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Cloning and tissue-specific expression of five voltage-gated potassium channel cDNAs expressed in rat heart

(ion-channel molecular biology/mammalian cardiovascular system/*Drosophila* Shaker and Shal genes)

STEVEN L. ROBERDS AND MICHAEL M. TAMKUN*

Departments of Pharmacology and of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232

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ABSTRACT **Five distinct K^+ channel cDNA molecules (RK1 to RK5) were cloned from either rat heart or rat aorta cDNA libraries. Four of the channels, RK1 to RK4, are similar or identical to Shaker-like K^+ channels previously identified in rat brain cDNA libraries. Major differences among RK1 to RK4 exist in the amino- and carboxyl-terminal regions and in amino acids representing potential extracellular sequence between the S1 and S2 hydrophobic domains. RK5 encodes a unique channel of 490 amino acids having six hydrophobic domains but only five basic residues in the putative voltage-sensing domain. Unlike RK1 to RK4, RK5 is a rat homologue of the *Drosophila* Shal family of K^+ channels, which have not been previously described in mammals. Although RK5 mRNA is present in cardiac atrium and ventricle, it is most abundant in brain. RK1, RK2, and RK3 transcripts are predominantly found in brain but are present also at lower levels in other tissues, such as heart and aorta. RK2 is absent from skeletal muscle whereas RK1 and RK3 are present in this tissue. RK4 mRNA is ubiquitous in electrically excitable tissue, being present at comparable levels in atrium, ventricle, aorta, brain, and skeletal muscle. The cloning of RK5 confirms the presence in mammals of all four *Drosophila* K^+ channel families: Shaker, Shab, Shaw, and Shal.**

Potassium channels constitute the most diverse group of voltage-gated ion channels. At least four general classes of K^+ channels have been defined by electrophysiological means, with each class being comprised of many subtypes. These classes include inward rectifiers, Ca^{2+}-activated K^+ channels, A-type K^+ channels, and delayed rectifiers (1). This diversity of K^+ channel electrophysiology suggests that these channels are also structurally diverse. The discovery of a *Drosophila* strain possessing a mutant K^+ channel prompted the cloning of the *Drosophila* Shaker gene (2), initiating the study of K^+ channels at the molecular level. To date, the complete cDNA sequences for 1 mouse (3), 2 human (4, 5), and 11 rat (6–15) K^+ channel cDNA clones have been reported. Heterogeneity in mammals appears to be generated primarily by the expression of a large number of genes, since known mammalian K^+ channel genes are intronless (4, 5, 12, 14, 16) and alternative splicing in mammals has not been reported.

Whereas previous K^+ channel cloning efforts have focused on the nervous system, this paper reports the sequence and tissue distribution of five K^+ channel clones (RK1 to RK5) from the rat cardiovascular system.† RK1 to RK4 are homologues of the *Drosophila* Shaker K^+ channel family. RK5 has not been described previously, to our knowledge, and is a mammalian homologue of the *Drosophila* Shal gene. This study further illustrates the diversity of structure and tissue-specific expression of mammalian K^+ channels.

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MATERIALS AND METHODS

cDNA Library Construction and Screening. Size-fractionated libraries [2- to 8-kilobase (kb) cDNA] were constructed according to standard protocols (17). PCR-generated cDNA fragments corresponding to nucleotides (nt) 681–1007 of RBK1 (7) and nt 685–1014 of RBK2 (8) and subsequent partial clones were used as radiolabeled probes. Recombinants were transferred to nitrocellulose and hybridized overnight at 42°C in 20% (vol/vol) formamide/4× SSPE (600 mM NaCl/40 mM NaH_2PO_4/4 mM EDTA, pH 7.4)/5× BFP [bovine serum albumin (1 g/liter)/polyvinylpyrrolidone 40 (1 g/liter)/Ficoll (1 g/liter)/0.001% sodium azide]/sonicated salmon sperm DNA (0.1 mg/ml)/0.2% SDS. Filters were washed for 2 hr with multiple solution changes at 42°C in 3× SSC (450 mM NaCl/45 mM sodium citrate, pH 7.0)/0.1% SDS. Low-stringency screening yielded several full-length clones: one of RK2 (2.4 kb), two of RK3 (3.5 and 3.6 kb), two of RK4 (2.5 and 2.7 kb), and two of yet another unique K^+ channel, RK5 (2.8 and 3.9 kb). Duplicate clones did not differ with respect to polyadenylylation sites. Low-stringency screening with the same probes of 2.2×10^6 plaques of a library constructed from size-fractionated rat aorta cDNA yielded a full-length clone of RK1 (2.7 kb).

