Heteromultimeric Assembly of Human Potassium Channels
Molecular Basis of a Transient Outward Current?

S. Po, S. Robersds, D.J. Sudiers, M.M. Tamkun, and P.B. Bennett

To gain insight into the molecular basis of cardiac repolarization, we have expressed K⁺ channels cloned from ventricular myocardium in Xenopus oocytes. A recently identified human cardiac K⁺ channel isoform (human Kv1.4) has properties similar to the 4-aminopyridine-sensitive calcium-insensitive component of the cardiac transient outward current. However, these channels recovered from inactivation much slower than native channels. Hybrid channels consisting of subunits from different K⁺ channel clones (delayed rectifier channels [Kv1.1, Kv1.2, and Kv1.5] and Kv1.4) were created by coinjection of cRNAs in oocytes. Multimeric channels consisting of Kv1.4:Kv1.1, Kv1.4:Kv1.2, and Kv1.4:Kv1.5 were expressed and compared. The hybrid channels displayed characteristics of heterotetrameric channels with kinetics that more closely resembled a native cardiac transient outward current. The inactivation and recovery from inactivation of the heteromeric channels indicated that the presence of a single inactivating subunit (Kv1.4) was probably sufficient to cause channel inactivation. The results demonstrate that expression of different K⁺ channel genes can produce channel protein subunits that assemble as heteromultimers with unique properties. It is shown that certain combinations of voltage-gated K⁺ channels probably do not contribute to native transient outward current. However, one combination of subunits could not be excluded. Therefore, this mechanism of channel assembly may underlie some of the functional diversity of potassium channels found in the cardiovascular system. (Circulation Research 1993;72:1326–1336)

KEY WORDS • K⁺ channels • transient outward current • antiarrhythmic drugs • heterotetramer • cardiac excitation–contraction coupling • heart

In the mammalian cardiovascular system, K⁺ channels display a variety of phenotypes including both transient and delayed outward currents.¹² ¹ Potassium currents from transient outward current (Iₒ) channels, which cause a rapid early repolarization of the action potential (phase 1) and can, in part, determine the membrane potential of the action potential plateau.³–⁵ This, in turn, can modify other ionic currents and has profound effects on action potential duration.⁶–⁹ Both rat and human Iₒ are composed of more than one component. One component appears to be a 4-aminopyridine (4-AP)-sensitive⁷ ¹⁰ ¹¹ K⁺ current with a time to peak under 20 msec and an inactivation time constant of approximately 40 msec at +20 mV.

Progress has been achieved recently on the molecular properties of K⁺ channels,¹² ¹⁴ resulting largely from studies on channels encoded by the Shaker locus of the fruit fly, Drosophila melanogaster. A number of different K⁺ channel genes with homology to Shaker have been identified in the mammalian cardiovascular and nervous systems.¹⁵ ²² The best available evidence suggests that a given K⁺ channel gene codes for a peptide that is one fourth of a functional channel protein.² Thus, it is widely believed that K⁺ channels assemble as tetramers with general membrane topological features that resemble voltage-gated Ca²⁺ and Na⁺ channels.²

A number of K⁺ channel cDNAs have been cloned from cardiac tissue, including delayed rectifiers (Kv1.2 and Kv1.5)¹⁸ ²⁰ and a rapidly inactivating 4-AP-sensitive K⁺ channel cloned from human ventricle (Kv1.4).¹⁷ ²² ²³ These cloned K⁺ channels have many functional characteristics similar to native myocytes.⁸ ²⁴ The human Kv1.4 clone expresses A-type K⁺ currents that resemble the 4-AP-sensitive cardiac Iₒ.³–⁸ However, one striking difference between this cloned channel and native Iₒ is in the rate of recovery from inactivation. Both rat and human Kv1.4, which are similar in primary amino acid sequence,¹⁶ ³⁸ display this slow recovery,¹⁶ ²² ²⁵ ²⁹ requiring many seconds to fully recover from inactivation. Recovery from inactivation of the voltage-gated 4-AP-sensitive component of Iₒ is at least an order of magnitude faster, depending on species and experimental conditions.

Analysis of expression of K⁺ channel mRNA in cardiac
tissues has revealed that Kv1.2, Kv1.4, and Kv1.5 are expressed in the atrium and ventricle. Thus, the possibility exists for the formation of heterotetrameric K⁺ channels from each of these distinct gene products in vivo. The properties of A-type transient K⁺ channels from rat brain are known to depend on the presence of more than one species of mRNA. Heterotetrameric channels have been suggested as a means of generating diversity in the mammalian nervous system. These channels could arise if multiple genes coding for K⁺ channel subunits are expressed in the same cell, and the formation of heterotetrameric channels has been demonstrated. The purpose of this study was to investigate the properties of heteromultimeric K⁺ channels by using four channels cloned from the mammalian cardiovascular system. We wish to determine whether the slow recovery from inactivation in human Kv1.4 channels is modified by channel subunit composition.

Materials and Methods

Oocytes were obtained from frogs purchased from Xenopus 1, Ann Arbor, Mich. Defolliculated oocytes were prepared for RNA injection and electrical recording by exposure to collagenase (1 mg/ml, type II, Worthington Biochemical Corp., Freehold, N.J.) for 1–2 hours. Oocytes were removed as they dissociated from the epithelial/follicular cell matrix. They were kept at a room temperature of 20–22°C for 3–24 hours before cytoplasmic injection of cRNA. After injection of 20–50 nl (10–40 ng) of 5’ capped cRNA, the oocytes were kept for 2–6 days at room temperature, during which time they were tested for expression of K⁺ current by two-electrode voltage clamp. Voltage-clamp methods consisted of a standard two-microelectrode arrangement and were identical to those described previously.

