinea pig ventricular myocytes. (*A*) Action potentials recorded during stimulation of a cell by 2-ms current pulses at a rate of 12 pulses/min before (*C*) and after exposure to 3 μM E-4031 (*E*). (*B*) Effect of E-4031 on outward K^+ currents. Current was measured in response to a depolarizing pulse to +10 mV for 10 s, followed by repolarization to −40 mV for 0.75 s. The voltage-pulse protocol is shown above the current traces. The activation phase of currents was fit with a two-exponential function: $I(t) = A_0 + A_1\exp[1\text{-}(t/\tau_1)] + A_2\exp[1 - (t/\tau_2)]$. For the control current (*C*), A_1 = 188 pA, A_2 = 329 pA, τ_1 = 771 ms, τ_2 = 5,778 ms. In the presence of 5 μM E-4031 (*E*), A_1 = 150 pA, A_2 = 342 pA, τ_1 = 820 ms, τ_2 = 6,477 ms. E-4031-sensitive current (*DIF*) was obtained by digital subtraction of the two traces in *A*. The time constant of the E-4031-sensitive tail current was 179 ms.

terminal rates (so-called phases 1 and 3) of repolarization of the action potential were unaffected, suggesting that E-4031 affects a current activated at plateau potentials.

In the presence of 200 nM nisoldipine, only outward K^+ currents were observed upon depolarization of the membrane potential from a holding potential of −40 mV. The outward currents included the steady-state outward current contributed by the inward rectifier K^+ current (I_{K1}) and I_K. As shown in Fig. 1 *B*, 5 μM E-4031 had no effect on the holding current at −40 mV (I_{K1}), but decreased the time-dependent current during the depolarizing pulse to +10 mV. The initial (within 5 ms) inward shift of current measured upon depolarization in this and other pulse protocols results from the negative slope conductance of I_{K1} in these cells at potentials > −50

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mV (Hume and Uehara, 1985; Kurachi, 1985). Digital subtraction of the two current traces revealed that E-4031 blocked a rapidly activating component of outward current and a component of the tail current (Fig. 1 *B, bottom trace*). Note that there was no inactivation of the drug-sensitive current during the 10-s voltage pulse. One possible interpretation of this experiment is that E-4031 blocks an outward current with activation kinetics much faster than the classical cardiac I_K.

Concentration-dependent Effects of E-4031

The concentration-dependent effects of E-4031 on outward current was assessed by measuring the decrease in tail currents at −40 mV, after a 225-ms pulse to −10 mV. This pulse protocol minimized the contribution of the slowly activating, drug-insensitive component of the total tail current, as can be seen in Fig. 6 *B*. Each cell was exposed to only one or two concentrations of E-4031 to minimize any time-dependent changes in K^+ current magnitude not related to the addition of drug. Measured in this manner, the IC_{50} for block of outward current by E-4031 was

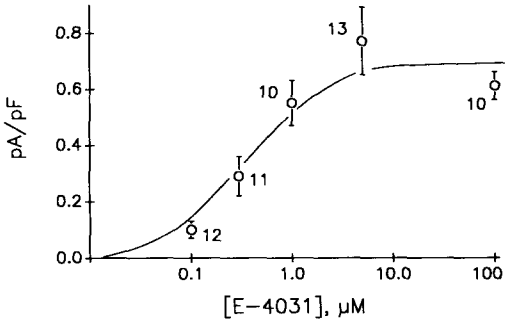

FIGURE 2. Concentration-dependent block of tail currents by E-4031. Drug-sensitive currents expressed relative to cell capacitance were measured upon repolarization to −40 mV, after a 225-ms pulse to −10 mV. The number of cells is given by each data point. The average data were fitted by the equation: $I = I_{max}/(IC_{50}/[\text{E-4031}] + 1)$, where I = drug-sensitive tail current. IC_{50} and I_{max} were estimated at 397 nM and 0.72 pA/pF, respectively ($R^2 = 0.91$).

estimated to be 397 nM (Fig. 2). In all remaining experiments we used 5 μM E-4031 to ensure complete block of the drug-sensitive component of I_K. We did not examine the possibility of voltage- or use-dependent block of outward current by nonsaturating concentrations of the drug.

Envelope of Tails Test

I_K of guinea pig ventricular myocytes is usually assumed to result from the activation of a single type of K^+ channel. However, we propose that the time-dependent outward current measured in guinea pig ventricular cells represents the activation of two distinct currents, one of which is specifically blocked by E-4031 and d-sotalol (Sanguinetti et al., 1989; Sanguinetti and Jurkiewicz, 1989). One test of this hypothesis is the envelope of tails test (Noble and Tsien, 1969). This test predicts that if I_K results from the conductance of a single type of channel, then the magnitude of tail currents after a given depolarizing pulse of variable duration should increase in parallel to the time course of activation of outward current during the pulse. In

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other words the ratio of tail current/time-dependent current ($\Delta I_{Ktail}/\Delta I_K$) should be constant, regardless of the pulse duration.

Cells were held at a holding potential of −40 mV and pulsed to +40 mV for a variable time (Δt), from 0.05 to 5 s. Sufficient time between test pulses was allowed for full deactivation of tail currents before application of another depolarizing pulse. In five experiments, the envelope of tails test was performed in the same cell before and after addition of 5 μM E-4031. These results were indistinguishable from those obtained in several other unpaired experiments. Cumulative data (10–14 cells) are shown in Fig. 3 *B*, where the $\Delta I_{Ktail}/\Delta I_K$ ratio is plotted as a function of the test pulse duration (Δt). The data were normalized in this fashion to eliminate cell-to-cell variability due to differences in current amplitudes. In untreated cells, tail currents were larger than time-dependent currents for very short pulses (<100 ms), but as the

