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Molecular and Functional Diversity of Cloned Cardiac Potassium Channels

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Summary. **Action potential duration is an important determinant of refractoriness in cardiac tissue and thus of the ability to propagate electrical impulses. Action potential duration is controlled in part by activation of K^+ currents. Block of K^+ channels and the resultant prolongation of action potential duration has become an increasingly attractive mode of antiarrhythmic intervention. Detailed investigation of individual cardiac K^+ channels has been hampered by the presence of multiple types of K^+ channels in cardiac cells and the difficulty of isolating individual currents. We have approached this problem by employing a combination molecular cloning technology, heterologous channel expression systems, and biophysical analysis of expressed channels. We have focused on six different channels cloned from the rat and human cardiovascular systems. Each channel has unique functional and pharmacological characteristics, and as a group they comprise a series of mammalian K^+ channel isoforms that can account for some of the diversity of channels in the mammalian heart. Each channel appears to be encoded by a different gene with little or no evidence for alternate splicing of RNA transcripts to account for the differences in primary amino acid sequence. In addition to the unique kinetic properties of these channel isoforms when expressed as homotetrameric assemblies, the formation of heterotetrameric K^+ channels is also observed. The formation of heterotetrameric channels from the different gene products to create new channels with unique kinetic and pharmacological properties might further account for cardiac K^+ channel diversity.**

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Key Words. **K^+ channel, I_{TO}, delayed rectifier, antiarrhythmic drugs, cardiac excitation-contraction coupling**

Ion channels in the plasma membrane of excitable cells represent the molecular elements of excitability in a wide range of different organ systems including skeletal muscle, brain, and heart [1,2]. Potassium ion channels in the cardiovascular system play a number of roles [3–6], including establishing the resting membrane potential [7] modulating the frequency and duration of action potentials, and serving as the target for several important antiarrhythmic drugs [8–12]. These various roles in cardiac electrical function appear to be subserved by a number of different potassium ion channels. Indeed, a large number of ionic current types have been recorded under voltage-clamp conditions in different cardiac membrane preparations. Because of this diversity and the presence of multiple overlapping potassium currents in native cardiac myocytes, the investigation of basic potassium-channel function and drug-channel interactions is greatly complicated. It is often necessary to apply sophisticated voltage-clamp protocols and to use numerous pharmacological agents in order to dissect out a particular current of interest. The difficulty in isolating potassium channel proteins has also hampered knowledge of the structure and function of these important macromolecules. The utilization of molecular cloning technology has allowed the identification of the primary structure of a number of potassium channels, including those cloned from the mammalian cardiovascular system, and has permitted the expression of the channel proteins in heterologous systems with detailed analysis of channel function and pharmacology. The ability to express a single protein type in a heterologous expression system is a significant advance in that it allows investigation of channel function in the absence of overlapping time and voltage-dependent current. To date, six distinct potassium channels cloned from the rat and human cardiovascular systems [13,14] have been expressed in both the *Xenopus* oocyte and a mammalian fibroblast L-cell expression system [15–20]. Studies are underway to functionally characterize their gating and pharmacology.

Methods

Preparation of Xenopus *oocytes*

Oocytes were obtained from frogs purchased from Xenopus 1 (Ann Arbor, MI). Defolliculated oocytes were prepared for RNA injection and electrical re-

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cording by exposure to collagenase (1 mg/ml; Worthington, Type II) for 1–2 hours. Oocytes were incubated at 20°C for 3–24 hours prior to cytoplasmic injection of cRNA. After injection of 20–50 nl (10–30 ng) of 5′ capped cRNA, the oocytes were kept for 2–8 days at 20°C, during which time they were tested for expression of K^+ current by two-electrode voltage clamp.

Preparation of RNA for oocyte injections

Cloning of channel cDNA from rat and human myocardium has been described in detail [13,14]. Templates for in vitro cDNA synthesis from Kv1.2 DNA were prepared by subcloning cDNA fragments into the *Eco*RI site of pOEV. A linearized pOEV construct was incubated with 300 U T7 RNA polymerase and 0.5 mM of CAP analog, $m^7G(5')ppp(5')G_m$, for 1 hour at 37°C in 40 mM Tris buffer at pH 7.5. The reaction mixture also contained 6 mM $MgCl_2$, 2 mM spermidine, 5 mM NaCl, 10 mM DTT, 0.1 mg/ml BSA, 2 U RNAsin, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, and 50 μM GTP. The RNA was then incubated for 30 minutes at 37°C with 4.5 U poly(A)polymerase, followed by ethanol precipitation, and then dissolved in H_2O.