In vitro cRNA was prepared as described previously. In vitro transcribed Kv1.2 and Kv1.4 cRNAs were mixed by vortexing immediately before coinjection. The ratio of cRNAs for different channels in a mixture was adjusted, depending on the concentration of RNA, to give approximately equal expression of each channel type. Approximately 40 nl total volume (2–40 ng RNA) of cRNA was injected into each oocyte. After injection, oocytes were placed in ND-96 solution at 20–22°C until use. In each batch of oocytes injected with channel cRNA, control oocytes were injected with RNase-free water and subsequently voltage-clamped to ensure that there were no endogenous ionic currents. If a significant endogenous current was seen, then all the oocytes in the batch were discarded.

Solutions

The bath solution (ND-96) contained (mM) NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, and HEPES 5. pH was adjusted to 7.50 at 22°C with NaOH.

Pulse Protocols

The holding potential was −120 mV for cells expressing Kv1.4 or Kv1.4 + Kv1.x channels and −80 mV for cells expressing delayed rectifier channels (Kv1.1, Kv1.2, and Kv1.5), unless otherwise indicated. Between −120 and −80 mV, only linear leak current was observed, and linear least-squares fits to these data were used for passive leak correction. All measurements were made with custom analysis programs designed to read and analyze pClamp data files. The voltage dependence of channel availability (‘inactivation curves’) was measured with a two-pulse protocol. The membrane potential was clamped to a level between −110 and 0 mV in 10-mV steps. Channel availability for opening was assessed by a subsequent test pulse to +30 mV. Peak current during the test pulse was plotted as a function of the prepulse clamp potential. Recovery from inactivation was assessed by first inducing inactivation with a 500-msec prepulse to 0 mV. The degree of recovery at different times after the prepulse was determined by a test pulse to +30 mV. A plot of the peak current during the test pulse as a function of the time duration after the prepulse gave the time course of recovery from inactivation.

Data Analysis

Activation and inactivation curves were fitted with a Boltzmann equation: y(V)=1/[1+e(V-V1/2)k]−1, where V is voltage, V1/2 is half-maximal voltage, and k is the slope factor. The time courses of the falling phase of the macroscopic K⁺ current ‘apparent inactivation’ were fitted with an exponential function: y(t)=A₀+B⋅exp(−t/τ). These equations were used as the simplest equations that could adequately parameterize the data for subsequent comparisons. We do not place any mechanistic importance for these equations. Curve fitting was based on a nonlinear least-squares algorithm (Marquardt-Levenberg). The fitted results were displayed in linear and semi-logarithmic formats together with a plot of the residuals. Goodness of fit was judged by visual inspection. The fitted parameters always produced an adequate representation of the data. Results are presented as mean ± SEM. Statistical significance was taken as p<0.05.

Computer Simulations of Homotetrameric and Heterotetrameric Channels

Simulations of channel behavior for homotetrameric (four identical subunits) and heterotetrameric channels were done in Microsoft QuickBasic (see Figures 8 and 9). Channels were assumed to assemble as tetramers from inactivating subunits (H subunits, Kv1.4) or noninactivating subunits (R subunits, Kv1.2). A homotetrameric channel consisted of four identical subunits, e.g., four H subunits. A heterotetrameric channel contained both types of subunits, e.g., R-R-H-H (see “Appendix”). For convenience, channel activation (a) was described by an equation for four subunits: a(t)=[(a₁−(aᵣ−a₀)exp(−t/τᵣ)]₄, where aᵣ is the steady state open gate probability, a₀ is the initial open gate probability, and τᵣ is the activation time constant for each subunit. The time constant for inactivation (τᵣ) was dependent on the number (n) of H-type (inactivating) channel subunits (0–4): τᵣ=1/(n+1), where k is the association rate constant, and I is the dissociation rate constant. τᵣ was modeled as a time constant for the interaction of an intracellular blocking particle (ball peptide) with the channel as described below (see “Appendix”). The time course of channel inactivation was described as h(t)=[h₁−(h₁−h₀)]exp(−t/τᵣ), where
Heterotetrameric $K'$ injection
gene

Monomeric membrane potential channels have $Kvl.1$, $Kvl.2$, rapidly monoexponential dependence and some are units (see "Discussion"). Thus, a homotetrameric channel with four identical H subunits had an association rate constant of $4k$; a heterotetrameric channel with one H subunit had an association rate constant of $k$. The dissociation rate constant $l$ was also dependent of the channel stoichiometry. A homotetrameric channel with four identical H subunits had the highest affinity for the inactivation particle with a dissociation rate constant of $l_3$. As H subunits were replaced in heterotetrameric channels by R subunits, the dissociation rate constant increased with each R subunit such that each additional R subunit progressively destabilized the binding site for the inactivating N terminus. A homotetrameric channel with four R-type subunits had the lowest affinity for the inactivation peptide ($l_3$) but did not inactivate because the association rate was zero (no N-terminal inactivation domains in R). A heterotetrameric channel with three R and one H subunit had the lowest observable affinity. The simulations were based on the following values:

\[ +40 \text{ mV: } k = 8 \text{ sec}^{-1}; l_1 = 0.25 \text{ sec}^{-1}; l_2 = 2.5 \text{ sec}^{-1} \]
\[ -100 \text{ mV: } k = 5 \times 10^{-8} \text{ sec}^{-1}; l_1 = 2.5 \times 10^{-4} \text{ sec}^{-1}; l_2 = 2.5 \times 10^{-3} \text{ sec}^{-1} \]

which were calculated from the following equations for the rate constants:

\[ l_1 = 0.00015 e^{-0.01 \times (V + 50)}; l_2 = 100 l_1; k = 0.003 e^{0.02 \times (V - 40)} \]