Northern Blot Analysis. Total RNA (5 μg) from each tissue was fractionated by electrophoresis through a 1% agarose/3% (vol/vol) formaldehyde gel in the absence of ethidium bromide. Application of equal amounts of RNA to each lane was confirmed by ethidium bromide staining of a parallel gel after electrophoresis. The gel was submerged for 5 min in 50 mM NaOH/1.5 M NaCl and then neutralized 30 min in 1 M Tris·HCl, pH 6.8/1.5 M NaCl, before an overnight transfer to Nytran (Schleicher and Schuell) by capillary action in 20× SSC. The filters were prehybridized overnight at 65°C in 20% formamide/10% (wt/vol) dextran sulfate/4× SSPE/5×BFP/sonicated salmon sperm DNA (0.1 mg/ml)/ yeast RNA (0.2 mg/ml)/5% (wt/vol) SDS. Filters were hybridized 24 hr at 65°C with an RK1-5 isoform-specific probe (10^6 cpm/ml) having a specific activity of 7.5×10^8 cpm/μg. The K^+ channel cDNA fragments used as probes, with the adenine of the initiating codon ATG being +1, were as follows: RK1, nt 945 to 1549; RK2, nt −40 to 1768; RK3, nt −530 to 3025; RK4, nt 1621 to 2199; RK5, nt −785 to 2007, drk1, nt 1310 to 2580 (10); rat brain Na^+ channel II, nt −77 to 1513 and nt 3362 to 5866 (18). These RK sequences were isoform-specific as judged by hybridization analysis. Filters were washed twice for 30 min at 65°C with 3× SSC/1% SDS, twice with 1× SSC/1% SDS, and twice with 0.2× SSC/1% SDS before autoradiography.

Abbreviation: nt, nucleotide(s).

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M59980).

RESULTS AND DISCUSSION

Cloning and Primary Sequence Analysis. Low-stringency library screening with the PCR-generated cDNA probes yielded five distinct cDNA clones from the rat cardiovascular system. The most distinct of these was designated RK5. The nucleotide and deduced amino acid sequences of RK5 are shown in Fig. 1. One open reading frame encoding 490 amino acids was identified. The initiating methionine was assigned because of its homology to the mammalian consensus sequence for initiation of translation, CCACCAUGG (20), and because it represents the first AUG positioned 3′ of termination codons in all reading frames. RK5 is predicted to encode a nascent polypeptide of 55 kDa. Hydropathy analysis of RK5 (data not shown) reveals six potential membrane-spanning domains similar to those found in other K^+ channels. The fourth hydrophobic domain, S4, has five basic amino acids at every third position, a motif unique among known rat K^+ channels but identical with that found in the *Drosophila* Shal family (19). In this region, the Shaker gene has seven basic residues, Shab has six, and Shaw has four (19). The amino- and carboxyl-terminal ends are proposed to be intracellular in accordance with prevailing models of K^+ channel structure (21). Two potential N-linked glycosylation sites (22) are identified at residues 46 and 408. However, according to generally accepted models of K^+ channel orientation in the membrane, both sites would be intracellular and, therefore, carbohydrate-free. One potential phosphorylation site exists at Thr-38 for cAMP-dependent protein kinase (23).

RK5 is a rat homologue of the *Drosophila* Shal K^+ channel family (19). Amino acids conserved from fly to rat are shaded in Fig. 1. RK5 and Shal are most homologous within the core region containing the six putative membrane-spanning domains. The 222 amino acids including S1 to S6 are 85% identical between the two channels. The fourth hydrophobic domain, S4, is identical in both channels. The amino-terminal 185 amino acids are 58% identical, and the carboxyl-terminal 84 residues are 44% identical. Of the final 50 carboxyl-terminal amino acids, only 8 (16%) are identical between the two proteins. Both proteins are rich in proline residues near the amino terminus, but only in RK5 are 5 of the 13 carboxyl-terminal residues proline. Rat homologues of Shab, drk1 (10), and Shaw, NGK2 (11) and RShIIIA (13), have been described by other investigators. The presence of mouse homologues of all four *Drosophila* gene families has been reported (19), but full sequences have not been published.