Templates for in vitro cRNA synthesis from human Kv1.4 DNA were prepared as follows. The Bst XI-*Eco*RI fragment of Kv1.4 (nucleotides 290–2800) was isolated, blunted with Klenow, and subcloned into the blunted Bgl II site of a *Xenopus* expression vector [21]. The construct was linearized with *Eco*RI, and cRNA was synthesized with SP6 polymerase using the Stratagene (LA Jolla, CA) transcription kit. For each batch of oocytes injected with channel cRNA, control oocytes were also injected with RNase-free water and were voltage clamped to ensure that there were no endogenous ionic currents. If significant endogenous current was seen at +40 mV, all the oocytes in the batch were discarded.

Transfection and cell culture

The K^+ channel cDNA-containing expression vectors were transfected into mouse *Ltk*$^-$ cells (L cells) using calcium phosphate precipitation. The mammalian expression vector contains a dexamethasone-inducible murine mammary tumor virus (MMTV) promotor, controlling transcription of the cDNA inserted at the polylinker site, and a gene conferring neomycin resistance driven by the SV40 early promotor. Selection for cells expressing the K^+ channels was carried out with 0.5 mg/ml G418 (a neomycin analog) for 2 weeks or until discrete neomycin resistant foci formed. Individual foci were isolated, maintained in 0.25 mg/ml G418, and screened for K^+ channel specific mRNA expression by Northern analysis, as previously described [13,14]. Cell lines transfected with the same vector, but lacking channel cDNA, were prepared in parallel, and served as negative controls (sham-transfected cell lines). The sham-transfected cells did not display specific mRNA hybridization on Northern analysis at high stringency with a channel-specific probe and did not express K^+ currents.

Cells were cultured in DMEM supplemented with 10% horse serum and 0.25 mg/ml G418 under a 5% CO_2 atmosphere. The cultures were passed every 4–5 days using a brief trypsin treatment. Prior to experimental use, subconfluent cultures were incubated with 2 μM dexamethasone for 24 hours to induce efficient channel expression. The cell suspension was stored at room temperature and used within 12 hours for all the experiments reported here. However, up to 24 hours of storage at room temperature did not yield significantly different results.

Electrical recording

Voltage commands were generated by a 12-bit D/A converter (Tecmar, Cleveland, OH), driven by custom software. Currents were filtered at 1 or 5 kHz (−3dB; 4 pole Bessel filter) and sampled at 10 kHz by a 12 bit A/D converter. Data were saved for subsequent analysis on the hard disk and were archived on an optical disk drive. The holding potential was −120 mV for cells expressing Kv1.4 and −80 mV for the other channels, unless indicated otherwise. Recovery from inactivation for Kv1.4 required over 10 seconds. Therefore the cycle time for any protocol was 15 seconds or longer (with the exception of specific pulse trains). Between −120 and −80 mV, only passive linear leak was observed. Linear least squares fits to these data were used for leak correction.

Two-electrode voltage clamp

Oocytes were voltage clamped using standard two microelectrode voltage-clamp techniques [22,23]. Electrodes were pulled from Radnoti starbore glass and filled with 3 M KCl. Electrode resistances were 1–5 mΩ for voltage-recording electrodes and 0.15–0.3 MΩ for current-passing electrodes. Membrane potentials were controlled by an AxoClamp voltage clamp amplifier (Axon Instruments, Foster City, CA). With this amplifier and the low-resistance current electrodes, currents up to 10 μA could be measured. A grounded metal shield was inserted between the two electrodes to minimize electrode coupling and to speed the clamp rise time.