These rate constants approximated the onset and recovery time constants observed (see Table 1 and Figure 8).

The time course of the current derived from heterotetrameric channels is a sum of the currents through five populations of channels made by the five possible subunit combinations, weighted by the binomial coefficients derived from the relative frequency of the subunits (see "Discussion"). The time constants for these different populations are closely clustered and dominated by the most abundant channel, resulting in apparent monoeponential behavior of ensemble current whose time constant is near that of the most abundant heterotetramer.

## Results

Figure 1 shows currents recorded during different membrane potential steps in oocytes expressing either $Kvl.1$, $Kvl.2$, $Kvl.4$, or $Kvl.5$ cRNA. $Kvl.4$ inactivates rapidly during a voltage step, whereas $Kvl.1$, $Kvl.2$, and $Kvl.5$ are delayed rectifier-like currents. The voltage dependence and some pharmacological aspects of these channels have been described.16,17,20,25,37

### Heterotetrameric $K'$ Channels

Many $K'$ channels are believed to assemble from four monomeric peptides to form a tetramer.29-32 A $K'$ channel gene codes for one of these monomers. In an artificial expression system, such as we have used with injection of a single cRNA species, channels can only assemble from identical protein subunits (homotetramers) because only one source of channel subunit (coded by the injected RNA) is available for expression. If RNA coding for both $Kvl.2$ and $Kvl.4$ peptides is expressed in the same cell, then the possibility for channel assembly from these different protein subunits exists. In other words, the channels may assemble as heterotetramers. Therefore, we attempted to express hybrid channels with subunits derived from each of two co-injected RNAs. Figure 2 compares the $K'$ currents recorded in oocytes injected with either $Kvl.4$ cRNA alone and or co-injected with a mixture of $Kvl.4$-$Kvl.1$, $Kvl.4$-$Kvl.2$, or $Kvl.4$-$Kvl.5$ cRNA. The current expressed in each of the co-injected oocytes resembles $Kvl.4$ more than $Kvl.1$, $Kvl.2$, or $Kvl.5$, since the currents now show near complete fast inactivation.

A property of the cardiac $I_{to}$ in native cells is a process of use-dependent inactivation, where channels accumulate in an inactivated state during a rapid pulse train. Figure 3A shows results from an experiment to test for this process in an oocyte expressing $Kvl.4$ channels. The top panel shows the peak outward $K'$
current recorded during pulse trains delivered at different rates. The current declined during the train, indicating that inactivated channels did not have sufficient time between pulses to recover from inactivation. In general, the extent of the inactivation that accumulated even at moderate rates (e.g., 1 Hz) was far greater than that seen in native channels. Analysis of the recovery from inactivation of Kv1.4 channels indicated that recovery could be described with an exponential function with a time constant of approximately 3 seconds (see Table 1). If the membrane potential was clamped to -120 mV or to more negative membrane potentials, then the recovery was somewhat faster; however, the recovery time constant was never smaller than 2 seconds (data not shown). This indicates that the rate of recovery from inactivation of the cloned channels was much slower than the native channels. In native human or rat cells, a recovery time constant at least an order of magnitude faster is observed. This discrepancy between the observed rates of recovery from inactivation for the cloned and native channels may provide insight into the mechanisms of channel inactivation. There is evidence for multiple genes expressing RNAs that code for different K+ channels in mammalian cells, and both Kv1.2 and Kv1.4 mRNA are expressed in cardiac tissue. Therefore, we considered the possibility that native cardiac K+ channels may assemble from different gene products and that these hybrid channels may have modified kinetic properties.

Figure 3 also compares peak outward currents recorded during train-pulse protocols in oocytes injected with pure Kv1.2 (panel B) and a mixture of Kv1.2 + Kv1.4 (panel C). The interpulse interval or cycle length of the train pulse is indicated in each panel. Whereas panel A shows the marked use-dependent inactivation of K+ current in an oocyte injected with Kv1.4, panel B indicates that there was little or no such inactivation during moderate rates in oocytes expressing only homomeric Kv1.2 K+ channels. Panel C was recorded from an oocyte cojected with Kv1.2 + Kv1.4 cRNA. In this case, much less use-dependent inactivation occurs during the pulse trains.

