Four different components contribute to outward current in rat ventricular myocytes

HIMMEL, Herbert M., ERICH WETTWER, QI LI, and URSULA RAVENS. Four different components contribute to outward current in rat ventricular myocytes. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H107–H118, 1999.—In rat ventricle, two Ca\(^{2+}\)-insensitive components of \(K^+\) currents have been distinguished kinetically and pharmacologically, the transient, 4-aminopyridine (4-AP)-sensitive \(I_{to}\) and the sustained, tetraethylammonium (TEA)-sensitive \(I_K\). However, a much greater diversity of depolarization-activated \(K^+\) channels has been reported on the level of mRNA and protein. In the search for electrophysiological evidence of further current components, the whole cell voltage-clamp technique was used to analyze steady-state inactivation of outward currents by conditioning potentials in a wide voltage range. Peak (\(I_{peak}\)) and late (\(I_{late}\)) currents during the test pulse were analyzed by Boltzmann curve fitting, producing three fractions each. Fractions \(a\) and \(b\) had different potentials of half-maximum inactivation (\(V_{0.5}\)); the third residual fraction, \(r\), did not inactivate. Fractions \(a\) for \(I_{peak}\) and \(I_{late}\) had similar relative amplitudes and \(V_{0.5}\) values, whereas size and \(V_{0.5}\) of fractions \(b\) differed significantly between \(I_{peak}\) and \(I_{late}\). Only \(b\) of \(I_{peak}\) was transient, suggesting a relation with \(I_{to}\), whereas \(a\), \(b\), and \(r\) of \(I_{late}\) appeared to be three different sustained currents. Therefore, four individual outward current components were distinguished: \(I_{to}\) (\(b\) of \(I_{peak}\)), \(I_{K}\) (\(a\), the steady-state current \(I_{ss}\) (\(r\)), and the novel current \(I_{Kx}\) (\(b\) of \(I_{late}\)). This was further supported by differential sensitivity to TEA, 4-AP, doflium, quinidine, dendrotoxin, heteropodatoxin, and hanatoxin. With the exception of \(I_{to}\) none of the currents exhibited a marked transmural gradient. Availability of \(I_{K}\) was low at resting potential; nevertheless, \(I_{K}\) contributed to action potential shortening in hyperpolarized subendocardial myocytes. In conclusion, on the basis of the electrophysiological and pharmacological evidence, at least four components contribute to outward current in rat ventricular myocytes.

isolated myocytes; rat ventricle; transient current; sustained current; heteropodatoxin; hanatoxin; dendrotoxin; cloned channels

**ACTION POTENTIAL WAVEFORMS** differ between atrial and ventricular myocytes as well as between subepicardial and subendocardial myocytes, as shown for many species including dog, rat, and human (3, 14). This heterogeneity can be traced back to differences in outward current (2, 21, 25, 42). In particular, the transient outward current (\(I_{to}\)) is more prominent in subepicardial than in subendocardial myocytes (rat ventricle; see Ref. 14). In rat ventricular myocytes two components of outward current are distinguished kinetically and pharmacologically (4, 14, 40), the rapidly activating and inactivating \(I_{to}\), which is blocked by 4-aminopyridine (4-AP), and the rapidly activating but slowly inactivating delayed-rectifier-like current (\(I_K\)), which can be blocked by tetraethylammonium (TEA). Furthermore, \(I_{to}\) and \(I_K\) differ with respect to the potential dependence of availability of the underlying channels (4). In some cardiac preparations, e.g., Purkinje fiber, dog ventricle, and human atrium, \(I_{to}\) can be subdivided into a cytosolic \(Ca^{2+}\)-insensitive, 4-AP-sensitive \(I_{to1}\) and a cytosolic \(Ca^{2+}\)-sensitive, 4-AP-insensitive \(I_{to2}\) (6). In other preparations such as rat ventricle, only the cytosolic \(Ca^{2+}\)-insensitive, 4-AP-sensitive \(I_{to3}\) has been described (23). In the present paper, only \(I_{to1}\) is considered, and for reasons of simplicity it is referred to as \(I_{to}\).

With molecular biological approaches, a multitude of depolarization-activated \(K^+\) channel genes of the \(Kv1, Kv2,\) and \(Kv4\) families and rat \(erg\) and \(KvLQT1\) have been identified in adult and embryonic rat ventricle, respectively (7, 16, 17). Among those genes, \(Kv4.2\) and \(Kv4.3\) encode for \(K^+\)-channel proteins with \(I_{to}\)-like properties (19, 39, 45), whereas three other gene products (\(Kv1.2, Kv1.5, Kv2.1\)) are channel proteins with \(I_K\)-like properties (9, 20, 22, 28, 36, 44). On the basis of these reports, more than two components of \(K^+\) outward current of rat ventricular myocytes are expected to be distinguishable, provided that the gene products differ in electrophysiological and/or pharmacological properties.

Here we report that it is in fact possible to differentiate at least four components of outward current by making use of steady-state inactivation kinetics. The sensitivity of these components to block by TEA, 4-AP, dendrotoxin (DTX), heteropodatoxin (HptX3), and hanatoxin and other tools yielded pharmacological profiles that were compared with those reported for cloned channels. Because the amplitude of total outward current declines from subepicardial to subendocardial cells within the ventricular wall, we have also characterized the contribution of each current component to this differential current distribution. Preliminary results have been published in abstract form (22a and 22b).