Whole cell patch clamp

Whole-cell recordings [24] were made with an Axopatch amplifier (Axon Instruments, Foster City, CA) as described previously [5]. Pipettes were pulled from Radnoti borosilicate glass and were heat polished. All data were obtained at room temperature. Electrode resistance was kept below 3.5 MΩ; the average resistance was 2.2 MΩ ($n = 46$) to improve voltage-clamp quality. Junction potentials were zeroed with the electrode in the standard bath solution. The average seal resistance was 17 GΩ (range 5–50 GΩ). Capacitance and series resistance compensation were used, and

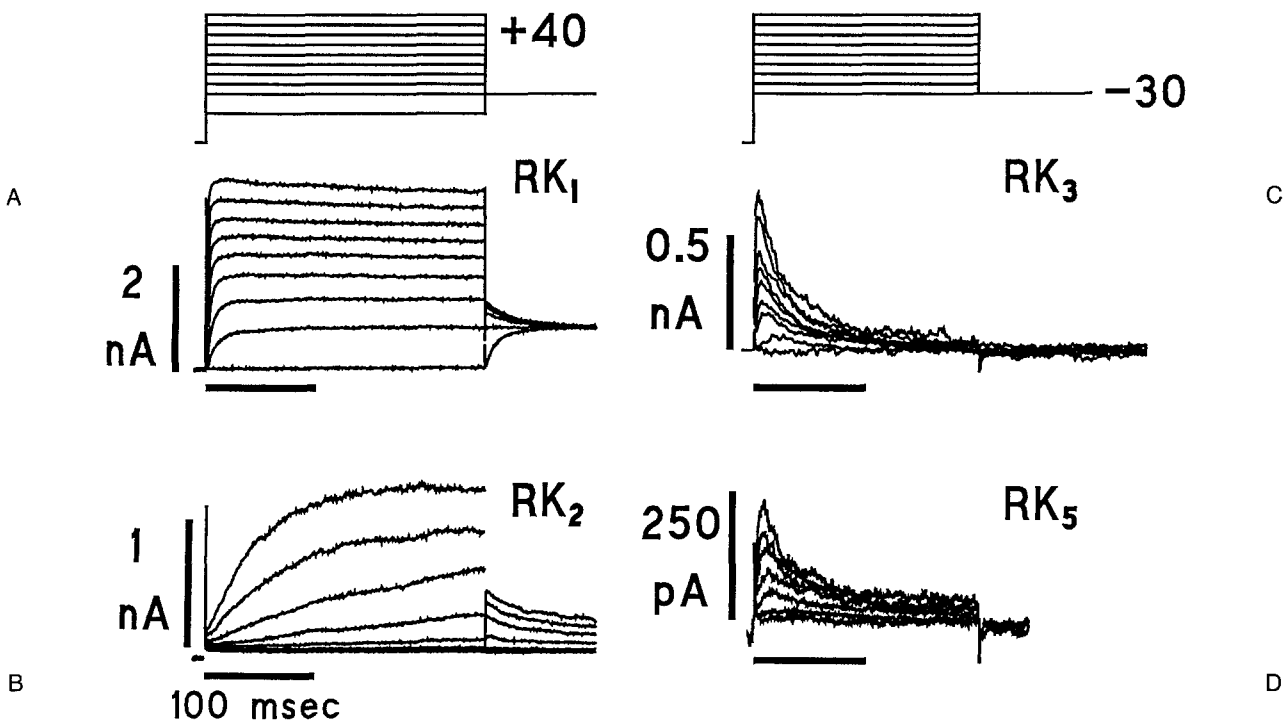

Fig. 1. *Expression of K^+ channel cDNAs cloned from the rat cardiovascular system in mouse fibroblasts. Each panel represents a cell from a different cell line that was transfected with a plasmid containing the putative channel coding region plus an antibiotic resistance gene that allowed survival and selection of cells that had expressed the channel. A: Kv1.1 (RK1) was cloned from a rat aortic library. (B) Kv1.2 (RK2), (C) Kv1.4 (RK3), and (D) Kv4.2 (RK5) were all isolated from rat ventricle.*

80% compensation was usually obtained. With an average current of 1.6 nA at +60 mV (i.e., worst-case analysis of largest currents associated with strongest depolarization), series resistance voltage errors were acceptably small (<5 mV). The low capacitance of the L cells enabled a relatively fast-clamp voltage clamp compared to native myocytes. The time constant for imposing a voltage step in these cells was approximately 100 μsec before series resistance compensation and was improved further after compensation.