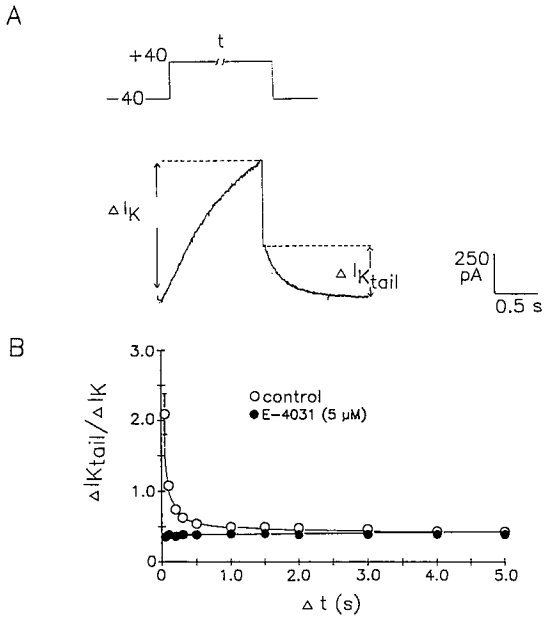

FIGURE 3. Envelope of tails test for I_K. (*A*) The procedure used to determine $\Delta I_{Ktail}/\Delta I_K$. Note, the current shown in the inset was recorded in the presence of E-4031. (*B*) Current ratio as a function of pulse duration. Data represent the mean ± SEM of 10 control and 14 E-4031-exposed cells. For most data points the SE bars are smaller than the symbol size. Line through E-4031 data points is: $\Delta I_{Ktail}/\Delta I_K = 0.384$.

pulse duration was lengthened the time-dependent current slowly increased in magnitude, such that for a 5-s pulse a ratio of 0.427 was attained. This resulted from an increase in (*a*) the magnitude of drug-insensitive current and (*b*) the contribution of this current to the total tail current measured upon repolarization. In cells treated with 5 μM E-4031, the $\Delta I_{Ktail}/\Delta I_K$ ratio was constant (0.384 ± 0.004), regardless of the duration of the depolarizing pulse to +40 mV. This is close to the predicted ratio (0.4), calculated from the ratio of the driving force at +40 and −40 mV for a nonrectifying, K^+-selective outward current. These properties are characteristic of I_K in frog atrial cells, a preparation with only a single outward K^+ current (Hume et al., 1986; Simmons et al., 1986). In the presence of 5 μM E-4031, a single outward K^+ current remains, which we refer to as I_{Ks} (slowly activating delayed rectifier K^+

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current). Thus, after pulses to +40 mV, the envelope of tails test is only satisfied in the presence of E-4031.

We also directly tested whether E-4031-sensitive current satisfied the envelope of tails test. In these experiments the depolarizing pulse of variable duration was to −10 mV. Rectification of drug-sensitive K^+ current is incomplete at this potential (see below), such that both time-dependent outward currents during the pulse and tail currents are measurable upon digital subtraction of currents recorded before and after exposure to 5 μM E-4031. The average drug-sensitive currents measured during pulses ranging from 25 to 900 ms ($n = 9$) are plotted in Fig. 4. As expected for a single current, the increase in tail currents paralleled the increase in time-dependent outward currents.

Thus, the time-dependent outward current (I_K) measured in untreated cells represents at least two distinct currents, whereas only one component (I_{Ks}) is present after exposure to drug. We refer to the drug-sensitive current as I_{Kr} ("rapidly-activating" delayed rectifier K^+ current), to distinguish it from I_{Ks}. The remainder of

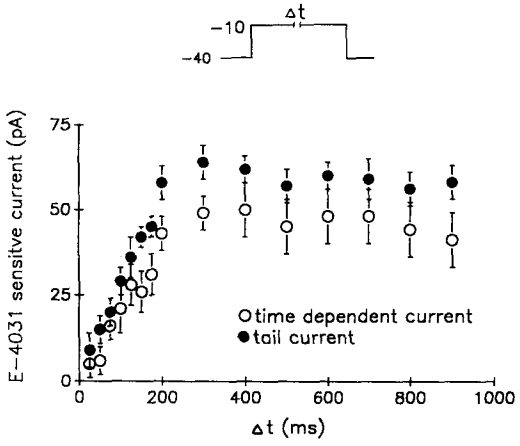

FIGURE 4. Envelope of tails test for E-4031-sensitive current. Time-dependent and tail currents blocked by 5 μM E-4031 were measured in response to pulses to −10 mV. The increase in tail current paralleled the increase in time-dependent current as the pulse duration was lengthened from 25 to 900 ms. Values represent mean ± SEM for nine cells.

this study is a characterization of I_{Kr} and I_{Ks}, with direct comparisons to the composite current, I_K.

Characterization of E-4031-sensitive (I_{Kr}) and -insensitive (I_{Ks}) Currents

Effect of E-4031 on outward K^+ currents. Activation of I_K in most cardiac preparations has a delayed onset, often referred to as a sigmoidal onset of activation. However, in guinea pig ventricular cells, sigmoidal activation of I_K was evident in control records only during pulses to test potentials >+30 mV (Fig. 5). Sigmoidal onset of activating outward current during depolarizations to potentials <+30 mV were only obvious after block of I_{Kr} by E-4031. As seen in Fig. 5, E-4031 had no effect on I_K during the pulse to +40 mV. In general, E-4031 only decreased time-dependent outward currents during depolarizing pulses to potentials <+40 or +50 mV. Even at concentrations as high as 100 μM, E-4031 did not significantly decrease time-dependent outward current measured during large depolarizing

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pulses. The time-dependent outward current measured at the end of 450-ms pulses to +40 mV was 4.46 ± 0.97 pA/pF before drug, and 4.50 ± 0.83 pA/pF after exposure to 100 μM E-4031 ($n = 5$). d-Sotalol (100 μM) also blocked time-dependent outward currents only during depolarizing pulses to potentials <+40 or +50 mV.

