Four different components contribute to outward current in rat ventricular myocytes

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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 Ca2+-insensitive components of K+ current have been distinguished kinetically and pharmacologically, the transient, 4-aminopyridine (4-AP)-sensitive Ito and the sustained, tetraethylammonium (TEA)-sensitive IK. 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 (Ipeak) and late (Ilate) 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 (V1/2); the third residual fraction, r, did not inactivate. Fractions a for Ipeak and Ilate had similar relative amplitudes and V1/2 values, whereas size and V1/2 of fractions b differed significantly between Ipeak and Ilate. Only b of Ipeak was transient, suggesting a relation with Ito, whereas a, b, and r of Ilate appeared to be three different sustained currents. Therefore, four individual outward current components were distinguished: Ito (b of Ipeak), IK (a), the steady-state current Is (r), and the novel current IKs (b of Ilate). This was further supported by differential sensitivity to TEA, 4-AP, dofetilide, quinidine, dantrolene, heteropodatoxin, and hanatoxin. With the exception of Ito, none of the currents exhibited a marked transmural gradient. Availability of IK was low at resting potential; nevertheless, IK contributed to action potential shortening in hyperpolarized subendocardial myocytes. In conclusion, on the basis of 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; dantrolene; 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 (Ito) 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 Ito, which is sensitive to blockade by 4-aminopyridine (4-AP), and the rapidly activating but slowly inactivating delayed-rectifier-like current (IK), which can be blocked by tetraethylammonium (TEA). Furthermore, Ito and IK 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, Ito can be subdivided into a cytosolic Ca2+-sensitive, 4-AP-sensitive Ito1 and a cytosolic Ca2+-sensitive, 4-AP-insensitive Ito2 (6). In other preparations such as rat ventricle, only the cytosolic Ca2+-sensitive, 4-AP-sensitive Ito3 has been described (23). In the present paper, only Ito1 is considered, and for reasons of simplicity it is referred to as Ito.

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 Ito-like properties (19, 39, 45), whereas three other gene products (Kv1.2, Kv1.5, Kv2.1) are channel proteins with IK-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, dantrolene (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 Ca2+-free saline solution (composition in mM: 100 NaCl, 10 KCl, 5.0 MgSO4, 1.2 KH2PO4, 20 glucose, 0.5 CaCl2, 11.5 HEPES, pH 7.4 at 37°C), for 5–7 min with nominally Ca2+-free saline solution (composition in mM: 100 NaCl, 10 KCl, 5.0 MgSO4, 1.2 KH2PO4, 20 glucose, 0.5 CaCl2, 11.5 HEPES, pH 7.4 at 37°C),
50 taurine, and 5.0 MOPS, adjusted to pH 7.0 with NaOH).

The rat hearts were then perfused for 15 min with collagenase-
50 taurine, and 5.0 MOPS, adjusted to pH 7.0 with NaOH).

H108 OUTWARD CURRENT COMPONENTS IN RAT VENTRICULAR MYOCYTES

was continued separately to obtain subepicardial and suben-
from the apex and the base of the heart, and their dissociation
5–15 min. On some occasions tissue batches were dissected
chopped into small pieces and dissociation was continued by
albumin (1 g/l). After enzyme perfusion, the hearts were
suction; on average, the seal resistance was 2.8 G
borosilicate filament glass (OD 1.5 mm, Hilgenberg, Malsfeld,
technique was applied. Heat-polished pipettes made from

clear striations were used. For action potential and mem-


within 12 h.

tation and the slope factor, respectively. However, a single

Measurement of outward K⁺ current. To measure outward
current in ventricular myocytes of rat heart, the bath was
perfused with a solution similar to that for action potential
recording, except that 0.6 mM CaCl₂ and 0.1 mM CdCl₂ were
used to block Ca²⁺ channels. Electrodes had tip resistances of
1.5–2.5 MΩ when filled with the same solution as used for
action potential recordings. Current-voltage relations (range
−40 to +60 mV) were measured with 300-ms clamp steps in
10-mV increments after Na⁺ current inactivation by a 40-ms
clamp step to −40 mV from the holding potential of −80 mV.
For steady-state inactivation, 2,000-ms conditioning clamp
steps (range −140 to +20 mV; 10-mV increments) were
followed by a test clamp step to +60 mV (duration 300 ms); a
step of 5 ms at −40 mV was interspersed between conditioning
and test clamp steps to keep step amplitude and capacitive
current constant.