**METHODS**

Cell isolation. All studies complied with the German home office regulations governing the care and use of laboratory animals. Male Wistar rats (body wt 200–250 g) were killed by cervical dislocation. As described previously (41), the hearts were perfused on a Langendorff apparatus at 37°C for 5–7 min with nominally \(Ca^{2+}\)-free saline solution (composition in mM: 100 NaCl, 10 KCl, 5.0 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 20 glucose,
50 taunine, and 5.0 MOPS, adjusted to pH 7.0 with NaOH). The rat hearts were then perfused for 15 min with collagenase-containing solution (collagenase type I, 0.5 g/l, Sigma C-0130, Munich, Germany) supplemented with CaCl₂ (200 µM) and albumin (1 g/l). After enzyme perfusion, the hearts were chopped into small pieces and dissociation was continued by gentle stirring of the tissue pieces in fresh enzyme solution for 5–15 min. On some occasions tissue batches were dissected from the apex and the base of the heart, and their dissociation was continued separately to obtain subepicardial and subendocardial myocytes, respectively (14). Single ventricular myocytes were collected in a low-Ca²⁺ solution, the Ca²⁺ concentration of which was slowly increased (0.2 mM steps in intervals of 10 min) until a final concentration of 0.6 mM was reached. The cells were stored at room temperature and used within 12 h.

Whole cell voltage-clamp technique. Myocytes were transferred to a small Perspex chamber (volume 0.5 ml) placed on the stage of an inverted microscope (Olympus IMT-2 or Zeiss Axiovert-10). The chamber was continuously perfused at a constant rate (1.2 ml/min). Only rod-shaped myocytes with clear striations were used. For action potential and membrane current recordings, the single-electrode voltage-clamp technique was applied. Heat-polished pipettes made from borosilicate filament glass (OD 1.5 mm, Hilgenberg, Malsfeld, Germany) were used to form gigahm seals with gentle suction; on average, the seal resistance was 2.8 GΩ (range 1–12 GΩ). The patched membrane was then disrupted by a pulse of suction to establish continuity of the interior of the electrode with the cytosol. Voltage or current clamp was achieved using a List L/M-EP/C-7 or an Axopatch 200 amplifier. For stimulus protocol design and data acquisition, the Axolab TL-125 interface and pCLAMP 5.5 software (Axon Instruments, Foster City, CA) were used.

To account for variabilities in cell size, the membrane capacitance was measured before compensation by means of fast depolarizing ramp pulses (from −40 to −35 mV, duration 5 ms) at the beginning of each experiment. Because the membrane conductance is very low and constant in this range, a change in current level is caused by the capacitive component. For steady-state inactivation, 2,000-ms conditioning clamp steps to −30 mV (duration 300 ms); a 5-ms pulse of suction to establish continuity of the interior of the electrode with the cytosol. Voltage or current clamp was achieved using a List L/M-EP/C-7 or an Axopatch 200 amplifier. For stimulus protocol design and data acquisition, the Axolab TL-125 interface and pCLAMP 5.5 software (Axon Instruments, Foster City, CA) were used.

Data analysis. Steady-state inactivation curves were obtained by plotting normalized current (|I|/I_max) at the test potential as a function of the conditioning potential (V_m) and fitting a Boltzmann function to the data points

$$I/|I|_{\text{max}} = 1/[1 + \exp[(V_m - V_{0.5})/k]]$$

where V_{0.5} and k are the potentials of half-maximal inactivation and the slope factor, respectively. However, a single Boltzmann function did not adequately describe the data, whereas in almost all cases the sum of two Boltzmann functions plus a residual component significantly improved the goodness of fit

$$I/|I|_{\text{max}} = (a/1 + \exp[(V_m - V_{0.5,a})/k_a]) + (b/1 + \exp[(V_m - V_{0.5,b})/k_b]) + r$$

where a and b are the fractional amplitudes of the two functions and r is the residual component, i.e., (1 – a – b). Fits of theoretical equations to the experimental data were performed using pCLAMP software (Clampfit) or Prism (Graphpad Software, San Diego, CA).

The results are expressed as means ± SE or SD of n experiments. Statistical differences were analyzed by means of Welch’s approximate f-test, which does not assume equal variances, or an appropriate nonparametric test for paired or grouped data. Correlation between two parameters was tested with the nonparametric rank test according to Spearman.
RESULTS

Steady-state inactivation of total outward current. A typical family of outward current traces after various \( V_m \) (Fig. 1A) revealed distinct differences in inactivation pattern for peak and late outward current (\( I_{\text{peak}} \) and \( I_{\text{late}} \), respectively). With a \( V_m \) of \(-140 \) mV (trace 1), the test current at \(+60 \) mV rapidly reached its peak value at the beginning of the clamp step (\( I_{\text{peak}} \)) and declined to 70\% of \( I_{\text{peak}} \) toward the end of the clamp step (\( I_{\text{late}} \)), indicating that a large fraction of outward current did not inactivate during the test clamp step. In the voltage range negative to the resting potential the amplitude of the test pulse current became smaller, with less decrease in \( I_{\text{peak}} \) than in \( I_{\text{late}} \), causing an apparent increase in the transient component (compare traces 1 and 2 in Fig. 1). After two to three conditioning steps with little change in test current giving rise to a plateau, \( I_{\text{peak}} \) was strongly diminished by \( V_m \) between \(-60 \) and \(-30 \) mV (trace 3). The remainder of the current was inactivated by \( V_m \) up to \(-20 \) mV, and the residual current positive to this potential was resistant to any inactivation (trace 4).

Because the pattern of voltage dependence appeared to be more complex than the simple sum of the known currents \( I_{\text{so}} \) and \( I_{\text{K}r} \), \( I_{\text{peak}} \) and \( I_{\text{late}} \) were evaluated separately. Normalization of data to the maximum outward current resulted in biphasic steady-state inactivation curves for \( I_{\text{peak}} \) and \( I_{\text{late}} \), which were best fitted by the sum of two Boltzmann functions with fractions a and b in addition to the residual fraction r (Fig. 1B; see METHODS). Because \( I_{\text{peak}} \) and \( I_{\text{late}} \) were evaluated separately and each consists of these three fractions, a total of six fractions, i.e., a, b, and r of \( I_{\text{peak}} \) and a, b, and r of \( I_{\text{late}} \), could be distinguished.