Current-voltage relationship of drug-sensitive currents. For these experiments, maximal effective concentrations (i.e., 5 μM E-4031 and 100 μM d-sotalol) were used to study the effects of these drugs on outward currents elicited by short depolarizing pulses, when the contribution of I_{Ks} to the total outward current would be minimized. Fig. 6 shows the current-voltage (*I-V*) plots for both time-dependent currents measured during the depolarization (*A*) and amplitudes of the tail currents measured upon repolarization to −40 mV (*B*). In each graph, the amplitudes of control currents, currents measured after exposure to E-4031, and the drug-sensitive currents are plotted. Note that exposure to E-4031 eliminates the outward hump of the control *I-V* curves. Fig. 7 shows examples of E-4031-sensitive currents obtained by digital subtraction of currents recorded in the presence of drug from control records. The drug-sensitive currents do not exhibit any obvious delay in

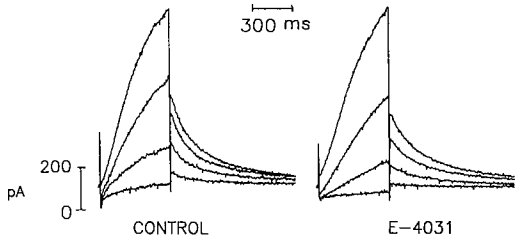

FIGURE 5. Outward K^+ currents recorded before and after exposure to 5 μM E-4031. Currents were recorded during 550-ms pulses to −20, 0, +20, and +40 mV from a holding potential of −40 mV. Note that the onset of I_K activation has an obvious delay only at +40 mV in control, whereas activation was delayed at all potentials in the presence of E-4031.

onset, and are fully activated during these short pulses at potentials ≥0 mV. At test potentials >0 mV, the currents during depolarizing pulses decrease, whereas the tail currents saturate. The results plotted in Fig. 6 *A* could be interpreted to mean that either (*a*) E-4031 blocks an inwardly rectifying component of the composite outward K^+ current or (*b*) I_K is a single current and E-4031 blocks it in a voltage-dependent manner. If E-4031 blocked I_K in a voltage-dependent manner, both the time-dependent and tail currents blocked by the drug should both display inward rectification. However, only the drug-sensitive time-dependent current rectifies, whereas the drug-sensitive tail currents saturate at ~+10 mV. Thus, consistent with the envelope of tails tests, E-4031 appears to block a distinct component of outward current (I_{Kr}) in these cells. I_{Kr} was measured during 225-ms steps in 12 other cells. The currents were normalized relative to cell size (total capacitance) by expressing current magnitude in pA/pF. The average *I-V* relation for all 13 cells is plotted in Fig. 8 *A*. The peak current at 0 mV varied from 0.5 to 1.2 pA/pF in this group of cells. The results from the same type of experiments with 100 μM d-sotalol ($n = 9$), a maximal effective concentration, are plotted in Fig. 8 *B*. Nearly identical results were obtained using this less potent benzenesulfonamide. In several experiments we

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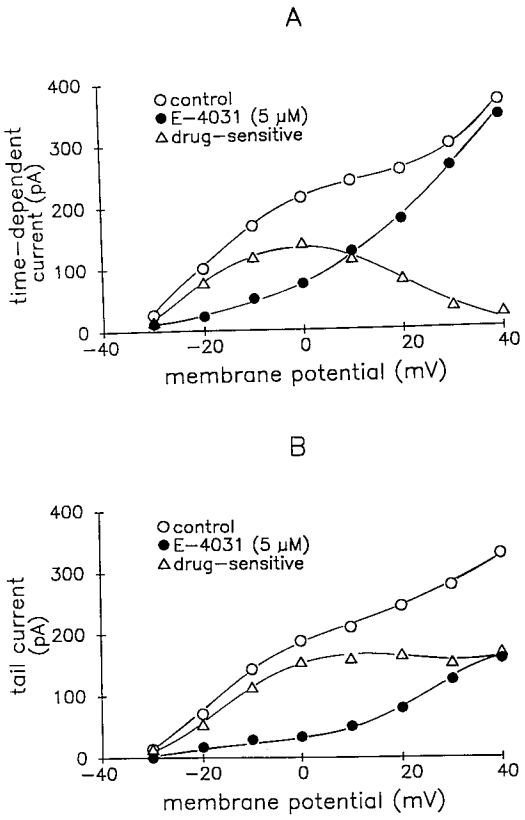

FIGURE 6. Current-voltage relationships recorded before and after exposure of a cell to E-4031. (*A*) Current-voltage plot of time-dependent outward currents measured during 225-ms pulses to the indicated membrane potentials. (*B*) Current-voltage plot of tail currents measured upon repolarization to −40 mV from the indicated membrane potentials.

found that in the presence of 100 μM d-sotalol, the addition of 5 μM E-4031 had no further effects (data not shown).

Voltage-dependent activation of I_K, I_{Kr}, and I_{Ks}. The voltage dependence of I_{Kr} activation was determined by measuring the amplitude of E-4031-sensitive tail currents after 550-ms pulses to test potentials ranging from −30 to +20 mV. This pulse duration was sufficient for nearly complete activation of I_{Kr} at the potentials

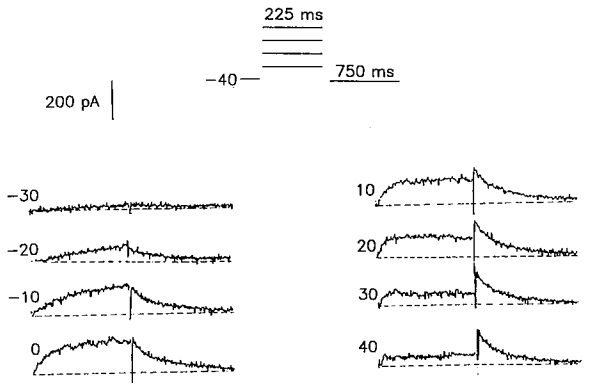

FIGURE 7. E-4031-sensitive currents. The drug-sensitive currents were measured at the indicated test potentials, ranging from −30 to +40 mV. Data are from same cell as Fig. 6.