Solutions

The bath solution for oocytes (ND-96) contained (mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 2.5 pyruvic acid, and 0.5 theophylline. pH was adjusted to 7.50 at 22°C with NaOH. Although there are numerous references in the literature of endogenous channels in *Xenopus* oocytes, we minimized endogenous channel activity by selecting batches of oocytes that showed no ionic currents in the absence of injected RNA [25].

For the mouse L-cell experiments, the "intracellular" pipette filling solution contained in mM: 110 KCl, 10 HEPES, 5 K_4BAPTA, 5 K_2ATP, and 1 $MgCl_2$, and was adjusted to pH 7.2 with KOH, to give a final intracellular K^+ concentration of 145 mM. The bath solution contained (mM): 130 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and was adjusted to pH 7.35 with NaOH.

Results

Figure 1 shows currents recorded using whole-cell voltage clamp for four of these clones expressed in mouse L-cells. Two basic phenotypes are observed when these cDNA clones where expressed in L-cells or oocytes. Kv1.1 (RK1), Kv1.2 (RK2), as well as Kv1.5 (HK2, not shown [13,14] all express predominantly as delayed rectifier-type currents. All of the delayed rectifier-type channels exhibit some degree of slow but incomplete inactivation during prolonged depolarizations (>500 msec). It is noteworthy that all of these channels appear to activate within less than 50 msec which is much faster than the very slow delayed rectifier that has been identified in guinea pig [5] and other tissues [1]. Kv1.4 (HK1 and RK3) and Kv4.2 (RK5) show very rapid and complete inactivation. Kv1.1 is the only channel of this group that is significantly inhibited by external TEA ($IC_{50} < 1$ mM); the remaining channels are resistant to TEA concentrations up to 100 MM. All of these channels are inhibited by internal TEA.