If heterotetramer formation is the molecular basis of native channels, the heterotetrameric channels should have pharmacological profiles consistent with that of native channels. The cardiac I_{To} is highly sensitive to 4-AP and is relatively insensitive to externally applied tetracetylaminium (TEA). Therefore, we investigated the sensitivity of the multimeric channels to these compounds. Among the homotetrameric-expressed channels, only Kv1.1 is sensitive to external TEA, with an IC50 of 0.5 mM. Figure 4 shows the effects of external TEA on Kv1.4 and Kv1.1. TEA has been used extensively to demonstrate the formation of heterotetrameric channels. Previous studies have combined TEA-sensitive and TEA-insensitive subunits to produce hybrid channels with unique TEA sensitivity. Our results show that the cardiovascular isoforms of these channels behave in the expected manner. Kv1.1 is inhibited 67% by 1 mM TEA (IC50, 0.5 mM), whereas human Kv1.4 is not blocked by 30 mM TEA. However, channels formed during coexpression inactivate like Kv1.4 and are more sensitive to TEA than heterotetrameric Kv1.4, but they are less sensitive than Kv1.1 (see Figure 4). Although this is the first such demonstration using Kv1.4, these data are consistent with previous studies using other members of the Shaker subfamily channels. They demonstrate that the new channels acquire both inactivation and TEA sensitivity. However, since native I_{To} is relatively TEA insensitive, the data suggest that Kv1.4:Kv1.1 channels are not involved.

Neither Kv1.2 nor Kv1.5 is sensitive to external TEA. However, they are both highly sensitive to inhibition by 4-AP (Figure 5). The concentration dependence of block of homotetrameric channels as well as channels formed by coexpression of Kv1.4:Kv1.2 or Kv1.4:Kv1.2 (Figure 5A) and Kv1.4:Kv1.5 (Figure 5B) is shown. Fifty percent of the Kv1.2 channels are inhibited by 0.3 mM 4-AP. This compares with an IC50 of 0.7 mM for Kv1.4 and 0.79 mM for Kv1.4:Kv1.2. Thus, the hybrid channels remain 4-AP sensitive. This contrasts with the results obtained with Kv1.5. Homotetrameric Kv1.5 was inhibited with an IC50 of 0.1 mM. The hybrid channels (Kv1.4:Kv1.5) were much less sensitive to 4-AP (IC50, 2.5 mM). Thus, the channels acquire a new and unique property that is qualitatively different from either of the parent channels. Although this proves that heteromultimeric channels are being formed, it also strongly suggests that Kv1.4:Kv1.5 hybrid channels do not underlie the native I_{To}. Thus, according to the pharmacological properties of the homotetrameric and hybrid channels, only Kv1.4:Kv1.2 channels retain 4-AP sensitivity and lack TEA sensitivity.
If the inactivation properties of hybrid channels are modified compared with the homotetrameric parent channels, this may also be reflected in the steady-state relation between inactivation and membrane potential (channel availability or inactivation curve). Figure 6 compares the voltage dependence of channel availability for opening after a prepulse for oocytes expressing Kv1.2, Kv1.4, or Kv1.4.Kv1.2. In these experiments, 2-second prepulses were used to induce sufficient slow inactivation in Kv1.2. These channels normally do not inactivate within several hundred milliseconds but do show partial inactivation (∼40%) after several seconds at potentials more positive than −50 mV. Kv1.4 channels show marked inactivation over a narrow voltage range.17,23,25 The average V1/2 and slope factor k for these channels are given in Table 2. The K⁺ current in the coinjected oocytes showed a V1/2 that was more positive than for Kv1.4 and a slope factor that was intermediate between the two homotetrameric parent channels.

Coinjection of two different channel RNAs can have two possible results. One possibility is assembly of channels from each of the two types of protein subunit (heteromultimers). Alternatively, the cell may assemble two sets of channels, each consisting of just one subunit—homomultimers. In the latter case, currents recorded will be the summation of current through the two populations of homomeric channels. Figure 6 also shows (as broken lines) the curves expected from the summation of currents through independent sets of homotetrameric channels. The examples shown are for the summation of three different ratios of homotetrameric channels. Neither the curve representing overexpression of homotetrameric Kv1.2 nor that of Kv1.4 resembles the observed data. Overexpression of Kv1.4 (0.8:0.2) results in a curve that is too negative. Equal expression of homotetrameric channels (1:1) also fails to account for the observed data.

### Table 1. Recovery From Inactivation for Kv1.4 and Heteromultimeric Channels

<table>
<thead>
<tr>
<th>Channel</th>
<th>Relative amplitude</th>
<th>Time constant (seconds)</th>
</tr>
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<tbody>
<tr>
<td>Kv1.4 (n=11)</td>
<td>0.99±0.01</td>
<td>3.2±0.40</td>
</tr>
<tr>
<td>Kv1.4:Kv1.1 (n=10)</td>
<td>0.88±0.03</td>
<td>1.38±0.13</td>
</tr>
<tr>
<td>Kv1.4:Kv1.2 (n=10)</td>
<td>0.95±0.018</td>
<td>0.71±0.1</td>
</tr>
<tr>
<td>Kv1.4:Kv1.5 (n=15)</td>
<td>0.64±0.06</td>
<td>0.71±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*Fitted fraction of total current that inactivated (in 500 msec) and recovered with the time constant shown.
The $K^+$ current observed after coinjection of Kv1.2 and Kv1.4 cRNA inactivates rapidly (Figures 2, 4, and 7A). This behavior more closely resembles Kv1.4 than Kv1.2 channels (see Figure 2). If this current were the result of the dominant expression of homotetrameric Kv1.4 over Kv1.2 channels, then we would expect a slow Kv1.4-like recovery ($\tau$=3 seconds) from inactivation. The recovery from inactivation is shown in Figure 7B for three different oocytes: one expressing Kv1.2 cRNA, one expressing Kv1.4 cRNA, and one oocyte that was injected with a mixture of Kv1.2 and Kv1.4. The averaged recovery parameters for these three conditions are summarized in Table 1, which gives the amplitudes and time constants of the dominant component that recovers from inactivation. On average, only 19% of the current was inactivated by a 2-second prepulse in the Kv1.2-expressing oocytes, and this recovered with a time constant of $\approx$0.5 seconds; a 0.5-second prepulse induced very little inactivation. In contrast, all of the current was inactivated by the 0.5-second prepulse in Kv1.4-expressing oocytes, and recovery was slow ($\tau$=3.2 seconds; see Table 1). In the oocytes injected with Kv1.2+Kv1.4 cRNA, the current was almost fully inactivated (95%), similar to Kv1.4-expressing cells, but recovered much faster from inactivation ($\tau$=0.7 seconds), indicating that the channels that inactivated were not homotetrameric Kv1.4 channels. This pattern of near complete inactivation during a 500-msec prepulse and the enhanced rate of recovery (compared with homotetrameric Kv1.4) was also observed by coinjecting Kv1.4 with Kv1.1 or Kv1.5 (Table 1).