The amino acid sequences of all five rat cDNA clones are compared in Fig. 2. RK1, isolated from a rat aorta cDNA library, is identical to RBK1 (7) and RCK1 (6) cloned by others from brain. RK2, isolated from a rat heart cDNA library, has coding sequence identical to the RBK2 channel cDNA cloned from rat brain by McKinnon (8). However, 52 base pairs (bp) of 5′ untranslated sequence (nt −114 through −165) of RBK2 are absent from RK2. RK2 and RBK2 differ from the RCK5 channel in rat brain (9) by 9 of 499 amino acids and contain somewhat different nucleotide sequences in untranslated regions, suggesting that the two channels may represent allelic variations. RK3 is identical to RHK1 reported by Tseng-Crank *et al.* (24) except for two amino acid substitutions at residues 42 (Leu → Arg) and 309 (Ala → Gly). Both RK3 and RHK1 differ by 6 additional amino acids from RCK4 (9), a K^+ channel cloned from rat brain. A human K^+ channel clone, HK1 (27), obtained in this laboratory from a human ventricular cDNA library, is more homologous to RK3 and RHK1 than to RCK4. RK4 is nearly identical to the K_v1 K^+ channel of rat brain reported by Swanson *et al.* (12). Two nucleotide differences within the coding region were found. AGC encodes Ser-553 in RK4, and TGC encodes Cys-553 in K_v1. Gly-228 is present in both clones, although