Figure 2 illustrates the current phenotype of two

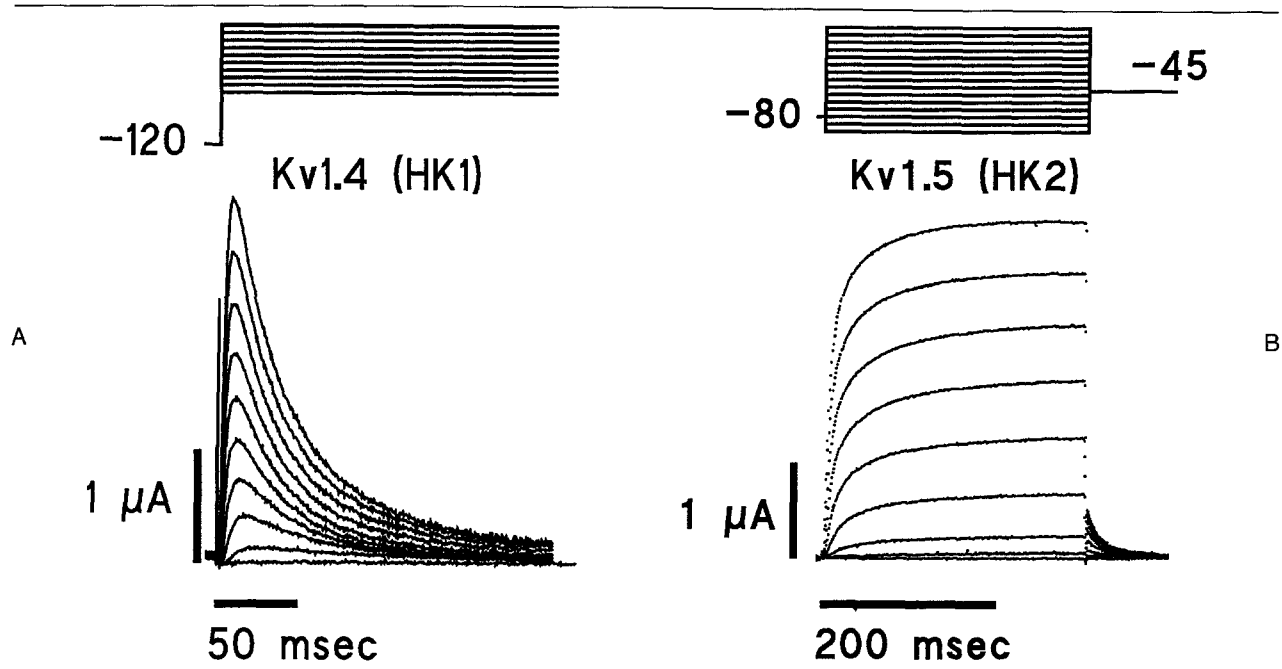

Fig. 2. Expression of K^+ channel cDNAs cloned from human ventricle. RNA was transcribed from cDNA templates in vitro and injected into Xenopus *oocytes. Newly synthesized K^+ channel current could be detected in approximately 8 hours and was usually recorded between 24 and 48 hours. Batches of oocytes were selected that show no endogenous voltage gated ion channel activity. In each panel oocytes were voltage clamped in a two-electrode arrangement. Voltage-clamp pulse protocols are shown above the current records. A: Human Kv1.4 expresses as a rapidly activating, rapidly inactivating A-type current. B: Kv1.5 shows a delayed rectifier phenotype.*

different human K^+ channels expressed in *Xenopus laevis* oocytes following microinjection of channel coding cRNA. Human Kv1.4 expresses as a rapidly inactivating A-type current [15–18]. These channels inactivate with time constants under 50 msec at 22°C. The current shows outward rectification in asymmetric potassium concentrations and recovers from inactivation with a time constant near 3 seconds [16]. Kv1.5 expresses a delayed rectifier-type channel that rapidly activates and shows little fast inactivation at room temperature. The channel does undergo a slow inactivation following prolonged depolarizations (>1 second), and the rate of inactivation increases with temperature. However, unlike Kv1.4, Kv1.5 does not completely inactivate, even for very long depolarizations.

Discussion

Table 1 shows the relative tissue distribution of expressed messenger RNA for these various clones in rat and human [13,14]. All of the channels listed in Table 1 have been expressed either transiently in *Xenopus* oocytes or in stable cell lines (mouse L-cells). Rat atrial levels of Kv1.4 mRNA appear equal or greater than those in brain, and both tissues show higher levels than aorta, ventricle, liver, or skeletal muscle. In rat, Kv1.5 shows high relative mRNA levels in heart compared to other tissues. Rat Kv1.5 mRNA levels are approximately equal in atrium and ventricle, and in aorta and all three of these tissues have much higher levels than in skeletal muscle or brain. In the human, in contrast to the rat, Kv1.4 mRNA appears in high levels in ventricle as well as in atrium, perhaps with slightly higher levels in ventricle compared to atrium. Thus, there is a distinction between expression of human Kv1.4 and rat Kv1.4 in ventricle. Human Kv1.4 mRNA levels are present in heart at approximately the same level as sodium channel message. Human Kv1.5 mRNA levels are much higher in atrium than in ventricle, but there is a significant presence of Kv1.5 mRNA in ventricle, being approximately equal to the levels of Kv1.4. We cannot assume necessarily that levels of message reflect levels of protein. However, at present, messenger RNA levels are the best available marker for these channels. When expressed in mouse L-cells, the antiarrhythmic agents quinidine [27] and clofilium block Kv1.5 channels at therapeutically relevant concentrations (quinidine IC_{50} = 6 μM; clofilium IC_{50} = 1 μM). This provides some preliminary evidence that this channel my serve as a target for these Class III antiarrhythmic agents.

Table 1. *Relative distributions of K^+ channel mRNA in different mammalian tissues*

cDNA clone	Original name	Relative tissue distribution	Drosophila subfamily
Rat			
Kv1.1	RK1,RCK1,RBK1	Brain >> atrium > SkM > aorta	*Shaker*
Kv1.2	RK2,BK2,RCK5,RAK	Brain > atrium > aorta = ventricle	*Shaker*
Kv1.4	RK3,RHK1,RCK4	Atrium > brain > aorta > ventricle = SkM	*Shaker*
Kv1.5	RK4	Atrium = ventricle = aorta > SkM > brain	*Shaker*
Kv4.2	RK5,mShal	Brain >> ventricle = atrium	*Shal*
Human			
Kv1.4	HK1	Ventricle = > atrium	*Shaker*
Kv1.5	HK2	Atrium >> ventricle	*Shaker*