The parameters derived from the steady-state inactivation curves of 141 myocytes are summarized in Table 1. Very few of these myocytes possessed monophasic behavior.

### Table 1. Parameters of steady-state inactivation curves of outward current

<table>
<thead>
<tr>
<th>Fraction</th>
<th>( I_{\text{peak}} )</th>
<th>( I_{\text{late}} )</th>
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<tr>
<td>a %</td>
<td>25 ± 10</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>pA/pF</td>
<td>6.7 ± 2.9</td>
<td>7.3 ± 2.0</td>
</tr>
<tr>
<td>( V_{50a} ), mV</td>
<td>-95 ± 9</td>
<td>-93 ± 8</td>
</tr>
<tr>
<td>( k_a ), mV</td>
<td>-9.8 ± 3.0</td>
<td>-9.3 ± 2.3</td>
</tr>
<tr>
<td>b %</td>
<td>47 ± 15</td>
<td>9 ± 4*</td>
</tr>
<tr>
<td>pA/pF</td>
<td>13.8 ± 8.0</td>
<td>2.4 ± 1.0*</td>
</tr>
<tr>
<td>( V_{50b} ), mV</td>
<td>-38 ± 6</td>
<td>-28 ± 8*</td>
</tr>
<tr>
<td>( k_b ), mV</td>
<td>-4.8 ± 1.4</td>
<td>-4.4 ± 2.1</td>
</tr>
<tr>
<td>r %</td>
<td>28 ± 9</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>pA/pF</td>
<td>7.4 ± 2.0</td>
<td>7.4 ± 2.0</td>
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Data were obtained from 141 rat ventricular myocytes. Because adequate curve fitting was not possible in all cases, data listed represent mean ± SD from \( n = 138, n = 137, \) and \( n = 139 \) cells for current fractions a, b, and r of peak current \( I_{\text{peak}} \) and late current \( I_{\text{late}} \), respectively. \( I_{\text{peak}} \) and \( I_{\text{late}} \) were evaluated separately and normalized to maximal outward current, and fitted with sum of 2 Boltzmann functions (see METHODS). \( V_{50a} \), half-maximum inactivation potential; \( k_a \), slope factor. Amplitudes of individual fractions are expressed as percentage of maximal outward current as well as current density.

*Statistically significant differences between \( I_{\text{peak}} \) and \( I_{\text{late}} \) (\( P < 0.01 \), Welch’s approximate t-test for grouped data).
steady-state inactivation curves that could not be fitted reliably with the sum of two Boltzmann functions because of very small a or b of I_{late}. In 138 cells, a contributed 25% of total outward current to I_{peak} and 27% to I_{late}. The V_{0.5} were also similar, i.e., -95 mV for I_{peak} and -93 mV for I_{late}. Therefore, on the basis of electrophysiological properties, a of I_{peak} and a of I_{late} could represent a single current component and be well defined as the delayed rectifier current (I_K). The residual current fraction r that did not inactivate even at very positive V_{m} amounted to 28% of the total outward current for both I_{peak} and I_{late} and thus could be defined as the steady-state current (I_{ss}). In marked contrast, b of I_{peak} and I_{late} differed significantly in size, i.e., 47% of total outward current (I_{peak}) versus only 9% (I_{late}), as well as in V_{0.5} values (I_{peak}: -38 mV; I_{late}: -28 mV). In addition to these differences, b of I_{peak} had a marked transient time course with a time constant of 59.5 ± 3.1 ms (+60 mV, n = 22) for its exponential current decay, so that at the end of the 300-ms test clamp step >99% of current had been inactivated (compare Ref. 40). Hence, b of I_{peak} and of I_{late} could well represent two separate current components.

This hypothesis was tested by correlation analysis of fractional current amplitudes (Fig. 2). If identical, a, b, and r of I_{peak} are expected to correlate significantly with the respective fractions for I_{late}. This was indeed the case for a (Fig. 2A) as well as for r (Fig. 2C). In the latter case, the regression line passed through the origin, providing convincing evidence for correlation of current fractions and hence for their identity as a single current component. In the case of the two fractions a, however, I_{peak} was always smaller than I_{late}, which could be interpreted as current activating during the clamp step. In marked contrast to the significant correlation between I_{peak} and I_{late} in a and r, respectively, the amplitudes of I_{peak} and I_{late} for b were not at all related to each other (Fig. 2B). From this analysis it is concluded that b of I_{peak} and of I_{late} represent two independent current components, the former apparently representing I_{ss} and the latter a putatively novel sustained current designated I_{Kx}. Therefore, on the basis of steady-state inactivation, a total of four outward current components, i.e., I_{K}, I_{ss}, I_{Kx}, and I_{x}, could be distinguished.

It must be pointed out, however, that the conditioning clamp step had to last long enough for complete inactivation of all current components. This was checked by varying the duration of conditioning clamp steps between 400 and 8,000 ms; the results are summarized in Table 2. The modified clamp protocol slightly affected the relative contribution of the individual components to the total outward current but never resulted in complete disappearance of any one current component. For instance, I_{ss} decreased from 33 ± 2% of total current after 400-ms conditioning steps to 16 ± 2% after 8,000-ms steps, whereas I_{x} increased from 20 ± 1% to 29 ± 2% under these conditions. The contribution of I_{ss} did not significantly depend on the duration of the conditioning clamp step, whereas I_{Kx} became larger after long-lasting conditioning pulses.