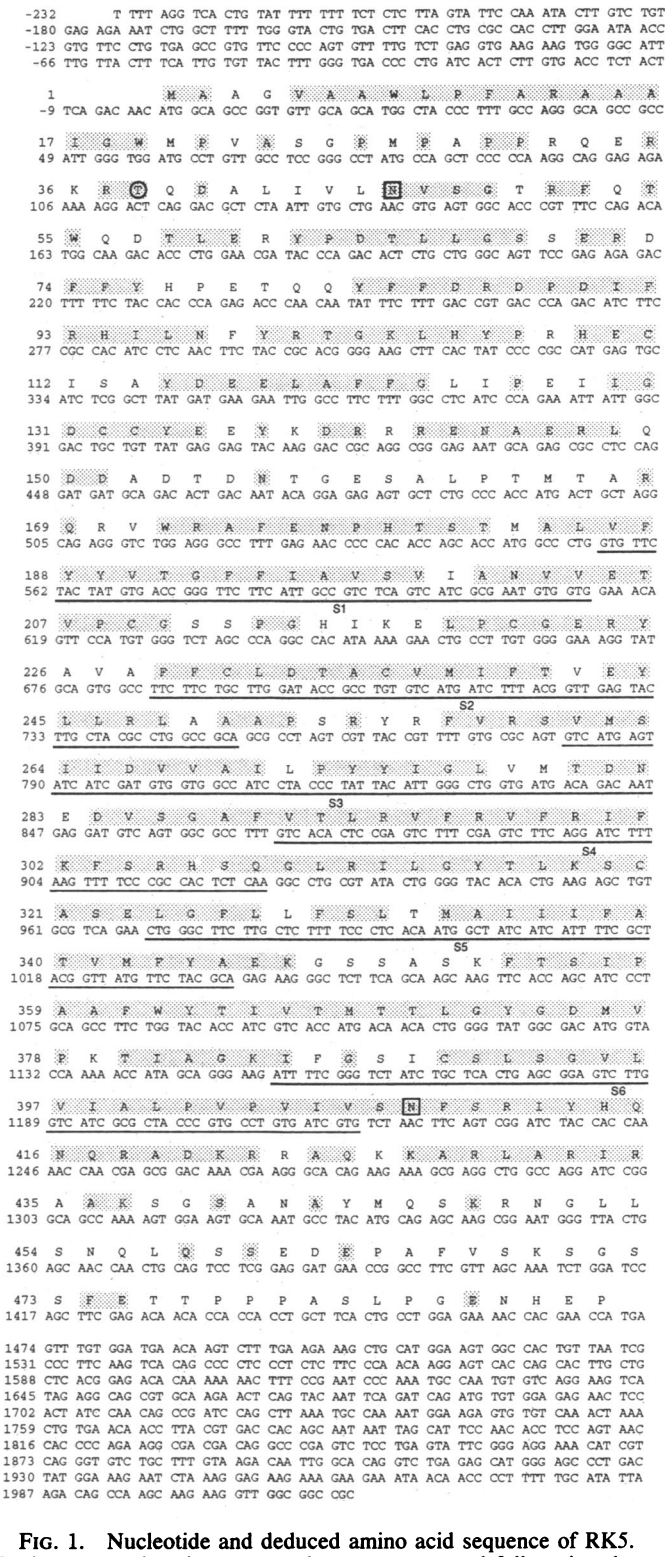

FIG. 1. Nucleotide and deduced amino acid sequence of RK5. Both sense and antisense strands were sequenced fully using the exonuclease III deletion method (17) and oligonucleotide primers. Six hydrophobic putative membrane-spanning domains are indicated by bars and labeled S1 to S6. Shaded amino acids are identical to the *Drosophila* Shal K^+ channel (19). Potential cAMP-dependent protein kinase phosphorylation sites are circled. Potential N-linked glycosylation sites are boxed.

it is encoded by GGA in RK4 and GGG in K_v1. In untranslated regions, a single nucleotide substitution 9 bp upstream of the initiation codon was observed (C → T), and the 8 bp at positions 12–19 downstream of the terminating TAA in K_v1 were absent in RK4. RK4 is most similar to RK1 and RK2 but