Initially, cloned vertebrate K^+ channels were named according the species from which they were derived: R indicated rat, M indicated mouse, H indicated human; a K indicated the fact that the gene coded for a K^+ channel, and numbers simply indicated the order in which they were identified. In some cases the tissue of origin was also indicated: RCK1 = rat cortex K^+ channel #1; RHK1 = rat heart K^+ channel #1. More recently, due to the increasing number of channels that have been identified and the increasing confusion in nomenclature, a naming system for cloned vertebrate K^+ channel cDNA hs been proposed [33]. Strictly speaking, these names refer to the cDNAs that code for a peptide that is one fourth of a functional K^+ channel. In this system, Kv is used to designate a voltage-gated K^+ channel. A number then designates the Drosophila subfamily from which the clone is most likely to be derived (*Shaker* subfamily = Kv1.). A second number after a period simply indicates the order in which a clone was identified. Thus, Kv1 identifies a channel that appears to be a homolog of the *Shaker* subfamily of potassium channels.

Comparison of K^+ channels cloned from the cardiovascular system with Drosophila Shaker K^+ channels

The first potassium channel whose structure and function has been characterized as a result of molecular cloning approaches was derived from a mutant strain of *Drosophila melanogaster* known as *Shaker* [28–30]. This gene locus codes for a potassium channel, which when deleted results in aberrant behavior of the fruit fly, including vigorous shaking upon exposure to ether. The *Shaker* cDNA encodes a 70,200 Da protein. Since the initial efforts in cloning the *Shaker* potassium channel, a large number of voltage-gated potassium channels have been cloned from various tissues, including brain, heart, aorta, and uterus [13–15,31]. Many of these channels have a fair degree of amino acid identity (~75%), with the *Shaker* potassium channel in the conserved core domains. Prominent features include six putative membrane-spanning domains. The fourth domain (S4) contains positively charged arginines or lysines in every third position and is believed to be involved in sensing changes in transmembrane electrical potential [2]. All of these channels have an extremely highly conserved region between the S5 and S6 membrane-spanning domains, which is believed to form the outer part of the ion conducting pore [2,30]. A cartoon outline some of the key features of *Shaker* subfamily K^+ channels is shown in Figure 3.

Initially by analogy to the primary structure of voltage-gated sodium and calcium channels, it was believed that a given potassium channel gene coded for a protein that represented one fourth of the channel structure [2]. In other words, a functional potassium channel is a tetramer consisting of four peptide subunits. A given gene such as Kv1.4 codes for one of these subunits. Additional work in *Drosophila melanogaster* has revealed the presence of numerous genes encoding potassium channels [30]. These can be classified into different subfamilies, which are referred to as *Shaker*, *Shab*, *Shal*, and *Shaw*. Each of these represents a different gene locus, and each of these codes for a distinct peptide. It has been demonstrated that peptides encoded by a gene subfamily such a *Shaker* can assemble to form a functional potassium channel. However, it is apparently not possible for subunits from different subfamilies, such as *Shaker* and *Shab*, to assemble into a functional channel protein [32]. Thus, there appears to be a molecular barrier at the protein level that prevents assembly of peptides that are derived from a different gene subfamily. Additional functional diversity of K^+ channels in Drosophila apparently arises from alternately spliced mRNA transcripts, where the final amino acid sequence can be modified at the level of RNA transcription, resulting in altered functional behavior of the channels. Many of the voltage-gated potassium channels derived from the mammalian cells represent *Shaker*-type channels. However, *Shal*-related channels have also been identified in the rat cardiovascular system [14]. An interesting apparent difference between these invertebrate channels and their mammalian homologues is that much of the diversity in mammals may be accounted for by the presence of multiple distinct genes, each of which codes for a different K^+ channel subunit within a single exon, with little evidence for alternative splicing of RNA transcripts.