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tested. The currents were normalized relative to the peak tail amplitude (assigned a value of 1.0) and this activation variable (n_∞) was plotted as a function of membrane potential (Fig. 9). The half-point of activation ($V_{1/2}$), which was determined by curve fitting the data to Eq. 1, was −21.5 mV, and the slope factor (k) was 7.5 mV.

$$n_\infty = 1/[1 + \exp[(V_{1/2} - E)/k]] \quad (1)$$

The voltage dependence of I_K and I_{Ks} activation was determined by measuring tail currents after 7.5-s test pulses to potentials ranging from −30 to +60 mV, from a

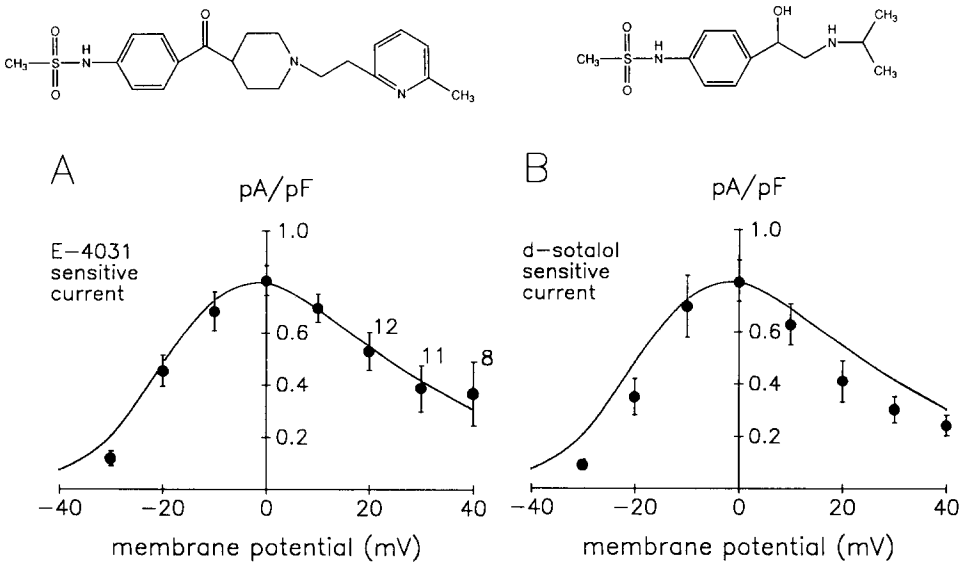

FIGURE 8. Current-voltage relation for I_{Kr}, defined as time-dependent current blocked by E-4031 (*A*) or d-sotalol (*B*). (*A*) Current blocked by 5 μM E-4031. Currents were normalized relative to cell capacitance. Data points represent the mean ± SEM of 13 cells, unless indicated otherwise. The average whole-cell capacitance for this group of cells was 94 ± 5 pF. The smooth curve is the calculated *I-V* relation based on Eq. 4 described in the Discussion. (*B*) Current blocked by 100 μM d-sotalol, also expressed relative to cell capacitance. Data represent the mean ± SEM of nine cells, except at +40 mV (n = 6). The average whole-cell capacitance for these cells was 86 ± 5 pF. The smooth curve is the same as the one plotted in *A*.

holding potential of −40 mV. Such long pulses were required to ensure nearly complete activation of I_{Ks}. Pulses were applied once every 16 s. In most cells direct comparison of activation curves measured before and after addition of drug was not possible since currents tended to run down during this procedure. Therefore, the voltage dependence of activation of I_K (before drug) and I_{Ks} (after drug) was determined in separate cells (Fig. 9). In the absence of drug, the steady-state activation curve of I_K had a half-point of +13 mV, and a slope factor of +14.1 mV (n = 11). In the presence of 5 μM E-4031, the steady-state activation curve of I_{Ks}

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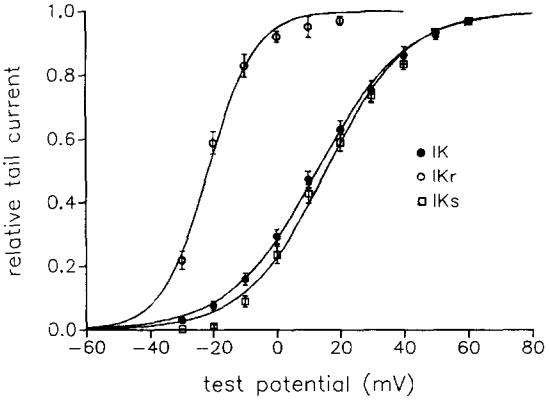

FIGURE 9. Voltage dependence of I_K, I_{Ks}, and I_{Kr} activation. As described in the text, E-4031-sensitive tail currents (I_{Kr}) were measured upon repolarization to −40 mV after a 550-ms test pulse to the indicated membrane potentials, then normalized relative to the maximum I_{Kr} tail current. In a separate set of experiments, tail currents were measured upon repolarization to −40 mV after a 7.5-s test pulse to the indicated membrane potential in the absence (I_K) or presence of 5 μM E-4031 (I_{Ks}). Tail current amplitudes were normalized relative to the maximum value. The smooth curves are least-squares fits of the averaged data to the Boltzmann distribution (Eq. 1). The half-activation voltage for I_{Kr} was −21.5 mV, and the slope factor was +7.5 mV (n = 9). The half-point (+13 mV) and slope factor (+14.1 mV) of the activation curves for I_K (n = 11) were similar to those determined for I_{Ks} (half-point = +15.7 mV; slope factor = +12.7 mV, n = 11).

had a half-point of +15.7 mV, and a slope factor of +12.7 mV (n = 11). The average amplitude of fully activated tail currents for I_K (11.6 ± 1.4 pA/pF, n = 11) was 11.4 times larger than the average value of fully activated I_{Kr} (1.02 ± 0.11 pA/pF, n = 17). For this reason the activation curves measured under the two conditions are only slightly different.