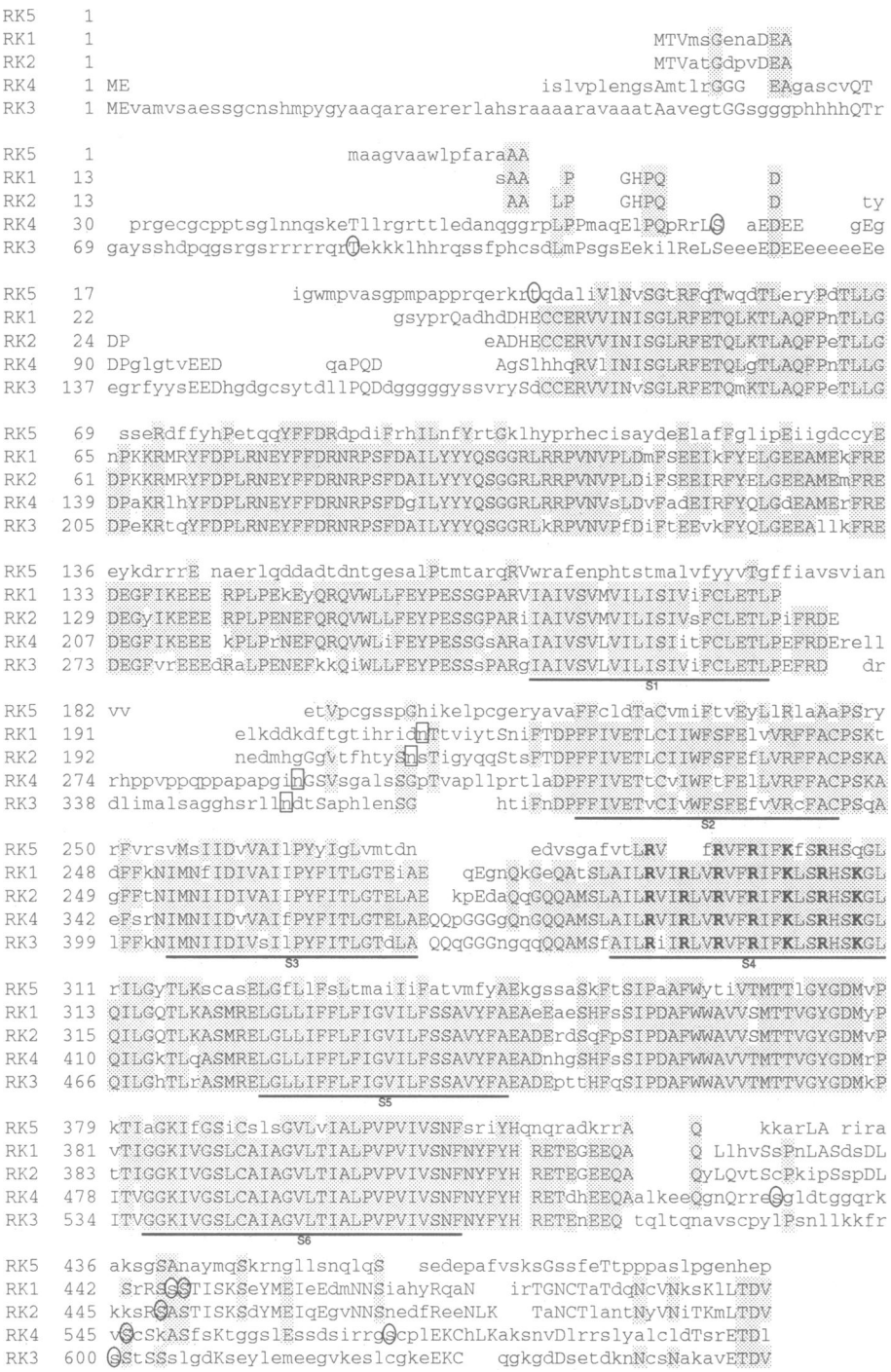

FIG. 2. Amino acid comparison of K^+ channel clones RK1 to RK5. RK1 to RK4 were sequenced extensively within the 5′ and 3′ untranslated regions and fully within the coding regions by using oligonucleotide primers. The six hydrophobic putative membrane-spanning domains are indicated by bars. Shaded amino acids are conserved among at least three clones. Potential cAMP-dependent protein kinase phosphorylation sites (23) hypothesized to be oriented toward the cytoplasm are circled. Potential N-linked glycosylation sites hypothesized to be oriented extracellularly are boxed. Basic amino acids within the S4 domain are in boldface type.

has 100 more amino acids 5′ of the S1 domain than either RK1 or RK2.

RK1 to RK4 are homologues of the *Drosophila* Shaker family of K^+ channels. Nucleotide differences among RK1 to RK4 are dispersed throughout the entire cDNA molecules, indicating that the clones are encoded by unique genes rather than differentially spliced transcripts of a single gene. Differences among isoforms are most prevalent in the amino- and carboxyl-terminal regions of the proteins, whereas K^+ channel core sequences encoding the putative membrane-spanning and S4 regions are well conserved. Like the Shaker family, RK1 to RK4 have seven basic amino acids in the S4 domain. The S4, S5, and S6 hydrophobic domains are identical in RK1, RK2, and RK4 and are well conserved in RK3. Less-conserved domains, such as the putative extracellular regions between S1 and S2, between S3 and S4, and the amino- and carboxyl-termini, may confer isoform-specific properties.

Tissue Distribution of K^+ Channel Isoforms. Northern blot analysis of K^+ channel expression in rat cardiac atrium and ventricle, aorta, brain, liver, and skeletal muscle (Fig. 3) was performed with isoform-specific probes for RK1 to RK5. Although each probe does not recognize any of the other four isoforms reported here, they may cross-hybridize with transcripts encoding undiscovered K^+ channel isoforms.