A list of potassium channels cloned from rat and

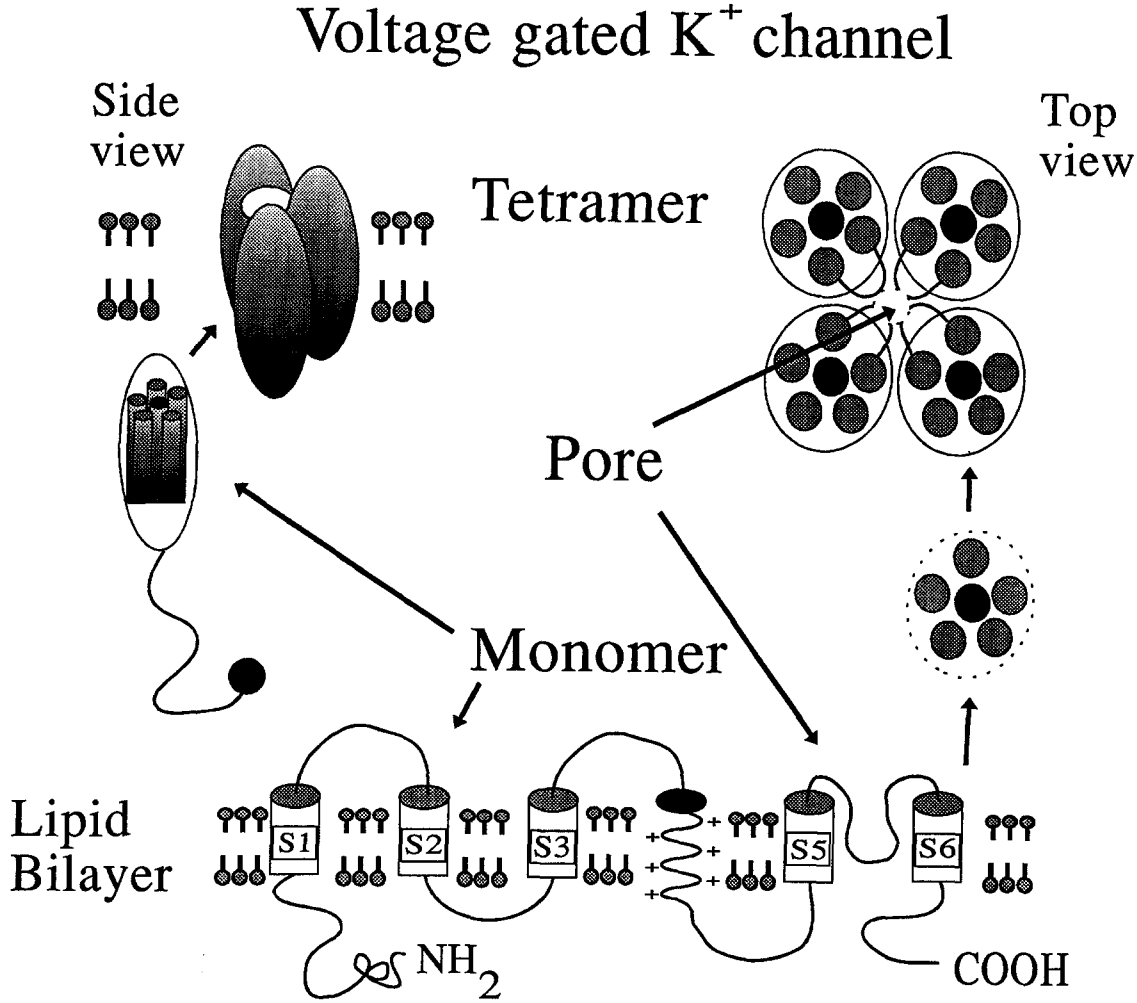

Fig. 3. Diagram of a voltage-gated cardiac potassium channel. Several unique genes code for a peptide containing 400–700 amino acids (bottom) have been described. These peptides all are predicted to have six major hydrophobic domains (S1-S6) that probably span the lipid bilayer. The amino and carboxy termini are intracellular. The fourth domain, S4, has a number of positively charged arginines or lysines, and may participate in sensing the transmembrane electrical field. It is believed that four of these peptides must assemble to form a functional K^+ channel. Presumably the more hydrophilic S4 domain would be surrounded and isolated from the membrane by the other more hydrophobic domains. A hydrophobic stretch of amino acids connecting the S5 and S6 domains probably forms part of the outer portion of the ion-conducting pore. Each subunit presumably contributes one of these loops, which dip into the membrane between the protein subunits and form the ion conducting pore. Some of the channels (e.g., Kv1.4) also have an elongated amino terminus, which presumably is involved in the fast inactivation of the channel. It has been proposed that this domain can block or plug the channel by binding in the inner mouth of the pore, thus inactivating it.