Reversal potential of I_{Kr} and I_K. To determine if I_{Kr} was selective for K^+, the reversal potential (E_{rev}) of this current was determined at two different $[K^+]_o$, 4 and 9 mM. E_{rev} was determined by measuring drug-sensitive tail currents recorded during repolarization to a range of potentials after an activating pulse (225 ms) to +20 mV, a voltage sufficient to fully activate the drug-sensitive current. The E_{rev} of I_{Kr} for $[K^+]_o$ = 4 and 9 mM was −93 ± 2 mV and −74 ± 0.8 mV, respectively (n = 6). Examples are shown in Fig. 10. This corresponds to a 54-mV change in E_{rev} for a

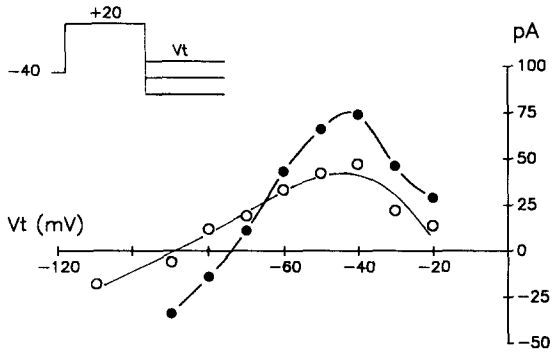

FIGURE 10. Reversal potential of I_{Kr}. E-4031-sensitive tail current amplitudes were measured at the indicated membrane potentials after an activating pulse to +20 mV for 225 ms. Two different cells were bathed in a solution with $[K^+]_o$ = 4 mM (O), or 9 mM (●). The reversal potentials of the drug-sensitive tail currents were −90 and −74 mV, respectively.

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10-fold change in $[K^+]_o$. Assuming $[K^+]_i = 140$ mM (Sheu et al., 1980; Desilets and Baumgarten, 1986), the E_{rev}'s predicted by the Nernst equation for $[K^+]_o = 4$ and 9 mM are -94 and -73 mV, respectively. Thus, I_{Kr} is selective for K^+.

E_{rev} of I_K was measured in the absence of drug after pulses to $+40$ mV for either 0.35 or 7.5 s in a solution with $[K^+]_o = 4$ mM. Tail current amplitude was measured relative to the current at time zero, determined by fitting tail currents to biexponential functions and extrapolating back to the beginning of the repolarization pulse. After an activating pulse to $+40$ mV, most of the tail current measured upon return to a given test potential, ranging from -60 to -95 mV, was due to deactivation of I_{Ks}. E_{rev} of I_K did not change significantly when determined after pulses of either 0.35 or 7.5 s; E_{rev} was $-75 + 2$ mV ($n = 7$) and $-77 + 3$ mV ($n = 7$), respectively. This indicates that under the conditions of our experiments significant accumulation/depletion of K^+ does not occur. In contrast to I_{Kr}, the E_{rev} of I_{Ks} indicates that this current is not purely selective for K^+. Thus, for $[K^+]_o = 4$ mM, E_{rev} of I_{Kr} is ~18 mV more negative than that measured for I_{Ks}.

Kinetics of activation and deactivation I_{Kr}. Based upon the findings that onset of I_{Ks} activation is sigmoidal and that deactivation is biexponential at some potentials, the activation variable of I_{Ks} is usually raised to the second power (Simmons et al., 1986; Matsuura et al., 1987). In contrast, the initial activation of the drug-sensitive current did not exhibit any obvious sigmoidal component (Figs. 1*B* and 7) and was well fit by a single exponential function. I_{Kr} tail current was adequately fit with a single exponential function in the majority of experiments, although some currents had a measurable slower component. This later component could result from a slight decline in I_{Ks} between the time the control and drug-exposed currents were recorded, or could represent a genuine second component. Considering the difficulty of accurately fitting the time course of very small and noisy currents (due to digital subtraction procedure), we chose to fit all deactivating currents with a single exponential function. The voltage-pulse protocol used to record activating and deactivating drug-sensitive currents is shown in Fig. 11, as are the average time constants determined as a function of test potential. Note that for the potentials (-20 and -30 mV) at which both activating and deactivating currents could be measured, the time constants measured by either pulse protocol were similar.

Fully activated I-V relation for I_{Kr}. The fully activated *I-V* relation for I_{Kr} was calculated based upon amplitudes of drug-sensitive currents divided by the expected increase in the activation variable for I_{Kr}: $I_{Kr}/[n_0 - n_\infty]$. For potentials ≥ -20 mV, I_{Kr} was determined as time-dependent, drug-sensitive currents measured during depolarization to a given test potential (protocol shown in Fig. 7). For potentials ≤ -20 mV, I_{Kr} was determined from drug-sensitive tail currents measured upon repolarization to a variable potential from $+20$ mV (protocol shown in Fig. 11 *A*). Fig. 12 is a plot of the fully activated I_{Kr}-*V* determined in this manner, expressed relative to cell capacitance. The I_{Kr}-*V* relationship is linear at voltages negative to -50 mV, and has a slope conductance of 22.5 S/F.

Specificity of E-4031

The usefulness of using E-4031 as a tool to dissect I_K into two distinct currents relies on the specificity of this drug. Therefore, we determined the effects of E-4031 on I_{Ca}

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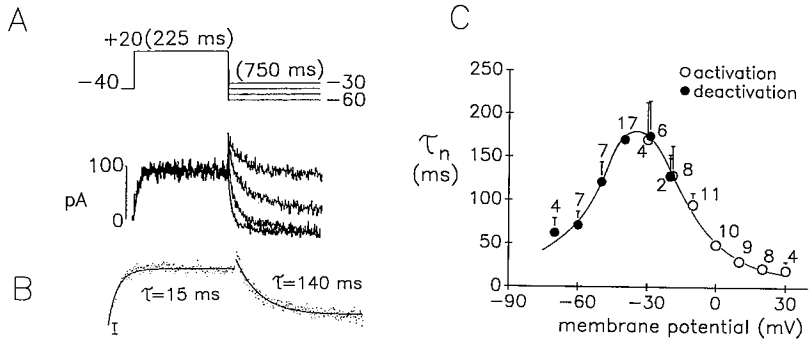

FIGURE 11. Time course of activation and deactivation of I_{Kr}. (*A*) E-4031-sensitive currents recorded during activation (at +20 mV) and deactivation. The drug-sensitive currents were fit to single exponential functions; activation: $I(t) = A_o + A_1[1 - \exp(1 - t/\tau_n)]$; deactivation: $I(t) = A_o + A_1\exp(-t/\tau_n)$. The time constants for decay of the tail currents were 134, 140, 72, and 39 ms for return potentials of −30, −40, −50, and −60 mV, respectively. (*B*) Examples of the least-squares fits are plotted as solid curves through point plots of I_{Kr} for the pulse to +20 and return to −40 mV (calibration bar, 20 pA). (*C*) Voltage dependence of the time constants for activation and deactivation of I_{Kr}. Number of cells used to obtain average τ_n is shown by each data point. Smooth curve was drawn by hand.

and I_{K1}, the only other currents normally activated upon depolarization from a holding potential of −40 mV in these cells. The effects of 5 μM E-4031 on Ca^{2+} currents were studied in cells exposed to the normal HEPES-buffered saline (without nisoldipine) at a temperature of 22°C. At this temperature time-dependent outward currents were not observed during the relatively short pulses (225 ms) used to activate Ca^{2+} currents. E-4031 had no significant effect on inward Ca^{2+} currents measured at test potentials ranging from −30 to +40 mV (Fig. 13, $n = 11$).