RK1 is most abundant in brain, less abundant in atrium and skeletal muscle, and barely detectable in aorta. No hybridization of the RK1 probe to ventricular or liver transcripts was detectable. Since atrium is more highly innervated than ventricle (25), RK1 may arise from nerve cells rather than muscle cells in these tissues. The RK1 cDNA probe hybridizes most strongly to a transcript of approximately 8 kb and

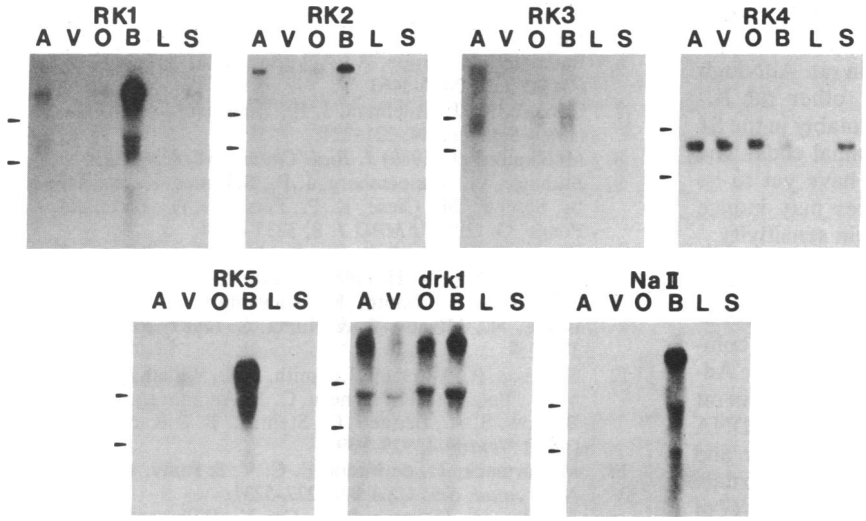

FIG. 3. Tissue distribution of RK1 to RK5 mRNAs. Each lane represents 5 μg of total RNA isolated from rat ventricle (lanes V), atrium (lanes A), aorta (lanes O), brain (lanes B), liver (lanes L), or skeletal muscle (lanes S). The autoradiograph of RK1 was exposed for 1 week at −70°C, whereas other autoradiographs were exposed for 3 days. No additional signals were detected after longer exposures. Positions of the large and small rRNA subunits, approximately 4.7 and 1.9 kb, respectively, are indicated. Hybridization of random-primer-labled phospho*enol*pyruvate-carboxykinase cDNA to a discrete rat liver transcript on reprobed blots confirmed that the liver mRNA in the above blots was intact.

less intensely to transcripts of approximately 3 and 4 kb. This is consistent with brain Northern blots of Beckh and Pongs (26), which revealed a primary transcript of 8.5 kb and multiple transcripts between 3 and 4.5 kb. Alternative mRNA splicing may occur in the long untranslated region(s), since the translated sequence consists of only 1500 bp. Additionally, alternative transcription start sites or polyadenylylation sites may be utilized.

RK2 is also most prevalent in brain. It is expressed at lower levels in atrium, ventricle, and aorta. RK2 is undetectable in liver and skeletal muscle. Levels of RK1 and RK2 in brain, atrium, and aorta are similar, but RK2, unlike RK1, is also present in ventricle. The RK2 probe, which contains the entire coding region, hybridizes primarily to a transcript of approximately 10 kb. This is consistent with RCK5-probed brain Northern blots (26), which revealed a single hybridizing transcript of approximately 11 kb. This is further evidence that RK2 and RCK5 represent different alleles of a single gene.

RK3 is most abundant in brain and atrium but is detectable at low levels in ventricle, aorta, and skeletal muscle. The RK3 probe hybridizes most strongly to a transcript approximately 4.5 kb long. RK3 also hybridizes to a very large transcript of approximately 15 kb and a smaller one of 1.5 kb. The primary transcript size is consistent with that observed for RHK1 in brain and heart, although RHK1 was not detected in aorta (24). Additionally, RHK1 was more abundant in brain than heart, perhaps because atrial RNA was diluted by ventricular RNA in the whole heart RNA preparation. In brain, RCK4 also hybridizes primarily to a 4.4-kb transcript (26).

RK4 is the most abundant cardiac channel of the five. It is present in nearly equal amounts in atrium, ventricle, aorta, and skeletal muscle, and to a lesser extent in brain. It is absent from liver. Swanson *et al.* (12) reported similar results in Northern blot analysis of K_v1, a clone nearly identical to RK4, in brain, heart, liver, and skeletal muscle. K_v1 was not analyzed with respect to smooth muscle or separate chambers of the heart. The RK4 probe hybridizes primarily to a 3-kb transcript. Although the relative amounts differ, RK4 is present in the same tissues as RK3. Given the 6-fold difference in length between RK3 and RK4 probes, RK4 is much more abundant in heart than RK3.