Figure 8 shows results from simulations with homotetrameric or heterotetrameric channels. In panels A and B, equal expression (1:1) of two sets of homotetrameric channels was assumed. In this case, during a voltage-clamp step to +50 mV, the observed current is the sum of the current through the two sets of independent channels. The current after coexpression is shown as the solid line. There is a large fraction of current that does not inactivate. In the recovery (panel B), a prepulse inactivates the current by $\approx$66%, and this fraction recovers at an intermediate rate compared with pure Kv1.2 and Kv1.4. Together (panels A and B), these simulations do not resemble the behavior in coinjected Kv1.2+Kv1.4 oocytes; the expressed current observed experimentally almost fully inactivated during the voltage step but recovered at a rate similar to Kv1.2. Panel C shows a simulation of overexpression of Kv1.4 (nine Kv1.4 versus one Kv1.2). In this case, the current largely inactivates (as is observed experimentally); however, the recovery is much too slow and resembles the pure Kv1.4 recovery. This is expected since Kv1.4 channels now dominate. Panels E and F show the results of simulated Kv1.2+Kv1.4 heterotetrameric channels. Here, the current is assumed to derive from five popu-
lations of channels (see "Discussion") that range from pure Kv1.2 (6.25%) to pure Kv1.4 (6.25%). The remainder of the channels contain one (25%) two (37.5%), or three (25%) Kv1.4 subunits and are thus heterotetramers. In this case, the current during the pulse largely inactivates but does so somewhat slower than the homotetrameric Kv1.4, as is observed experimentally (see Figure 2). The recovery from near complete inactivation (panel F) now occurs much faster than Kv1.4; this also simulates the experimental observation.

FIGURE 5. Graphs showing block of Kv1.4 (HK1), Kv1.2 (RK2), Kv1.5 (HK2), and hybrid channels by 4-aminopyridine (4-AP). Panel A: Percent inhibition of Kv1.4, Kv1.2, or Kv1.4:Kv1.2 currents. Symbols represent the means of three to 10 different cells in which one or more concentrations of 4-AP were used. Standard errors are shown if they are larger than the symbol. The curves represent the best least-squares fits of a single-site binding model to the pooled data. Panel B: Similar analysis as in panel A using Kv1.5 and Kv1.4:Kv1.5 channels.

FIGURE 6. Graph showing voltage dependence of channel availability for opening for pure Kv1.2 and Kv1.4 channels or after coexpression in oocytes. $V_{\text{pre}}$, prepulse voltage. A standard two-pulse voltage-clamp protocol was used to assess the availability of channels for opening during a test pulse after a prepulse to the membrane potential indicated on the abscissa. The duration of the prepulse was 2 seconds. The test pulse was a step to +30 mV. The solid lines are the best-fitting Boltzmann functions for each data set. The broken lines represent the expected curves derived from the summation of two sets of independent homomeric channels. Data from additional experiments are summarized in Table 2.
TABLE 2. Inactivation of Kv1.4, Kv1.2, and Heteromultimeric Channels

<table>
<thead>
<tr>
<th></th>
<th>(V_{1/2}) (mV)</th>
<th>(k) (mV)</th>
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<tbody>
<tr>
<td>Kv1.2 ((n=10))</td>
<td>-20.4±2.9</td>
<td>9.5±0.7</td>
</tr>
<tr>
<td>Kv1.4 ((n=12))</td>
<td>-66.3±3.1</td>
<td>4.1±0.17</td>
</tr>
<tr>
<td>Kv1.4:Kv1.1 ((n=10))</td>
<td>-51.0±0.9</td>
<td>4.8±0.2</td>
</tr>
<tr>
<td>Kv1.4:Kv1.2 ((n=11))</td>
<td>-41.4±2.1</td>
<td>7.4±0.27</td>
</tr>
<tr>
<td>Kv1.4:Kv1.5 ((n=4))</td>
<td>-26.2±4.2</td>
<td>9.5±0.76</td>
</tr>
</tbody>
</table>

Parameters are from fitted Boltzmann distribution: \(y(V) = [1 + e^{(V-V_{1/2})/k}]^{-1}\), where \(V\) is voltage, \(V_{1/2}\) is half-maximal voltage, and \(k\) is the slope factor. Values are mean±SEM.