Chemicals. Samples of HpTx3 and hanatoxin were kindly
provided by NPS Pharmaceuticals (Salt Lake City, UT) and
Dr. Kenton Swartz (National Institutes of Health, Bethesda,
MD), respectively. Clofylline tosylate was a gift of Eli Lilly
(Indianapolis, IN), and quinidine hemisulfate was from Merck
(Darmstadt, Germany). All drugs were dissolved in H₂O;
all aliquots of concentrated stock solutions were stored at −20 C
until use. Enzymes used for cell isolation (collagenase type I)
and BSA were obtained from Sigma Chemicals. All other
chemicals were purchased from commercial suppliers and
were of laboratory grade.

Data analysis. Steady-state inactivation curves were ob-
tained by plotting normalized current (I/Vₐ₉₀) at the test
potential as a function of the conditioning potential (Vₐ₉₀) and
fitting a Boltzmann function to the data points

where Vₐ₉₀ and k are the potentials of half-maximal inactiva-
tion 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

where a and b are the fractional amplitudes of the two functions
and r is the residual component, i.e., (1 – a – b).

The results are expressed as means ± SE or SD of n
experiments. Statistical differences were analyzed by means
of Welch’s approximate t-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 $I_a$ and $I_K$, $I_{\text{peak}}$ 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 properties.

### 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>
</tr>
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</table>

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, 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 \( I_{\text{late}} \). In 138 cells, a contributed 25% of total outward current to \( I_{\text{peak}} \) and 27% to \( I_{\text{late}} \). The \( V_{0.5} \) were also similar, i.e., −95 mV for \( I_{\text{peak}} \) and −93 mV for \( I_{\text{late}} \). Therefore, on the basis of electrophysiological properties, a of \( I_{\text{peak}} \) and a of \( I_{\text{late}} \) could represent a single current component and be well defined as the delayed rectifier current (\( I_r \)). 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 \( I_{\text{peak}} \) and \( I_{\text{late}} \) and thus could be defined as the steady-state current (\( I_{ss} \)). In marked contrast, b of \( I_{\text{peak}} \) and \( I_{\text{late}} \) differed significantly in size, i.e., 47% of total outward current (\( I_{\text{peak}} \)) versus only 9% (\( I_{\text{late}} \)), as well as in \( V_{0.5} \) values (\( I_{\text{peak}} \) = −38 mV; \( I_{\text{late}} \) = −28 mV). In addition to these differences, b of \( I_{\text{peak}} \) 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 \( I_{\text{peak}} \) and of \( I_{\text{late}} \) 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 \( I_{\text{peak}} \) are expected to correlate significantly with the respective fractions for \( I_{\text{late}} \). 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, \( I_{\text{peak}} \) was always smaller than \( I_{\text{late}} \), which could be interpreted as current activating during the clamp step. In marked contrast to the significant correlation between \( I_{\text{peak}} \) and \( I_{\text{late}} \) in a and r, respectively, the amplitudes of \( I_{\text{peak}} \) and \( I_{\text{late}} \) for b were not at all related to each other (Fig. 2B). From this analysis it is concluded that b of \( I_{\text{peak}} \) and of \( I_{\text{late}} \) represent two independent current components, the former apparently representing \( I_{ss} \) and the latter a putatively novel sustained current designated \( I_K \). Therefore, on the basis of steady-state inactivation, a total of four outward current components, i.e., \( I_K \), \( I_{ss} \), \( I_{\text{Kv}} \), and \( I_{\text{so}} \), 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 \( I_K \) 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 \( I_K \) 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., \( I_K \), \( I_{ss} \), \( I_{\text{Kv}} \), and \( I_{\text{so}} \). 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
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_{to} )</th>
<th>( I_{kk} )</th>
<th>( I_{ls} )</th>
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<tr>
<td>400 (n = 10)</td>
<td>( 0.20 \pm 0.01 )</td>
<td>( 0.53 \pm 0.05 )</td>
<td>( 0.04 \pm 0.01 )</td>
<td>( 0.33 \pm 0.02 )</td>
</tr>
<tr>
<td>2,000 (n = 11)</td>
<td>( 0.25 \pm 0.02 )</td>
<td>( 0.58 \pm 0.04 )</td>
<td>( 0.10 \pm 0.01 )</td>
<td>( 0.23 \pm 0.02 )</td>
</tr>
<tr>
<td>4,000 (n = 7)</td>
<td>( 0.26 \pm 0.02 )</td>
<td>( 0.65 \pm 0.04 )</td>
<td>( 0.14 \pm 0.02 )</td>
<td>( 0.19 \pm 0.03 )</td>
</tr>
<tr>
<td>8,000 (n = 5)</td>
<td>( 0.29 \pm 0.02 )</td>
<td>( 0.60 \pm 0.04 )</td>
<td>( 0.14 \pm 0.02 )</td>
<td>( 0.16 \pm 0.02 )</td>
</tr>
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Values are means ± SE from n experiments. Amplitudes of sustained, tetraethylammonium-sensitive \( I_K \), transient, 4-amino-pyridine-sensitive \( I_{to} \), and steady-state \( I_{ks} \) 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.