Voltage dependence of activation of outward current components. The steady-state inactivation data presented so far allow us to distinguish a total of four outward current components, i.e., I_{K}, I_{ss}, I_{Kx}, and I_{x}. To obtain data on the activation kinetics and voltage dependence of the four current components, outward currents were activated by stepping to voltages in the range of -40 to +60 mV from the three V_{m} of -140, -70, and -20 mV (Fig. 3, A–C) followed by digital subtraction of current tracings. This procedure was aimed at further characterizing the individual current components (Fig. 3, D–E). Because channel availability...
should be at its maximum with a $V_m$ of $-140 \text{ mV}$ (compare Fig. 1), the amplitude of activated outward current is large (Fig. 3A). Both $I_{\text{peak}}$ and $I_{\text{late}}$ are decreased after conditioning steps to $-70 \text{ mV}$ (Fig. 3B), where steady-state inactivation of $I_K$ should be complete (Fig. 1; Table 1). Therefore, digital subtraction of these two sets of current tracings should result in the isolation of $I_{\text{K}}$ (Fig. 3, D and F). Currents activated from a $V_m$ of $-20 \text{ mV}$ should reflect activation of $I_{\text{ss}}$ (Fig. 3, C and H). Hence, the difference between $V_m$ of $-20$ and $-70 \text{ mV}$ should represent the sum of $I_{\text{to}}$ and $I_{\text{Kx}}$ (Fig. 3, E and G), which cannot be separated electrophysiologically because steady-state inactivation occurs in overlapping voltage ranges (Fig. 1; Table 1).

$I_K$ appears as a rapidly activating and slowly inactivating current with an activation threshold negative to $-30 \text{ mV}$ (Fig. 3, D and F). Assuming $K^+$ as the major, but not only, charge carrier (compare Fig. 6) and a reversal potential ($E_{\text{rev}}$) of $-65 \text{ mV}$ (Ref. 41; tail current analysis cannot be conducted with difference currents), $I_K$ is half-maximally activated at $-34 \pm 6 \text{ mV}$ (slope $14 \pm 2 \text{ mV}$, $n = 12$). Current activation accelerated at more depolarized potentials and could be approximated by a third-order power function yielding an activation time constant of $3.5 \pm 0.8 \text{ ms}$ at $+40 \text{ mV}$. Current inactivation was voltage independent and followed a monoexponential time course with an inactivation time constant ($\tau_{\text{in}}$) of $205 \pm 32 \text{ ms}$ at $+40 \text{ mV}$. On the contrary, $I_{\text{to}}$ activates and inactivates rapidly with an activation threshold positive to $-30 \text{ mV}$ (Fig. 3, E and G). On average, activation of $I_{\text{to}}$ is 2.6 times faster than that of $I_K$ (activation time constant of $I_{\text{to}}$: $1.0 \pm 0.1 \text{ ms}$ at $+40 \text{ mV}$). Current inactivation of $I_{\text{to}}$ was voltage independent to $0 \text{ mV}$, $-3.9 \text{ times}$ faster than $I_K$, and followed a monoexponential time course with a $\tau_{\text{in}}$ of $48 \pm 7 \text{ ms}$ at $+40 \text{ mV}$. Half-maximal activation of $I_{\text{Kx}}$ (b of $I_{\text{late}}$) occurred at $-8 \pm 2 \text{ mV}$ (slope $12 \pm 2 \text{ mV}$) and for $I_{\text{to}}$, at $+1 \pm 2 \text{ mV}$ (slope $13 \pm 1 \text{ mV}$, $n = 12$), inactivating b of $I_{\text{peak}}$. Finally, $I_{\text{ss}}$ (Fig. 3, C and H) was characterized by an almost instantaneous activation and no inactivation within $300 \text{ ms}$, an activation threshold positive to $-10 \text{ mV}$, and half-maximal activation at $+7 \pm 3 \text{ mV}$ (slope $14 \pm 1 \text{ mV}$). In conclusion, the current components $I_K$, $I_{\text{to}}$, and $I_{\text{ss}}$ isolated by means of a subtraction approach display distinct differences in terms of their activation and inactivation kinetics and voltage dependence. For components $I_{\text{to}}$ and $I_{\text{Kx}}$, however, the kinetic differences are discrete, and therefore pharmacological tools are required for current separation.

Sensitivity of outward current components to pharmacological tools. So far, electrophysiological evidence in support of four outward current components has been presented. In another approach to channel differentiation, we made use of several pharmacological tools that selectively block individual currents. For instance, 4-AP selectively blocks $I_{\text{to}}$ (4, 12), TEA attenuates $I_K$ (4, 35), and quinidine or clofilium reduces both current components (10, 26, 35). DTX is a potent blocker of a delayed-rectifier-like current flowing through the cloned Kv1.2 channel (13, 36). HPTx3 selectively blocks cloned and native Kv4.2 channels (32), whereas hanatoxin blocks both Kv4.2 and Kv2.1 channels, as shown in a Xenopus expression system (36). Because the selectivity and efficacy of these agents are usually maintained after expression of cloned channels in mammalian cell lines, the sensitivity profile can be used for channel identification (6, 22).