Discussion

The results described in this article demonstrate heteromultimeric K\(^+\) channel formation with channels cloned from human ventricle and raise the possibility that Kv1.4 or a heterotetrameric channel with Kv1.4 subunits may give rise to \(I_{TO}\) in native cells in the mammalian cardiovascular system. The data indicate that a heterotetrameric channel consisting of Kv1.4 and Kv1.5 is unlikely, since the new channels are too insensitive to 4-AP. In addition, a hybrid channel with Kv1.4 and Kv1.1 is ruled out because these channels are blocked by external TEA and \(I_{TO}\) is insensitive to external TEA. Our results do not exclude Kv1.4:Kv1.2 heteromultimers, since the hybrid channels are appropriately blocked by 4-AP, they are insensitive to external TEA, they show appropriate rates of onset of macroscopic inactivation, and their recovery from inactivation is much faster than that for Kv1.4 alone.

Comparison of Expressed K\(^+\) Current to \(I_{TO}\) in Native Cardiac Cells

The expressed cloned K\(^+\) channel known as Kv1.4 has many functional characteristics similar to a component of the cardiac ionic current referred to as \(I_{TO}\). This A-type current has been observed in a number of different species and cardiac tissues, including rabbit and human atrium, canine and rat ventricle, and rabbit crista terminalis. Analysis of expression of K\(^+\) channel RNA in various tissues revealed that human Kv1.4 was approximately equally abundant in the atrium and ventricle. The macroscopic activation and inactivation of these channels was time and voltage dependent, and the channels were blocked by low concentrations of 4-AP. The recovery of channels from inactivation was time dependent, and the rate of recovery increased when the membrane potential was held at increasingly negative values. Because of the time required for the channels to recover from inactivation, it was possible to induce significant reductions in the current if a voltage-clamp pulse train was applied too rapidly.

Shibata et al\(^7\) have successfully recorded action potentials and \(I_{TO}\) in human atrial cells. Block of \(I_{TO}\) by 4-AP (0.5 mM) had dramatic effects on the action potential, not only on phase 1 but also on duration. Shibata et al also observed use-dependent changes in \(I_{TO}\) amplitude at different train rates (from 0.2 to 3.57 Hz), suggesting that this current plays a significant role in the modulation of action potential shape and duration by changes in heart rate. However, the rate of recovery from inactivation in the human atrial cell \((\tau=141\) and 54 msec at \(-60\) or \(-80\) mV, respectively) was faster than \(I_{TO}\) and others\(^16,21\) have observed in the cloned human or rat channels. Thus, it is possible and perhaps likely that still other unidentified factors may modify or regulate the channel kinetics.

Comparison With Other Cloned K\(^+\) Channels

The deduced primary amino acid sequence of human Kv1.4 channels is similar but not identical to a channel cloned from rat brain\(^23\) (RCK4). Human cardiac Kv1.4 is more similar but still not identical to the rat cardiac channel described by Tseng-Crank et al,\(^16\) which they originally referred to as RHK1. The human channel is 97% homologous to the rat heart channel, with 18 amino acid differences. Both the rat and human channels belong to the Shaker family of K\(^+\) channel genes. All of these channels are highly conserved in the putative pore domain, which is made up of the amino acids linking S5 and S6. Hoshi et al\(^16\) have shown that the N-terminal 20 amino acids are involved in Shaker K\(^+\) channel inactivation. They proposed that this region acts as a blocker of the open K\(^+\) channel pore similar to the “ball-and-chain” model originally proposed by Armstrong.\(^38\) Recent evidence in Shaker K\(^+\) channels has also implicated the domain linking putative transmembrane segments S4 and S5 as being part of the internal mouth of the pore and the receptive site for the N-terminal ball peptide.\(^39\) Thus, in a tetrameric Kv1.4 K\(^+\) channel, each peptide subunit would contribute one N-terminal ball peptide and one S4–S5 domain for a total of four each.

![Figure 7](http://circres.ahajournals.org/)

**Figure 7.** Panel A: Currents expressed in oocytes injected with a Kv1.4 or a mixture of Kv1.4:Kv1.2 RNA. Panel B: Graph showing recovery from inactivation in oocytes injected with Kv1.4, Kv1.2, or Kv1.4+Kv1.2 cRNA. The inactivation induction and recovery voltage-clamp protocols were as described in “Materials and Methods.” The solid curves represent the nonlinear least-squares fit of an exponential function as described in “Materials and Methods.” Data from additional experiments are summarized in Table 1. Note that the recovery that is shown is derived from the current that inactivated completely in 250 msec; thus, any homomeric Kv1.2 channels are excluded.
Heterotetrameric \( K^+ \) Channels

Because of the similarity between the \( Shaker \) gene product and one of the four internal repeats of voltage-gated \( Na^+ \) and \( Ca^{2+} \) channels, it has been assumed that \( K^+ \) channels also consist of four subunits. \( K^+ \) channel RNAs code for peptides with deduced amino acid numbers between 400 and 800, approximately one-fourth that of a voltage-gated \( Na^+ \) channel. This then suggests that \( K^+ \) channels are formed by the assembly of four peptide subunits. This could occur by assembly of four identical copies of the same subunit (heterotetramers) or by the assembly of nonidentical subunits (heterotetramers). It has been proposed\(^{28} \) that heterotetrameric channels could arise if multiple genes coding for \( K^+ \) channel subunits are expressed in the same cell. Evidence for heterotetrameric \( K^+ \) channels has been seen by coexpression in \( Xenopus \) oocytes of channel-specific RNAs from brain\(^{29,30} \) by \( drosophila \) \( Shaker \) \( K^+ \) channels\(^{31} \), and by experiments with fractionated poly(A)\(^+\) mRNA.\(^{27} \) Furthermore, MacKinnon\(^{32} \) has provided evidence that the channel subunit stoichiometry is four.