At 5 μM, E-4031 also had no significant effect on resting membrane potential or I_{K1}, measured as instantaneous current at potentials between −120 and −40 mV ($n = 10$). However, 100 μM E-4031 decreased both inward and outward I_{K1}. Peak outward I_{K1} measured between −60 and −70 mV was 2.56 ± 0.41 pA/pF in control, and 0.92 ± 0.24 pA/pF after 100 μM E-4031 ($n = 5$). Inward I_{K1}, measured at a test potential of −120 mV was decreased from −27.72 ± 2.22 pA/pF in control

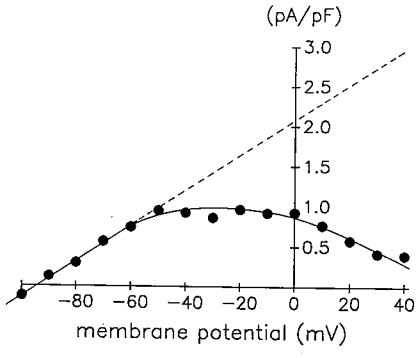

FIGURE 12. Fully-activated *I-V* relation for I_{Kr}. The method used to determine this relation is described in the text. The dotted line is an extrapolation of the linear region of this relationship. The maximum chord conductance (slope of line) is 22.5 S/F.

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to −18.25 ± 1.82 pA/pF after 100 μM E-4031 (n = 5). Thus, E-4031 decreased outward I_{K1} (64%) more than inward I_{K1} (34%) at this high concentration. As expected, block of I_{K1} by 100 μM E-4031 caused a depolarization in the resting membrane potential from −86.6 ± 0.2 mV to −83.0 ± 0.8 mV (n = 5).

DISCUSSION

Comparison of I_{Kr} with Other Cardiac Currents in Guinea Pig Ventricular Cells

It is important to discriminate between the possibilities of the drug-sensitive current measured in this study (I_{Kr}) being a distinct K^+ current, or a consequence of some complex voltage-dependent interaction of E-4031 and d-sotalol with one or more currents previously described in guinea pig ventricular myocytes. Na^+ and Ca^{2+}

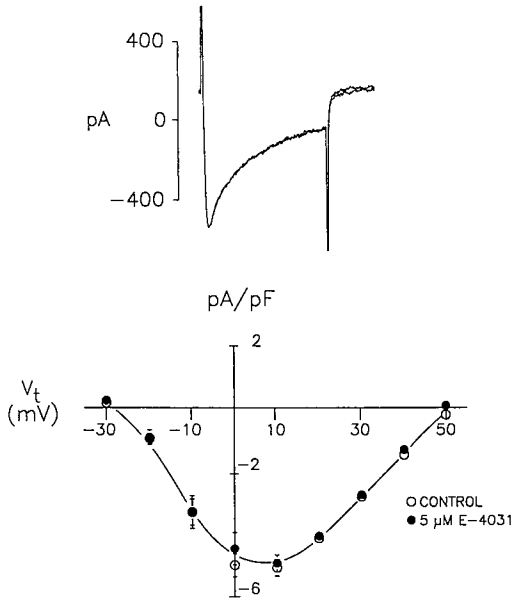

FIGURE 13. Lack of effect of E-4031 on peak inward Ca^{2+} current measured at 22°C. Upper panel shows currents measured during a pulse to 0 mV before and after the addition of 5 μM E-4031. Lower panel is a plot of peak inward currents, relative to zero-current level (n = 11). For most data points the SEM bars are smaller than the symbol size.

currents can easily be ruled out since both were eliminated in these experiments. Clamping the membrane at a holding potential of −40 mV was used to voltage inactivate Na^+ current, and nisoldipine was used to block L-type Ca^{2+} current (Kass, 1982; Sanguinetti and Kass, 1984). In addition, E-4031 at 5 μM had no effect on Ca^{2+} current. The possibility that another inward current was enhanced, or that a Cl^- current was blocked by E-4031 and d-sotalol was eliminated by experiments demonstrating the reversal potential of the drug-sensitive current was highly dependent on $[K^+]_o$. In addition, E-4031 was shown to decrease membrane conductance during brief depolarizing pulses (data not shown), which is consistent with block of an outward current.

Thus, ample evidence exists that E-4031 blocks an outward K^+ current in guinea pig ventricular cells. E-4031 at 5 μM had no effect on I_{K1}. Furthermore, the largest

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drug-sensitive currents were recorded at potentials (−10 to 10 mV; $[K^+]_o$ = 4 mM) where I_{K1} does not contribute to maintained outward current in guinea pig ventricular cells due to intense inward rectification (Hume and Uehara, 1985; Kurachi, 1985). These agents do not block ATP-sensitive K^+ current. Action potentials shortened by hypoxia or exposure to cromakalim, an activator of these channels (Escande et al., 1988; Sanguinetti et al., 1988), are not lengthened by either drug (Siegl et al., 1989). It is unlikely that I_{Kr} represents the macroscopic equivalent of the single K^+ channel current (i_{Kp}), activated at depolarized potentials (Yue and Marban, 1988). Unlike I_{Kr}, i_{Kp} does not exhibit inward rectification and activates much more rapidly.