Table 2. Modulation of outward current components by duration of conditioning clamp steps

<table>
<thead>
<tr>
<th>Duration (ms)</th>
<th>$I_K$†</th>
<th>$I_{\text{to}}$†</th>
<th>$I_{\text{Kx}}$†</th>
<th>$I_{\text{ss}}$†</th>
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<tr>
<td>400 ms</td>
<td>0.20 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>2,000 ms</td>
<td>0.53 ± 0.05</td>
<td>0.58 ± 0.04</td>
<td>0.65 ± 0.04</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>4,000 ms</td>
<td>0.04 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>8,000 ms</td>
<td>0.33 ± 0.02</td>
<td>0.23 ± 0.02</td>
<td>0.19 ± 0.03</td>
<td>0.16 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE from n experiments. Amplitudes of sustained, tetraethylammonium-sensitive ($I_{\text{Kx}}$), transient, 4-aminopyridine-sensitive ($I_{\text{to}}$), novel ($I_{\text{Kx}}$), and steady-state ($I_{\text{ss}}$) current components were expressed as fraction of maximal outward current at 2,000-ms conditioning pulses vs. conditioning clamp steps of variable duration, i.e., 400, 2,000, 4,000, and 8,000 ms. Statistically significant differences (P < 0.05) to reference value at 2,000 ms: *ANOVA and Dunnett’s post hoc test.
however, the interpretation of the latter effect as block of Kv2.1 must remain preliminary. We were unable to use higher concentrations to achieve a more complete block because hanatoxin is a very rare toxin.

Besides HpTx3 and hanatoxin, several other K\(^+\)-channel blockers were tested, and their effects on I\(_K\), I\(_{to}\), I\(_{Kx}\), and I\(_{ss}\) (i.e., a, b, and r of I\(_{peak}\) and I\(_{late}\)) are summarized in Fig. 5. 4-AP (100 µM and 1 mM) hardly affected I\(_K\), slightly reduced I\(_{ss}\), and blocked both I\(_{to}\) and I\(_{Kx}\) in a concentration-dependent manner. With 1 mM 4-AP, block of I\(_{Kx}\) was significantly larger than block of I\(_{to}\). TEA showed a complex blocking pattern in the concentration range of 1–10 mM: the compound reduced I\(_K\) and I\(_{ss}\) in a concentration-dependent manner with a larger maximum block of I\(_K\) than of I\(_{ss}\), i.e., reduction to <20% vs. 70% of predrug control. I\(_{to}\) and I\(_{Kx}\) were affected differently by 10 mM TEA: I\(_{Kx}\) was reduced to some 20% of predrug control, whereas I\(_{to}\) was not significantly impaired at this concentration. Clofilium (3 µM) significantly reduced all current components, which confirms the nonselective nature of this blocker. With 30 µM clofilium, a and b of I\(_{late}\) were reduced to a larger extent than were the respective fractions of I\(_{peak}\).
Fig. 4. Heteropodatoxin-3 (HpTx3, 2 µM) shifts steady-state inactivation curve. A: superimposed current tracings elicited by stepping to test potential of +60 mV after conditioning clamp steps in range of −140 (trace 1) to +20 (trace 4) mV; traces 2 and 3 correspond to Vm of −60 and −20 mV, respectively. Dashed line marks zero current level. For clamp protocol see inset of Fig. 1. B: both Ipeak (top) and Ilate (bottom) were normalized to Imax and plotted as a function of Vm; data obtained under control conditions (n = 7 experiments) and after exposure to HpTx3 (2 µM) were fitted by sum of 2 Boltzmann functions (see METHODS; best line fit). Note that, in range of −60 to 0 mV, monophasic steady-state inactivation of Ipeak under control conditions becomes biphasic in presence of HpTx3 (arrows). Parameters of Boltzmann curve fitting for Ilate were V0.5, a2 97 6 3 mV, k a 2 9 6 1 mV, a 1 5 6 2 %, V0.5, b 2 3 6 6 2 mV, k b 2 4 6 1 m V, b 9 6 6 %, and r 14 6 3 %; although a and r remained unaffected, HpTx3 shifted a portion of b resulting in V0.5,b1 1 2 1 4 1 m V, k b 1 2 4 1 6 1 m V, b 1 1 6 3 %, V0.5,b2 2 8 6 1 m V, k b 2 2 4 6 1 m V, and b 2 2 3 6 2 %.

Fig. 5. Effects of pharmacological tools 4-amino-pyridine (4-AP), tetraethylammonium (TEA), clofilium (Clof), quinidine (Quin), and dendrotoxin (DTX) on various components of outward current expressed in percentage of respective control values. Top: sustained TEA-sensitive current component (I K; component a). Middle transient 4-AP-sensitive current component (Ito; fraction b of Ipeak) and novel current component (IKx; fraction b of Ilate). Bottom: steady-state current component (I ss; component r). Concentrations used were 100 µM (n = 7 experiments) and 1 mM (n = 10) for 4-AP, 1 (n = 8), 3 (n = 7), and 10 (n = 8) mM for TEA, 3 (n = 7) and 30 (n = 11) µM for clofilium, 5 µM (n = 8) for quinidine, and 100 mM (n = 7) for DTX. Mean values ± SE of Ipeak (cross-hatched bars) and Ilate (solid bars)-derived data are shown for test potential of +60 mV. *Statistically significant differences between Ipeak- and Ilate-derived data (P < 0.05, Student's t-test for paired data). #Statistically significant differences vs. control level (= 100%) (calculated with 1-sample t-test; P < 0.05).
must be interpreted with caution because clofilium is known to cause time-dependent block (10, 26). Quinidine (5 µM) reduced \( I_K \) but did not significantly impair the relative amplitudes of components \( I_{to} \), \( I_{Kx} \), or \( I_{ss} \). However, quinidine accelerated the apparent inactivation of \( I_{to} \) (not shown). Even at the maximum effective concentration of 100 nM, DTX neither blocked nor reduced any of the outward current components, suggesting the functional absence of Kv1.2 in rat ventricular myocytes. Therefore, of the investigated \( K^+ \)-channel blockers, only TEA (10 mM) selectively blocked \( I_{Kx} \) without any effect on \( I_{to} \).