RK5 is most abundant in brain and is present at lower levels in ventricle, atrium, and aorta. It is second only to RK4 in abundance in the ventricle, although other channels have equal or higher levels in atrium and aorta. Like RK2, RK5 is present in cardiac and smooth muscles, but not skeletal muscle. Shorter autoradiograph exposure times showed the primary transcripts in brain to be approximately 6 and 7 kb, but some hybridization was also observed to a 4-kb transcript. The atrial and ventricular transcripts were each represented by a band at approximately 6 kb, as opposed to the 6 and 7 kb transcripts observed in brain. This pattern was also seen when the 5′ 900 bp of the RK5 cDNA, containing 600 bp of untranslated sequence, which is unlikely to be conserved among Shal-like isoforms, was used as a probe (data not shown). This may indicate that RK5 mRNA from a single gene is transcribed or processed differently in cardiac tissue than in brain tissue.

The transcripts for two additional ion channels were analyzed. A probe for the Shab-like delayed rectifier, drk1 (10), of rat brain was used to compare RNA levels to a third class of voltage-gated K^+ channels. Probes for the rat brain type II voltage-gated Na^+ channel (18) were used for comparison with voltage-gated channels selective for other ions. The transcripts for drk1 are ubiquitous among electrically excitable tissues, much like RK4. drk1 is slightly more abundant in brain and aorta than in atrium, ventricle, and skeletal muscle. Two transcripts, approximately 4 and 10 kb, hybridize equally well to the drk1 probe. Rat brain Na^+ channel II is nearly brain specific, but it is present at low levels in aorta and atrium. The primary transcript is approximately 10 kb. This is consistent with analysis of brain, heart, liver, and skeletal muscle poly(A)$^+$ RNA by Noda *et al.* (18). This Na^+ channel is present in brain at an equal or lower level than RK1 and RK5.

Table 1. *Drosophila*-like rat K^+ channels and their relative abundance in tissues

K^+ channel	Equivalent nomenclature	Relative tissue abundance
Shaker		
RK1	RBK1 (7), RCK1 (6)	B >> A = S > O
RK2	RBK2 (8), RCK5* (9)	B > A > O = V
RK3	RHK1* (24), RCK4* (9)	B ≈ A > O = S > V
RK4	K_v1 (12)	A = V = O = S > B
Shal (RK5)	None	B >> V = A
Shab [drk1 (10)]	None	B > A = O > V = S
Shaw		
NGK2 (11)	None	ND
RKShIIIA (13)	None	ND

Numbers in parentheses refer to references. A, atrium; V, ventricle; O, aorta; B, brain; S, skeletal muscle; ND, not determined. Channels are arranged by homology to *Drosophila* classes.
*Some amino acid differences have been identified in this clone, perhaps indicating an allelic difference (see text for discussion).

Significance. With the description here of RK5, homologues of all four *Drosophila* K^+ channel families—Shaker, Shab, Shaw, and Shal—have been identified in rat. Although RK5 shares some structural features with other rat K^+ channels, it is unique in many regions, most notably in the S4 domain and in the amino- and carboxyl-terminal ends. The functional implications of these differences have yet to be determined. Even small sequence differences may induce variations in channel function or drug or toxin sensitivity.

We have also demonstrated that six K^+ channels, RK1 to RK5 and drk1, are expressed in the heart. Table 1 summarizes the rat clones described here and their relative abundance in six tissues. The Shaw family is included for completeness, although it is not investigated in this paper. Additionally, at least 12 unique K^+ channels are expressed in rat brain as determined by Northern blot analysis or cDNA cloning. K^+ channel expression in the cardiovascular and nervous systems is clearly complex. Although cloning to date has resulted in a preponderance of Shaker-like isoforms (7 of 12), it is reasonable to predict that the other three families may be equally diverse in mammals. A thorough investigation of the functional and pharmacological properties of each isoform, and those of constructed mutants, is necessary to expand our understanding of K^+ channel structure–function relationships.

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