The envelope of tails test showed that in the absence of drug the delayed outward K^+ current consisted of more than one component. After block of a component of outward current (I_{Kr}) by E-4031, the same test indicated that the remaining outward current was composed of a single current type, I_{Ks}. The envelope of tails test was also satisfied for the E-4031-sensitive current. The rapid activation, and rectification of the *I-V* relation for the drug-sensitive currents are inconsistent with an action on the slowly activating I_{Ks} of these cells. Activation of guinea pig I_{Ks} is best fit with a 2 or 3 exponential function with time constants that are at least 10 times larger than that measured for I_{Kr} (Roden et al., 1989). The fully activated *I-V* relationship for I_{Ks} shows little, if any rectification (Hume et al., 1986; Matsuura et al., 1987), whereas I_{Kr} rectifies at potentials >−50 mV and has a region of negative slope conductance. In this respect I_{Kr} is more similar to I_K of cat ventricular cells (Kleiman and Houser, 1989). In addition, the slope and half-point of the activation curves for I_{Ks} and I_{Kr} are very different. The slope factor for the voltage dependence of I_{Ks} in guinea pig cells has been estimated to be 12–14 mV (Matsuura et al., 1987; this study), compared with 7.5 mV for I_{Kr}. The half-point of the activation curves for the two currents differs by 37 mV. Finally, I_{Ks} is less selective for K^+ than I_{Kr}. The E_{rev} for I_{Ks} (and I_K) is 10–17 mV more positive than the K equilibrium potential (E_K) predicted by the Nernst equation (Bennett et al., 1985; Matsuura et al., 1987), whereas the E_{rev} for I_{Kr} is equal to the predicted E_K.

Comparison of I_{Kr} with Similar K^+ Currents from Other Cardiac Cell Types

I_{Kr} shares many characteristics with the relatively rapid activating I_K of rabbit atrial pacemaker cells (Nakayama et al., 1984; Shibasaki, 1987), I_{x1} of sheep Purkinje fibers (Noble and Tsien, 1969), and I_{x1} of chick atrial cells (Shrier and Clay, 1986). I_{x1} of chick atrial cells is characterized by rapid activation kinetics, is half activated at −30 mV, and exhibits intense inward rectification and a negative slope conductance for potentials >30 mV positive to E_K (Shrier and Clay, 1986). In these cells a slowly activating current (I_{x2}) is also present, and has properties similar to the I_{Ks} of guinea pig ventricular cells.

The high K^+ selectivity, voltage dependence of activation, rates of activation and deactivation, and inward rectification of the *I-V* relation at potentials >0 mV for I_{Kr} are similar to I_K of rabbit atrial node cells (Shibasaki, 1987). The two currents differ with respect to the degree of inward rectification. The steady-state inactivation variable for the rabbit I_K was estimated based upon single-channel currents recorded in a high K_o solution ($[K^+]_o$ = 150 mM).The calculated half-point for the steady-

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state inactivation variable of I_K in rabbit nodal cells was −68 mV. However, it should be noted that the fully activated *I-V* relation for the rabbit whole-cell I_K in normal $[K^+]_o$ (Fig. 10 of Shibasaki, 1987) is linear over the same voltage range as that recorded for I_{Kr} (Fig. 12). In cat ventricular cells, the fully activated *I-V* relationship of I_K also has a region of negative slope conductance (Kleiman and Houser, 1989), but this current activates much slower than I_{Kr}.

Mechanism of Inward Rectification of I_{Kr}

Inward rectification of outward K^+ currents has been demonstrated to result from either a voltage-gated inactivation process, or from voltage-dependent block of outward current through the channel by an internal ion, usually Mg^{2+}. The model for rectification of I_{x1} in chick atrial cells assumed the presence of an unknown blocking particle that impeded outward movement of ions in a voltage-dependent manner (Shrier and Clay, 1986). Inward rectification of I_{K1} in guinea pig ventricular cells has been shown in single-channel recordings to result in large part from the voltage-dependent block of outward K^+ ions through I_{K1} channels by internal Mg^{2+} (Matsuda et al., 1987; Vandenberg, 1987). To a lesser degree, rectification of I_{Ks} recorded from frog atrial cells is caused by block of outward current by internal Mg^{2+} ions (Duchatelle-Gourdon et al., 1989).

Another mechanism for inward rectification of outward K^+ currents has recently been reported. Shibasaki (1987) demonstrated that inward rectification of I_K in rabbit nodal cells results from a very rapid, voltage-gated inactivation process. Shibasaki (1987) was able to study the kinetics and voltage dependence of rapid inactivation of I_K in rabbit nodal cells by measuring single-channel and macroscopic currents in the presence of a high external K^+ concentration. I_K is an inward current under these conditions. In guinea pig ventricular cells, this approach is not feasible due to the presence of the large inward rectifier K^+ current (I_{K1}). Thus, we were unable to directly study the nature of the process that results in intense inward rectification of I_{Kr} by measuring macroscopic currents. The mechanism of deviation of the fully activated *I-V* relation for I_{Kr} from the ohmic properties observed at potentials negative to −50 mV (Fig. 12) is uncertain, and may be due to either a channel-gating event (i.e., rapid inactivation) or block of the channel by some internal ion. Ideally, single-channel recordings are needed to determine the cause of this rectification. However, reliable single-channel recordings of a delayed rectifier K^+ current in guinea pig ventricular cells have not yet been reported.

Whatever the exact mechanism of rectification, its voltage dependence can be determined by examination of the fully activated *I-V* relationship as described below. We refer to the value of the voltage-dependent, steady-state rectification factor as R. The value of R is given by:

$$R = I_{Kr}/[n_0 - n_\infty]\,\bar{I} \tag{2}$$

where $I_{Kr}/[n_0 - n_\infty]$ is the voltage-dependent values of fully activated I_{Kr} plotted in Fig. 12, and $\bar{I}$ represents fully activated I_{Kr} predicted to occur in the absence of any rectification (dotted line in Fig. 12). The calculated values for R were a sigmoidal

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function of voltage (Fig. 14) and were well fitted to a Boltzmann distribution:

$$R = 1/(1 + \exp[(E - V_{1/2})/k]). \quad (3)$$

The half-rectification potential ($V_{1/2}$) was estimated at −9 mV, and the slope factor (k) was +22.4 mV.