In conclusion, the pharmacological data presented so far are in line with our electrophysiological data and appear to support the hypothesis that four components contribute to outward current in rat ventricular myocytes. In particular, outward current fraction \( b \) of \( I_{peak} \) and component \( r \) resemble the well-characterized currents \( I_{to} \) and \( I_K \), respectively (4, 6, 14). The residual component \( r \) is similar to a steady-state current \( (I_{ss}) \), which has been occasionally mentioned in the literature but has never received much attention (4, 35, 42). Finally, the HptX3-insensitive fraction \( b \) of \( I_{late} \) appears to be a novel sustained current, which we have termed \( I_{Kx} \).

Ion selectivity of outward current components. Ion selectivity is another criterion to distinguish between different ion channels. Other ions than \( K^+ \) could contribute as charge carriers in generating the four current components. In particular, a nonselective cation current carried by \( K^+ \), \( Na^+ \), and \( Ca^{2+} \) or an anion background current carried by \( Cl^- \) could be involved. To test for ion selectivity, the intracellular ion concentration was varied by substituting \( K^+ \) in the pipette solution with either \( Cs^+ \) or TEA, both of which permeate poorly through \( K^+ \) channels, or by lowering extracellular \( Cl^- \) from 163 to 13 mM by substituting sodium methanesulfonate for NaCl.

The results of the respective experiments are summarized in Fig. 6. Replacement of \( K^+ \) by \( Cs^+ \) in the pipette solution significantly depressed \( I_K \), abolished \( I_{Kx} \) \((b \) of \( I_{late} \)), and reduced the amplitude of \( I_{to} \) \((b \) of \( I_{peak} \)) and \( I_{ss} \) to <50%. The latter current components were further decreased when TEA was present in the pipette solution. With 90% of extracellular \( Cl^- \) replaced by the membrane-impermeant methanesulfonate, only \( I_{ss} \) was reduced to 70%, whereas the other current components were not affected. These results suggest that the majority of outward current was carried by \( K^+ \) and that \( I_{ss} \) consisted of two separate currents, one of which appeared to be carried by \( K^+ \) and the other of which was most likely a \( Cl^- \) current.

Transmural distribution of outward current components. Within the ventricular wall, \( I_{to} \) was found to be more prominent in subepicardial than in subendocardial myocytes of rat ventricle (14), and a similar distribution has been reported for \( K^+ \)-channels at the mRNA and protein levels (7, 16). Myocytes of different transmural localization can be obtained from rat hearts by isolating myocytes separately from the apex and the base, yielding subepicardial and subendocardial cells, respectively (14). Using this approach, we consistently observed that subepicardial myocytes possessed a large rapidly inactivating transient outward current, whereas subendocardial myocytes were characterized by a small \( I_{to} \) component (data not shown).

When the size of \( I_{to} \) in absolute values (pA/pF) was plotted against \( I_{to} \) expressed as a fraction of total outward current (Fig. 7A), the data points from subepicardial myocytes clustered at the lower part and those from subendocardial cells at the upper part of the relation, as expected from the known transmural gradient of \( I_{to} \). In addition, not all myocytes presently investigated have been isolated according to their origin within the ventricular wall. In fact >70% of the total of 141 cells were obtained from the whole free left ventricular wall. The majority of these myocytes are expected to stem from the midmyocardial region, but some of them could also be derived from either subendocardial or subepicardial regions, and this was confirmed indirectly by the widespread distribution of
their amplitudes in the center part of this plot. Therefore, $I_{to}$ appears to possess a strong transmural gradient.

If the size of any of the other current components also depends on the site of origin within the ventricular wall, these fractions should correlate with $I_{to}$, which is used as a marker for the transmural gradient. Of the currents tested, $I_K$ and $I_{Kx}$ did not correlate significantly with $I_{to}$ (Fig. 7, B and C), whereas $I_{ss}$ showed a small but significant negative correlation with $I_{ss}$ (Fig. 7D). From these results it is concluded that the transmural gradient of outward current is caused by the differences in $I_{to}$.

Outward current component $I_K$ and repolarization of action potential. The outward current components $I_{to}$, $I_{K}$, $I_{Kx}$, and $I_{ss}$ should contribute to the shape of the action potential, as judged by the potential range of their availability. For component $I_K$, however, $V_{0.5}$ was $-93$ mV (see Table 1), and therefore this current component should be largely inactivated at normal RMP. Hence, its role for the action potential is less obvious than with the other current components. Here we tested whether increasing the availability of $I_K$ by hyperpolarizing the membrane could influence the shape of the action potential. Action potentials recorded from subendocardial myocytes are much longer than those from subepicardial myocytes because of their profound difference in $I_{to}$ (Fig. 8A). Action potentials measured at hyperpolarized potentials were markedly shortened in duration, and this effect was significantly greater in subendocardial cells than in subepicardial cells (Fig. 8B). This observation was consistent with the theoretically expected increase in $I_K$ availability under hyperpolarizing conditions. Because $I_K$ was half-inactivated at $-93$ mV (Table 1), its availability at a normal resting potential of $-70$ mV amounted to $8\%$. Average hyperpolarization by $12$ mV should have increased the availability to $24\%$, and this additional repolarizing current strongly reduced APD in subendocardial myocytes. The much lesser effect on APD in subepicardial cells was probably caused by the larger repolarizing force of $I_{to}$. Conversely, the blocking effect of TEA (10 mM) on outward currents did not produce any prolongation in APD in subepicardial myocytes (data not shown), whereas action potentials in subendocardial cells were markedly prolonged at both normal and hyperpolarized resting potentials (Fig. 8C). These data suggest that $I_K$ may contribute to repolarization at least in subendocardial myocytes.