human cardiovascular system is shown in Table 1. Also shown in Table 1 are the relative tissue distributions of the mRNAs that code for each of these channels. The presence of mRNA in a tissue indicates that the channel is expressed there, but since there is cellular heterogeneity in a given tissue, the cellular localization cannot yet be assigned. Kv1.1 (Table 1) has been cloned from rat aorta but has also been identified in whole brain and in cortex [31]. Kv1.2 has been isolated from rat heart and rat brain. Kv1.4 has been cloned from rat heart and from rat brain [31]. Human Kv1.4 is 98% identical to the rat Kv1.4 and has been cloned from a human ventricular library, and Kv1.5 has been cloned from a rat heart, as well as rat brain, and a human version of Kv1.5 has been cloned from human ventricle. The human heart clone is only 86% identical to the rat Kv1.5. Thus, although the new naming system in some ways serves to reduce the proliferating number of names for these channel clones, it does not identify structural and functional differences among the expressed proteins in different species and cells.

Comparison of voltage-gated cloned potassium channels within endogenous cardiac potassium currents

Kv1.1, Kv1.2, and Kv1.5 are all delayed rectifier-type currents that show little inactivation within a few hundred milliseconds, under voltage clamp at room temperature. Although Kv1.1 was cloned from an aortic library, the best available evidence suggests that it is primarily a channel of the nervous system [13,31]. Even less is known about Kv1.2, but it and the channel referred to as Kv1.5, which is present at high levels in rat and human heart, resemble a noninactivating current that has recently been described in rat atrium [34]. Paulmichael et al. [35] have suggested that Kv1.2 codes for channels represent a channel that corresponds to this atrial channel described by Boyle and Nerbonne [34]. This is supported by the fact that both the endogenous current and Kv1.2-generated currents are sensitive to 4-amino-pyridine and are relatively insensitive to external tetraethyl ammonium. Human Kv1.5 has similar rapid activation kinetics and is even more sensitive to external 4-amino-pyridine, raising the possibility that this channel may also correspond to the native channel, which is supported by the relatively high presence of Kv1.5 mRNA in cardiac tissue [14]. Additional evidence will be required to resolve and identify the correspondence between these cloned channels and a particular native channel.

The two inactivating channel clones Kv1.4 and Kv4.2 belong to the *Shaker* and a *Shal* Drosophila subfamily of channels, respectively, and both may be candidates for the A-type current that is seen in heart (I_{TO}) [36–40]. Kv1.4 has an appropriate voltage dependence and a relatively high sensitivity to 4-amino-pyridine ($IC_{50} < 1$ mM). Both rat and human Kv1.4 recovery from inactivation ($\tau = 3$ seconds) much much slowly than the native current. Kv4.2 recovers from inactivation much faster that Kv1.4. On the other hand, Kv4.2 is not very sensitive to 4-amino-pyridine ($IC_{50} > 5$ mM). The native channel is sensitive to low concentrations of 4-amino-pyridine ($IC_{50} < 1$ mM) and recovers from inactivation with a time constant less than 500 msec. A possible explanation for the difficulty in identifying a particular channel clone with a native channel may be that native channels consist of multiple heterogenous subunit types from different genes. The formation of such heterotetrameric K^+ channels has been demonstrated to occur in *Xenopus* oocytes upon coinjection of two different types of K^+ channel mRNA (e.g., Kv1.2 and Kv1.4) [18,41,42]. These hybrid channels can have properties of either or both parent channels, and this may be an additional explanation of the remarkable diversity of K^+ channels in the cardiovascular system and elsewhere.

Conclusions

In summary, although we have come a long way towards identifying the molecular details of cardiac excitability proteins, clearly much more remains to be done. A more complete identification between a cloned molecule and a native channel protein is needed. To accomplish this, high-resolution monoclonal antibodies and immuno-histochemistry will be required to localize channels to a particular cell type. In addition, techniques of in situ hybridization or microcloning techniques from single cells may provide insights into the channel types expressed in those cells. We have only begun to investigate the detailed gating and pharmacology of these channels that can now be expressed in a pure system, allowing for the first time very detailed kinetic analysis of these important proteins.

Acknowledgments

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