should be at its maximum with a \( V_m \) of −140 mV (compare Fig. 1), the amplitude of activated outward current is large (Fig. 3A). Both \( I_{peak} \) and \( I_{late} \) are decreased after conditioning steps to −70 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 \( I_K \) (Fig. 3, D and F). Currents activated from a \( V_m \) of −20 mV should reflect activation of \( I_{ss} \) (Fig. 3, C and H). Hence, the difference between \( V_m \) of −20 and −70 mV should represent the sum of \( I_{to} \) and \( I_{kk} \) (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 mV (Fig. 3, D and F). Assuming \( K^+ \) as the major, but not only, charge carrier (compare Fig. 6) and a reversal potential \( (E_{rev}) \) of −65 mV (Ref. 41; tail current analysis cannot be conducted with native currents), \( I_K \) is half-maximally activated at −34 ± 6 mV (slope 14 ± 2 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 ± 0.8 ms at +40 mV. Current inactivation was voltage independent and followed a monoeponential time course with an inactivation time constant \( (\tau_{in}) \) of 205 ± 32 ms at +40 mV. On the contrary, \( I_{to} \) activates and inactivates rapidly with an activation threshold positive to −30 mV (Fig. 3, E and G). On average, activation of \( I_{to} \) is 2.6 times faster than that of \( I_K \) (activation time constant of \( I_{to} \): 1.0 ± 0.1 ms at +40 mV). Current inactivation of \( I_{to} \) was voltage independent to 0 mV, −3.9 times faster than \( I_K \), and followed a monoeponential time course with a \( \tau_{in} \) of 48 ± 7 ms at +40 mV. Half-maximal activation of \( I_{kk} \) (b of \( I_{late} \)) occurred at −8 ± 2 mV (slope 12 ± 2 mV) and for \( I_{ls} \) at +1 ± 2 mV (slope 13 ± 1 mV, \( n = 12 \)), inactivating b of \( I_{peak} \). Finally, \( I_{ss} \) (Fig. 3, C and H) was characterized by an almost instantaneous activation and no inactivation within 300 ms, an activation threshold positive to −10 mV, and half-maximal activation at +7 ± 3 mV (slope 14 ± 1 mV). In conclusion, the current components \( I_K \) and \( I_{ss} \) isolated by means of a subtraction approach display distinct differences in terms of their activation and inactivation kinetics and voltage dependence. For components \( I_{to} \) and \( I_{kk} \), 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 \( I_{to} \) (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). HtPtx3 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).