DISCUSSION

Outward current in rat ventricular myocytes consists of at least four different components that are distinguished on the basis of time course, potential range of steady-state inactivation, sensitivity to pharmacological blockers, and gradient of amplitude within the ventricular wall. In addition to $I_{to}$, two delayed rectifier-like currents ($I_K$, $I_{Kx}$) and at least one noninactivating background component ($I_{ss}$) were identified.

Dissection of outward current components. In native rat ventricular myocytes, two major outward current components are regularly detected, i.e., the transient, 4-AP-sensitive $K^+$ current $I_{to}$ and the sustained TEA-sensitive $K^+$ current $I_K$ (4, 6, 14). For $I_{to}$, half-maximum steady-state inactivation ($V_{0.5}$) is found at potentials between $-29$ and $-46$ mV (4, 43). This difference in $V_{0.5}$ values from the various studies may be caused in part by divalent cations (i.e., Cd$^{2+}$ or Co$^{2+}$) that are used to block Ca$^{2+}$ current and are known to shift steady-state inactivation curves to the right (1). The steady-state inactivation of $I_K$ has a more shallow potential dependence than $I_{to}$; $V_{0.5}$ values are reported between $-77$ and $-114$ mV (4, 11). Occasionally, a noninactivating residual outward current is observed that persists in the presence of 4-AP and TEA and contributes $10$–$30\%$ to peak outward current (4, 19, 35, 40, 42).

In our experiments, trial protocols to estimate steady-state inactivation of outward current revealed that conditioning steps of $-100$ mV were not sufficiently
negative for complete current availability. This was achieved only with strongly negative conditioning pulses to \(-140\) mV. The pattern of voltage dependence observed under these conditions appeared to be more complex than the simple sum of the well-characterized currents \(I_\text{to}\) and \(I_K\). Therefore, we decided to evaluate \(I_\text{peak}\) and \(I_\text{late}\) separately and to temporarily use a special nomenclature for the various current components. Normalized steady-state inactivation curves exhibited three distinct current fractions (a, b, and r) for each of the separately analyzed \(I_\text{peak}\) and \(I_\text{late}\). Because the amplitudes of a of \(I_\text{peak}\) and \(I_\text{late}\) were found to correlate significantly, they were supposed to represent a single current component. By analogy, r of \(I_\text{peak}\) and \(I_\text{late}\) were also considered as one current component. This reduced the number of distinguishable outward current components to four: a, r, b of \(I_\text{peak}\) and b of \(I_\text{late}\).

It should be pointed out that the significant correlation, \(I_\text{peak}\) of a was smaller than \(I_\text{late}\), according to the regression line. However, only in the case of an ideal nonactivating current (\(I_\text{peak} = I_\text{late}\)), should one expect a positive correlation, with the regression line characterized by a slope of 1 and an intercept at the origin. In the case of an inactivating current as shown here (\(I_\text{peak} > I_\text{late}\), Fig. 3D), a significant positive correlation should also be observed, albeit with a different regression line (slope < 1 but > 0, intercept at origin). A Cole-Moore shift might also contribute to the fact that the regression line misses the origin. In the majority of the cells, this led to the impression of an increase in transient current with less negative \(V_m\) (between \(-140\) and \(-80\) mV). Differences in both activation and inactivation time constants of \(I_K\) and \(I_\text{to}\) could confound the time course; using a subtraction approach, we found that \(I_K\) apparently activated and inactivated more slowly than \(I_\text{to}\) (Fig. 3; see Voltage dependence of activation of outward current components). Therefore, \(I_\text{to}\) determines peak current amplitude, whereas the slower-activating \(I_K\) is underestimated. Indeed, it has been reported that the delayed rectifier \(I_K\) activates 10-fold more slowly than the transient outward current \(I_\text{to}\) (4).

\(I_K\) was a delayed rectifier-like current with a shallow steady-state inactivation curve at rather negative potentials (\(V_{m,-93}\) mV; Table 1, Figs. 1, 3); it was insensitive to 4-AP but was concentration dependently blocked by TEA in low millimolar concentrations (Fig. 5). Furthermore, \(I_K\) was inhibited by quinidine and by dofyllium (Fig. 5). Although the block by dofyllium (30 \(\mu\)M) of \(I_\text{late}\) was significantly stronger than that of \(I_\text{peak}\), this was not an argument against a single current component but could be explained on the basis of the time-dependent blocking mechanism of dofyllium (10, 26).

Therefore, the properties of \(I_K\) resemble those previously reported (4, 11). The transient current component \(I_\text{to}\) had a steep steady-state inactivation curve with a midpoint at \(-38\) mV (Table 1, Fig. 1), was blocked by millimolar concentrations of 4-AP and by HptX3 (Fig. 4), but was insensitive to TEA (Fig. 5). In addition, this component was predominant in subepicardial myocytes. Such properties are identical with the published characteristics of the transient outward current (4, 6, 12).

In addition, we have presented evidence for another current component termed \(I_{Kx}\) that was partially superimposed on \(I_\text{to}\) but clearly distinct from it. \(I_{Kx}\) and \(I_\text{to}\) differed significantly with respect to the midpoints of steady-state inactivation curves, i.e., \(-28\) vs. \(-38\) mV (Table 1). Their relative amplitudes did not correlate (Fig. 2); 10 mM TEA blocked \(I_{Kx}\) but did not affect \(I_\text{to}\) (Fig. 5), whereas HptX3 blocked \(I_\text{to}\) but did not influence \(I_{Kx}\) (Fig. 4). These data suggest that \(I_{Kx}\) is a separate entity and cannot be considered as a nonactivating part of \(I_\text{to}\). On the other hand, the differences in effects of 4-AP or dofyllium on \(I_{Kx}\) and \(I_\text{to}\) amplitudes did not allow this conclusion. With 4-AP the difference of block was too small, and with dofyllium the difference could be attributed to time-dependent channel block (10, 26). \(I_{Kx}\) could represent the small, sustained outward current inhibited by nanomolar concentrations of isoproterenol (33). However, \(I_{Kx}\) was not altered by the adenyl cyclase activator forskolin (data not shown). Furthermore, \(I_{Kx}\) does not resemble the sustained...
outward current $I_{so}$ present in human atrial myocytes, because $I_{so}$ was absent in ventricular cells and was TEA insensitive (2).