Reconstruction of Currents and I-V Relation

Time-dependent I_{Kr} (in picoamps per picofarads) can be calculated according to Eq. 4, where $\bar{g}I$ is the maximum conductance of I_{Kr} (22.5 S/F), E_{rev} is the reversal potential (−94 mV) of I_{Kr} (for $[K^+]_o = 4$ mM), and $n(t)$ is described by Eq. 5 from the Hodgkin and Huxley model (1952):

$$I_{Kr}(t) = \bar{g}In(t)R(E - E_{rev}) \quad (4)$$

$$n(t) = n_\infty + (n_0 - n_\infty) \exp(-t/\tau_n) \quad (5)$$

The calculated I-V relation for $t = 225$ ms is plotted as the smooth curve in Fig. 8, *A* and *B*.

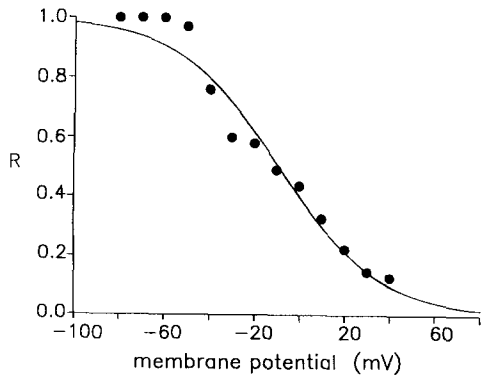

FIGURE 14. Voltage dependence of rectification. The values of R were determined as described in the text and Eq. 2. The solid curve is the least-squares fit for the Boltzmann distribution, Eq. 3. The half-point of this relationship was estimated at −9 mV, and the slope factor was 22.4 mV.

The values for n_0, n_∞, and R were calculated using the Boltzmann distribution fits shown in Figs. 9 and 14. The data shown in Fig. 11 were used for activation and deactivation time constants (τ_n). Essentially identical simulations were obtained by assuming that rectification was due to a rapid inactivation process that was much faster ($\tau < 2$ ms) than could be resolved with our whole-cell recordings of the relatively small drug-sensitive currents. In this case, R of Eq. 4 was replaced by $h(t)$, and the equations describing the open and closed rate constants of the fast inactivation gate determined by Shibasaki (1987) were used to compute τ_h at a given test potential. The voltage-dependent R values (Fig. 14) were used to estimate "h_∞" and "h_0." Fig. 15 shows the results of these simulations for 225-ms test pulses to −10, +10, and +30 mV from a holding potential of −60 mV, followed by a return to −40 mV for 750 ms. The simulated currents are reasonably similar to the drug-sensitive currents recorded in this study (compare with Fig. 7).

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Relative Importance of I_{Ks} vs. I_{Kr} in Repolarization of Ventricular Action Potentials

Fully activated I_{Ks} at +10 mV is about 10 times larger than the drug-sensitive currents measured in this study (e.g., Figs. 1 *B*, 5). Furthermore, intense inward rectification of the drug-sensitive current would minimize its contribution to total outward K^+ current at the most depolarized potentials of a cardiac action potential (phase 1 repolarization). However, activation of I_{Ks} is slow relative to APD, such that both currents would be expected to play a role in repolarization. This is obviously the case in guinea pig ventricular myocytes since both E-4031 and d-sotalol prolong APD without having any effect on I_{Ks}. One obvious concern raised by the results of this study is whether interpretation of results from previous studies of I_K in guinea pig ventricular cells were complicated by the presence of two currents. Most

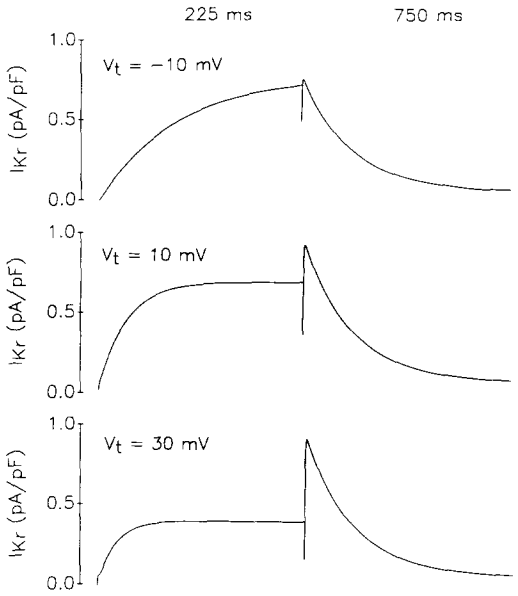

FIGURE 15. Simulation of I_{Kr}. Time-dependent currents were calculated as described in the text using Eqs. 4 and 5. Currents were simulated for depolarizing pulses from −60 to −10, +10, or +30 mV for 225 ms, followed by return to −40 mV for 750 ms.

previous studies of I_K in guinea pig cells have used long pulses to very positive potentials, conditions where the contribution of I_{Kr} to total time-dependent outward current is negligible. It should be noted that not all Class III antiarrhythmic agents are specific blockers of I_{Kr} in guinea pig cells. For example, we have confirmed the findings of Arena and Kass (1988) that clofilium blocks the slowly activating component of I_K.

In agreement with an earlier report (Balser and Roden, 1988), we also find that addition of extracellular La^{3+} to an otherwise normal bathing solution eliminates the discrepancy between the envelope of tail currents and time-dependent outward I_K. Moreover, E-4031 has no effect on I_K of cells pretreated with 0.1 mM La^{3+}. However, in the absence of extracellular Ca^{2+} and Na^+ (replaced with equimolar Tris) E-4031 still blocks a component of outward current with the properties

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described for I_{Kr} (data not shown). Thus, it appears that La^{3+} directly blocks I_{Kr}, rather than simply interfering with some extracellular Ca^{2+} binding site that regulates the gating of I_K.

Further studies are required to determine the mechanisms of rectification and the sensitivity to neurohormones of I_{Kr}, and to determine if "I_K" of other cardiac cells results from activation of more than one type of K^+ current, as originally proposed by Noble and Tsien (1969).

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