To investigate the effects of HtPtx3 and the other pharmacological tools on the electrophysiologically distinct current components, we examined outward currents at test steps to +60 mV after selected \( V_m \), i.e., −140, −60, −30, and +20 mV (see traces 1–4 in Fig. 4A) within the range of −140 to +20 mV. The control current traces in the absence of any blocker mark the transitions between the current components (i.e., from \( I_K \) to \( I_{ss} \); compare Fig. 1). In comparison with control recordings, the Kv4.2 blocker HtPtx3 (2 μM; Fig. 4A) had little effect on current components \( I_K \) and \( I_{ss} \) (no reduction of current amplitude between traces 1 and 2 and between traces 3 and 4, respectively). However, HtPtx3 markedly reduced the amplitude of \( I_{to} \) (trace 2) and in addition shifted the steady-state inactivation of \( I_{to} \) to less negative potentials, as evidenced by the persistence of a transient current in the presence of the toxin (trace 3 in Fig. 4A). Between traces 2 and 3, however, both peak and late current decreased to a similar extent in the presence of HtPtx3, suggesting that a sustained current component, presumably \( I_{kk} \), undergoes steady-state inactivation there. This is supported by the quantitative analysis of steady-state inactivation curves displayed in Fig. 4B. Relative amplitudes, \( V_{0.5} \) values, and \( k \) were not affected by HtPtx3, except for \( I_{peak} \) in the range of −60 mV to 0 mV (Fig. 4B). The transient current component \( I_{to} \) was reduced in its amplitude to 33 ± 4% of control and shifted on the voltage axis by some 30 mV to the right. However, the block of \( I_{ss} \) was incomplete with 2 μM of HtPtx3 and amounted to 72 ± 5% at +20 mV but only 54 ± 2% at +60 mV (data not shown). Therefore, a portion of \( I_{to} \) is expected to remain unblocked in the presence of HtPtx3 (Fig. 4).

Using the Kv4.2 and Kv2.1 blocker hanatoxin (500 nM; data not shown), we observed a distinct decrease of the amplitude of \( I_{to} \) to 49 ± 9% of control and a significant amplitude reduction of \( I_K \) to 62 ± 11% of control (n = 6 experiments). The former blocking effect appears to confirm the results obtained with HtPtx3;
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 $I_{peak}$. This difference in sensitivity to block

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Fig. 3. Outward current activated from various $V_m$. A–C: superimposed current tracings elicited by stepping to potentials between −40 and +60 mV (see inset) after 2,000-ms conditioning clamp steps to −140 (A), −70 (B), and −20 (C) mV, respectively. Zero current level at horizontal bar. D and E: difference currents obtained by digital subtraction of tracings displayed in A–C: D = A − B, and E = B − C. Vertical calibration bar starts at 0 nA. F–H: voltage (V) dependence of current activation after digital subtraction of tracings recorded at $V_m$ of −140 and −70 mV (F) and −70 and −20 mV (G) and of tracings measured at $V_m$ of −20 mV (H). Nos. in parentheses indicate no. of experiments.
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 V_m of −60 and −20 mV, respectively. Dashed line marks zero current level. For clamp protocol see inset of Fig. 1. B: both I_{peak} (top) and I_{late} (bottom) were normalized to I_{max} and plotted as a function of V_m; 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 I_{peak} under control conditions becomes biphasic in presence of HpTx3 (arrows). Parameters of Boltzmann curve fitting for I_{late} were V_{0.5} = 97 ± 3 mV, k_a = 9 ± 1 mV, a_2 = 266 ± 2%, V_{0.5} = 37 ± 1 mV, k_b = 4 ± 1 mV, b_0 = 68 ± 6%, r = 15 ± 3%; although a and r remained unaffected, HpTx3 shifted a portion of b resulting in V_{0.5} = 41 ± 1 mV, k_{b1} = 6 ± 1 mV, b_1 = 16 ± 3%, V_{0.5b2} = 8 ± 1 mV, k_{b2} = 4 ± 1 mV, and b_2 = 23 ± 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 (I_{To}; fraction b of I_{peak}) and novel current component (I_{Kx}; fraction b of I_{late}). 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 nM (n = 7) for DTX. Mean values ± SE of I_{peak} (cross-hatched bars)- and I_{late} (solid bars)-derived data are shown for test potential of +60 mV. *Statistically significant differences between I_{peak}- and I_{late}-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 mM) 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 \( K_{V1.2} \) 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 \( u \) 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 location 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 isolated from the base of the heart 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 subendocardial myocytes clustered at the lower part and those from subepicardial 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