\( Kvl.1, Kvl.2, \) and \( Kvl.5 \) are delayed rectifier-type channels that lack the amino terminal domain that is believed to be involved in fast inactivation.\(^{33} \) \( Kvl.4 \), which rapidly inactivates, does have the extra amino acids in the amino terminal domain, and fast inactivation is removed if this region is deleted or if cysteine 13 becomes oxidized.\(^{40} \) These channels are all highly homologous and are vertebrate members of the \( Drosophila \) \( Shaker \) subfamily of potassium channel genes. \( Kvl.2 \) and \( Kvl.4 \) differ by two amino acids in the putative receptor site for the inactivation amino terminus (\( S4-S5 \)) interdomain).\(^{39} \) Hybrid channels will differ from homotetrameric channels in the number of inactivating amino termini per channel and in the composition of the putative binding domain for the inactivation peptide.

One possible model is that the amino acids linking putative transmembrane domains \( S4 \) and \( S5 \) form one fourth of the receptor site for the \( N \)-terminal inactivation peptide.\(^{39} \) In this case, when the four subunits assemble into a channel, the inner mouth of the pore and the peptide receptive site are created by the apposition of the four \( S4-S5 \) domains.\(^{42} \) This is similar to the hypothesis for the external \( TEA \) receptor, where each subunit contributes to the \( TEA \)-receptive site.\(^{42} \) If this model holds for the \( N \)-terminal inactivation of \( Kvl.4 \) channels, then in a heterotetrameric channel made from \( Kvl.2 \) and \( Kvl.4 \) peptide subunits, the affinity of receptive site for the \( N \)-terminal peptide may depend on the number of \( Kvl.4 \) subunits in the channel. This is the model we have used to simulate our data (see “Materials and Methods” and Figure 8). Figure 9 depicts such heterotetrameric channels. Each additional \( Kvl.4 \) subunit contributes an inactivating amino terminus (ball and chain). A pure homotetrameric \( Kvl.4 \) channel (squares in Figure 9) has four such inactivating particles, and the association rate constant is four times greater than a channel with only one \( Kvl.4 \) subunit. Likewise, the binding site for the inactivation peptide (i.e., the inner mouth of the pore consisting of the \( S4-S5 \) domain)\(^{39} \) is progressively altered by addition of \( Kvl.2 \) subunits (circles in Figure 9). Each additional \( Kvl.2 \) subunit increases the inactivation peptide dissociation rate constant by altering the binding site. \( Kvl.2 \) and \( Kvl.4 \) differ by two amino acids in the putative \( N \)-terminal receptive site (\( S4-S5 \)). The amino acids at \( Kvl.4 \) positions 469 (H→Q) and 472 (R→K) are differ-

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**Figure 8.** Simulated expression of homotetrameric and heterotetrameric channels. Panel A: Simulated time course of current during a voltage-clamp step. Pure \( Kvl.1.2 \) or pure \( Kvl.1.4 \) channel currents are shown as dashed lines. The summation of current through the independent homotetrameric channels with equal expression levels is shown as the solid curve. The summed currents are divided by 2. Note the large amount of noninactivating current at the end of the voltage-clamp pulse. Panel B: Recovery from inactivation for independently expressed homotetrameric channels. Conditions were as in panel A. Pure \( Kvl.1.2 \) inactivates little and recovers rapidly. \( Kvl.1.4 \) inactivates fully and recovers slowly. The current seen if both types of homotetrameric channels are expressed independently appears to inactivate to an intermediate level and recovers at an intermediate rate. Panel C: Overexpression of \( Kvl.1.4 \) and \( Kvl.1.2 \) currents were summed together (9:1 [90\% \( Kvl.1.4 \) and 10\% \( Kvl.1.2 \)]) and are plotted as the solid line. The dashed lines show pure \( Kvl.1.2 \) and pure \( Kvl.1.4 \) for reference. Overexpression of \( Kvl.1.4 \) (inactivating current) might account for the experimentally observed large amount of inactivating current seen in the coinjected cells. Panel D: Recovery of homotetrameric channels after overexpression (9:1) of \( Kvl.1.4 \). The summed current almost fully inactivates but recovers from inactivation like \( Kvl.1.4 \). Panel E: Expression of heterotetrameric \( Kvl.1.2+Kvl.1.4 \) channels. Heterotetrameric channels inactivate to a large extent. Panel F: Recovery of heterotetrameric channels. Channels are almost fully inactivated (like \( Kvl.1.4 \)) but recover from inactivation much faster (like \( Kvl.1.2 \)).
ent in Kv1.2. This could result in a destabilization of the inactivation peptide binding interaction and could speed the recovery from inactivation in the heterotetramers containing Kv1.2 subunits. A homotetrameric Kv1.4 channel would have the highest affinity for the peptide and thus would recover the most slowly from inactivation. Heterotetrameric channels with one or more Kv1.2 subunits are expected to have a lower affinity and to recover faster from inactivation.