In every myocyte, >25% of total outward current persisted as $I_{ss}$ at $V_m$ positive to −20 mV (Table 1, Fig. 1). This current component was attenuated by the K\(^+\)-channel blockers 4-AP, TEA (10 mM), and delfinium (Fig. 5), was markedly reduced by substituting Cs\(^+\) or TEA for K\(^+\) in the intracellular solution (Fig. 6), and was inhibited by lowering the extracellular Cl\(^-\) concentration (Fig. 6). These findings suggest that K\(^+\) and Cl\(^-\) contribute to $I_{ss}$. At present, we can only speculate about its nature. For instance, a Ba\(^{2+}\)-sensitive background K\(^+\) current has been described to be active at action potential plateau, albeit in guinea pig ventricular myocytes (5). However, in our cells the relative amplitude of the residual current was only slightly reduced on exposure to Ba\(^{2+}\) (1 mM, −15%; $n$ = 4 experiments). Nonspecific currents carried by monovalent cations have been reported in human atrium (2, 15) and in rat ventricle (27). In rat, this current is blocked in a voltage-dependent manner by extracellular Ca\(^{2+}\) and could therefore contribute to $I_{ss}$ under our conditions. $I_{ss}$ was significantly reduced after substitution of extracellular Cl\(^-\) with methanesulfonate (Fig. 5B), indicating that a Cl\(^-\) conductance contributes to background current (see also Ref. 24). However, the poor selectivity of Cl\(^-\)-channel blockers precludes more detailed characterization of the Cl\(^-\)-conducting pathway (Ref. 24; unpublished observations).

The data presented so far support the hypothesis that outward current in rat ventricular myocytes consists of more than two distinct components, i.e., $I_{K}$, $I_{10}$, $I_{Kx}$, and $I_{ss}$. These components are distinguished on the basis of their time courses, potential dependence of availability, and pharmacological profile. The properties of $I_{K}$ and $I_{10}$ are consistent with published data. However, the sustained K\(^+\) current $I_{Kx}$ and the noninactivating steady-state current $I_{ss}$ appear to be novel phenotypes that could nevertheless match those identified by K\(^+\)-channel genes.

Relation to cloned voltage-dependent K\(^+\) channels. In rat ventricle, a multitude of depolarization-activated K\(^+\) channels have been identified at the mRNA level, whereas only two current phenotypes, $I_{10}$ and $I_{K}$, have been distinguished (7, 13, 16, 30). Heterologous expression of Kv channels allows their pharmacological profiling. Our data on fraction $b$ of $I_{peak}$ (transient time course, inactivation kinetics, pharmacological profile, transmural gradient) are consistent with the idea of its identity to $I_{10}$ and confirm the role of proteins of the Kv4 family in generating $I_{10}$ in rat ventricle (compare HpfTx3 data). The kinetic properties of component a resemble those of the delayed rectifier $I_{K}$. However, the present data and our indirect experimental approach do not allow a definite conclusion about the nature of the Kv channel responsible for $I_{K}$. In particular, component a, i.e., $I_{K}$, is a sustained current without transmural gradient and is sensitive to block by TEA and hanatoxin (blocker of Kv2.1 and Kv4.2; Ref. 36) but is insensitive to 4-AP, dendrotoxin (blocker of Kv1.2; Ref. 13), and HpfTx3 (blocker of Kv4.2; Ref. 32). This pattern could give rise to the hypothesis that Kv2.1 might underlie $I_{K}$. Finally, although the current components $I_{Kx}$ and $I_{ss}$ were unaffected by either of the toxins used, this lack of effect cannot be interpreted in terms of absence of the respective Kv gene products (particularly Kv1.2). Moreover, the reason for this finding is unclear and requires further investigation. In any case, Kv channel gene products can only be related to native currents with great caution, because of the inherent differences in heteromultimeric composition and accessory subunits of K\(^+\) channels between expression systems and native myocytes (31).

In conclusion, the great diversity in expression of K\(^+\) channels in myocardial cells determines the regional variability of cardiac action potential waveform (6, 8). The underlying K\(^+\) channels are subject to developmental change, to modulation by neurotransmitters, or to differential pathophysiological alteration (e.g., hypertrophy-associated action potential prolongation because of decreased $I_{to}$ and diminished expression of Kv4.2/3; Refs. 34, 37, 38). The possible consequences include increased susceptibility to arrhythmias and altered pump function of the heart. Under physiological conditions, the observed diversity of K\(^+\) currents and action potential waveforms has pronounced effects on patterns of myocyte shortening and the inotropic state (18, 33).

We have shown that outward current in rat ventricular myocytes consists of more than the two previously described currents. In addition to $I_{10}$ and $I_{K}$, a small sustained K\(^+\) current ($I_{Kx}$) and a noninactivating steady-state current ($I_{ss}$) contribute to total outward current. Knockout of individual K\(^+\)-channel genes by means of antisense oligonucleotides in cultured myocytes should provide further insight into rat ventricular outward current components and their (patho)physiological roles in cellular repolarization and modulation of contractility.

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