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**Fig. 6.** Alteration of ionic composition of pipette or bath solution affects size of current components. \( K^+ \) in electrode solution (140 mM, control = 100%; \( n = 11 \) experiments) was completely replaced by \( Cs^+ \) (\( [Cs^+]_e \), \( n = 5 \)) or by TEA (\( [TEA]^+ \), \( n = 3 \)); mean dialysis time was 7 ± 1 (\( Cs^+ \)) and 9 ± 1 (TEA) min after transition into whole cell mode. Current amplitudes of \( [Cs^+]_e \) and [TEA] groups were normalized to average maximal current of \( K^+ \) group. \( [Cl^-]_o \) in bath solution of \( [Cl^-]_o \) was lowered from 163 (control) to 13 (n = 6) mM by substituting sodium methanesulfonate for \( NaCl \). Test potential +60 mV; layout and statistics as in Fig. 5.
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_{to} (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_{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 were much longer than those from subepicardial myocytes because of their profound difference in membrane capacitance (4-AP-sensitive K current and are known to shift steady-state inactivation (1)).

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, and b of \(I_{\text{peak}}\) and b of \(I_{\text{late}}\).

It should be pointed out that despite the significant correlation, \(I_{\text{peak}}\) of a was smaller than \(I_{\text{late}}\) according to the regression on line. However, only in the case of an ideal noninactivating 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 (\(V_{\text{m,50}} \sim 93\) 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 dofylline (Fig. 5). Although the block by dofylline (30 µM) 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 dofylline (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, 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 blocked by quinidine and by dofylline (Fig. 5). Although the block by dofylline (30 µM) 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 dofylline (10, 26).

In addition, we have presented evidence for another current component termed \(I_{\text{Kx}}\) that was partially superimposed on \(I_{\text{to}}\) but clearly distinct from it. \(I_{\text{Kx}}\) 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 \(I_{\text{Kx}}\) but did not affect \(I_{\text{to}}\) (Fig. 5), whereas HpTx3 blocked \(I_{\text{to}}\) but did not influence \(I_{\text{Kx}}\) (Fig. 4). These data suggest that \(I_{\text{Kx}}\) is a separate entity and cannot be considered as a noninactivating part of \(I_{\text{to}}\). On the other hand, the differences in effects of 4-AP or dofylline on \(I_{\text{Kx}}\) and \(I_{\text{to}}\) amplitudes did not allow this conclusion. With 4-AP the difference of block was too small, and with dofylline the difference could be attributed to time-dependent channel block (10, 26). \(I_{\text{Kx}}\) could represent the small, sustained outward current inhibited by nanomolar concentrations of isoproterenol (33). However, \(I_{\text{Kx}}\) was not altered by the adenylyl cyclase activator forskolin (data not shown). Furthermore, \(I_{\text{Kx}}\) 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). None of the selective 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., $I_{K}$, $I_{to}$, $I_{Kx}$, 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 $I_{to}$ 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, $I_{to}$ and $I_{K}$, have been distinguished (7, 13, 16, 30). Heterologous expression of $K$ 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 $I_{to}$ and confirm the role of proteins of the Kv4 family in generating $I_{to}$ in rat ventricle (compare HptX3 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 $K^+$ 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, dendrotin (blocker of Kv1.2; Ref. 13), and HptX3 (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 $K$ gene products (particularly Kv1.2). Moreover, the reason for this finding is unclear and requires further investigation. In any case, $K^+$ 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 $I_{to}$ 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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