If the channel subunits derived from these cRNAs are expressed equally and the subunits combine randomly to form heterotetrameric channels, then the mixing of channel subunits can be predicted. The expected combinations of two types of subunits (A and B) assembling to form tetrameric channels is given as

\[(A+B)^4 = A^4 + 4 \cdot A^3 \cdot B + 6 \cdot A^2 \cdot B^2 + 4 \cdot A \cdot B^3 + B^4\]

where \(A^4 (A \times A \times A \times A)\) is a tetramer of pure Kv1.4 and \(B^4\) is a tetramer of pure Kv1.2. Thus, 1/16, calculated as 1/(1+4+6+4+1), or 6.25% of the channels would be pure Kv1.2 homotetramers. Likewise, 1/16 would be pure Kv1.4 channels. The remainder of the channels would then be heterotetramers with 4/16 consisting of three Kv1.2 subunits and one Kv1.4 subunit, 6/16 consisting of two Kv1.2 and two Kv1.4 subunits, and 4/16 consisting of one Kv1.2 and three Kv1.4 subunits. If only one of the four subunits needs to be a Kv1.4 subunit to cause the channel to inactivate, then 15/16 (93.75%) of the expressed channels will inactivate. If four Kv1.4 subunits are required for channel inactivation, then only 1/16 of the channels will inactivate. Figures 2 and 5 show that over 90% of the expressed current inactivates. Figures 7 and 8 indicate that the current that inactivates has a unique recovery rate. Thus, the expressed K⁺ current results not from overexpression of homotetrameric Kv1.4 but from expression of new heteromultimeric channels. This suggests that only one Kv1.4 subunit is required per channel to cause inactivation. Even if the assumptions of equal expression and random assembly are not completely valid, the results cannot be explained on the basis of summation of currents through separate homotetrameric channels.

Although our results demonstrate the formation of hybrid channels in the Xenopus expression system, it is important to point out that these results do not prove the existence of heterotetrameric channels in vivo. However, heterotetrameric channel assembly can explain the apparent anomalous recovery rate of human and rat cardiac Kv1.4 channels compared with native I_{K1α}. Additional insights into the relation between cloned and native channels will require isolation of native channel proteins, in situ hybridization studies with subcellular resolution to determine the presence of multiple K⁺ channel mRNA species, and high-affinity site-directed antibodies to identify the presence of heterologous subunits in a given channel.

Appendix

We assume N-type inactivation with a ball-and-chain model for Kv1.4, which is consistent with the elongated amino terminus of this channel relative to Kv1.1, Kv1.2, or Kv1.5. For subunit i, let \(k_i\) represent the binding rate of a single inactivating particle (ball) and \(l_i\) represent the unbinding rate. Let \(i = 1\) for the inactivating subunits (Kv1.4) and \(i = 2\) for the subunits of the delayed rectifier channel (Kv1.2). In the case of a single subunit, the inactivation rate is

\[\lambda = k_i + l_i\]  

(1A)

If one subunit is sufficient for induction of inactivation and if only one inactivation particle binds at a time, then the overall inactivation rate for a homomultimeric channel becomes

\[\lambda = n \cdot k_i + l_i\]  

(2A)

where \(n\) is the number of subunits forming the channel (e.g., 4 in the case of Kv1.4 homotetrameric channels). At depolarized membrane potentials, inactivation proceeds to completion, implying that \(l_i\) is small compared with the binding rate (\(n \cdot k_i\)), which reduces the expression for the apparent inactivation rate (\(\lambda_{max}\)) at positive potentials to

\[\lambda_{max} = n \cdot k_i\]  

(3A)

At negative potentials, the recovery from inactivation (\(\lambda_{max}\)) is complete (no steady-state inactivation), which implies that \(n \cdot k_i \cdot l_i\) is small compared with \(l_i\), reducing Equation 2A at negative potentials to

\[\lambda_{max} = l_i\]  

(4A)

For a heterotetrameric channel, the number of inactivation particles is equal to the number of inactivating subunits that are present in the channel. We assume that each inactivation particle has equal access to the binding site (\(k_i = k_j\)). Therefore, the overall rate of inactivation for a heterotetrameric channel is given by Equation 3A. We assume that the receptor for the inactivation particle consists of the inner mouth of the pore (S4–S5) and that each subunit contributes to the binding site. The stability of the receptor complex with the particle is
altered by the presence of Kv1.2 versus Kv1.4 subunits (l1≠l2). A homotetrameric Kv1.4 channel has a dissociation rate of l1, whereas a homotetrameric Kv1.2 channel has a dissociation rate of l2. A homotetrameric Kv1.4 channel forms the most stable complex with the ball peptide; therefore, the dissociation rate l1 is much smaller than l2. The recovery from inactivation is determined by an off rate that incorporates these two rate constants (l1 and l2), weighted by the number of each type of subunit:

\[ \lambda_{\text{on}} = \frac{n(n-1)}{4} l_1 + \frac{(4-n)(4-n)}{4} l_2 \]  

(A)

Acknowledgment

We thank Craig Short for excellent technical assistance.

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Heteromultimeric assembly of human potassium channels. Molecular basis of a transient outward current?

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doi: 10.1161/01.RES.72.6.1326

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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