Molecular Cloning and Characterization of LKv1, a Novel Voltage-Gated Potassium Channel in Leech

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ABSTRACT: We have cloned a novel voltage-gated K channel, LKv1, in two species of leech. The properties of LKv1 expressed in transiently transfected HEK293 cells is that of a delayed rectifier current. LKv1 may be a major modulator of excitability in leech neurons, since antibody localization studies show that LKv1 is expressed in the soma and axons of all neurons in both the central and peripheral nervous systems. Comparison of the biophysical and pharmacological properties of LKv1 with native voltage-gated conductances in leech neurons suggests that LKv1 may correspond to the previously characterized delayed rectifier current, I_K. Phylogenetic analysis of LKv1 shows that it is related to the Shaker subfamily of voltage-gated K channels although it occupies a separate branch from that of the monophyletic Shaker clade composed of the flatworm, Aplysia, Drosophila, and mammalian Shaker homologs as well as from that of two recently identified Shaker-related K channels in jellyfish. Thus, this analysis indicates that this group of voltage-gated K channels contains several evolutionarily divergent lineages. © 1999 John Wiley & Sons, Inc. J Neurobiol 38: 287–299, 1999

Keywords: Shaker; leech; immunocytochemistry; K channels; phylogeny

A particularly advantageous organism in which to address questions of how the distribution and properties of ion channels relate to the biological function of neurons can be found in the leech (Johansen, 1991). The leech central nervous system (CNS) is very simple, consisting of a fixed number of 33 ganglia: 21 similar midbody ganglia, four ganglia fused into a head brain, and seven fused into a tail brain, and a supraesophageal ganglion (Muller et al., 1981). Each ganglion contains very few neurons, <400, most of which are bilaterally symmetrically arranged, leaving no more than about 200–300 types of different neurons per ganglion (Muller et al., 1981; Macagno, 1980). The ganglia are to a high degree segmentally reiterated, further limiting the total number of different neurons within the CNS. These neurons are relatively large, ranging in diameter from 20 to 100 μm. The neurons surround a central neuropil forming a single layer, making each individual neuron easily accessible for electrophysiological recording. For these reasons, many leech neurons are functionally identified (Muller et al., 1981), and several neuronal networks mediating a number of behaviors—for example, swimming (Stent et al., 1978), control of heartbeat (Stent et al., 1979), and the bending and withdrawal reflexes (Krستان, 1982)—have been described in great detail. Furthermore, applications of voltage-clamp techniques have allowed the identification and
characterization of many of the conductances underlying the regulation of excitability in these neurons (Johansen and Kleinhaus, 1986, 1990; Johansen et al., 1987; Arbas and Calabrese, 1987; Stewart et al., 1989). As a beginning to analyze these currents on the molecular level, we here describe the cloning of a leech voltage-gated K channel, LKv1, and its expression in transiently transfected HEK-293 cells. The properties of LKv1 expressed in these cells are that of a delayed rectifier current. Antibody localization studies show that LKv1 is expressed in the soma and axons of all known neurons in the CNS and peripheral nervous system (PNS), suggesting that this current may constitute a major regulator of excitability in leech neurons. Although phylogenetic analysis of LKv1 shows that it is related to the Shaker subfamily of voltage-gated K channels, its sequence is highly divergent with many unusual features. Consequently, LKv1 is placed on a branch that is separate both from that of the monophyletic Shaker clade composed of the flatworm (Kim et al., 1995), Aplysia (Pfaffinger et al., 1991), Drosophila (Papazian et al., 1987), and mammalian Shaker homologs (Tempel et al., 1988; Pongs, 1993), as well as from that of the two Shaker-related voltage-gated K channels recently described in jellyfish (Jeglal et al., 1995). Thus, this group of voltage-gated K channels appear to be heterogeneous and to contain several evolutionarily divergent lineages.

**MATERIALS AND METHODS**

**Experimental Preparations**

For the present experiments, we used the three hirudinid leech species: *Hirudo medicinalis, Haemopis marmorata,* and *Macrobdella decora*. The leeches were either captured in the wild or purchased from commercial sources. Dissections of nervous tissue and embryos were performed in leech saline solutions with the following composition (in millimoles): 110 NaCl, 4 KCl, 2 CaCl₂, 10 glucose, 10 Hepes, pH 7.4. In some cases, 8% ethanol was added and the saline solution cooled to 4°C to inhibit muscle contractions. Breeding, maintenance, and staging of leech embryos at 22–25°C were as previously described (Fernández and Stent, 1982; Macagno et al., 1983; Jellies et al., 1987). Embryonic day 10 (E10) was characterized by the first sign of a tail sucker, while E30 is the termination of embryogenesis.

**Molecular Cloning and Sequence Analysis**

Degenerate universal primers to regions conserved in the four subfamilies of voltage-gated K⁺ channels: Shaker, Shal, Shab, and Shaw NEYFFD (T1 assembly domain) and TMTTVG (pore-lining region) were used in a polymerase chain reaction (PCR)-based screen. *Haemopis* genomic DNA was amplified using low-stringency annealing conditions (37°C) for 30 cycles using the Perkin-Elmer GeneAmp kit according to the manufacturer’s instructions. The degenerate oligonucleotide primers were synthesized on the basis of conserved sequences in *Drosophila* and included *EcoRI* and *SalI* restriction linker sequences for cloning purposes: 5’-AGTCTAGATTCCACC/T1AICCA5’/G1GTNTGCAT-3’ and 5’-GACTGCACTGGAC-AA5’/C1GA5’/A1TA5’ cTTGTTCTAAGT/1G15’/G-3’ (the primers were the generous gift of Dr. A. Wei and Dr. L. Salkoff). The PCR reaction products were digested with *EcoRI* and *SalI*, treated with calf intestinal alkaline phosphatase to prevent multimerization, and subcloned into M13 mp19 according to standard protocols (Sambrook et al., 1989) to generate a minilibrary of PCR reaction products. This minilibrary was plated, transferred to nitrocellulose filters, and probed using low-stringency conditions (Sambrook et al., 1989). A mixed *Drosophila* K⁺ channel probe was made by PCR amplification, as described above, of *Drosophila* Shaker, Shal, Shal, and Shaw K⁺ channel clones (generous gift of Dr. A. Wei and Dr. L. Salkoff). The probe was radioactively labeled using the Prime-A-Gene kit (Promega) according to the manufacturer’s protocol. A partial LKv1 genomic sequence was identified in this screen and subsequently used to identify clones from *Haemopis* genomic and cDNA libraries as well as from a *Hirudo* CNS-enriched cDNA library. The *Haemopis* genomic library was constructed in BamHI-cut *ΔASH* (Stratagene) using size-selected Sau 3A partial digestion products from high-molecular-weight genomic DNA according to standard protocols (Ausubel et al., 1987; Stratagene ΛDASH protocols). The *Haemopis* cDNA library (Johansen and Johansen, 1995) and the *Hirudo* CNS-enriched cDNA library (Huang et al., 1997) have been previously described. Positive clones were either subcloned or excised into pBluescript (Stratagene). From these screens, a full-length *Hirudo* LKv1 cDNA was obtained, but the largest *Haemopis* cDNA extended only to the S1 transmembrane domain. The missing S5 coding region was obtained by mapping and sequencing the S5’ end of a 15-kb *Haemopis* genomic clone, and exonic sequences were identified which contained the full S5’ coding region.

DNA sequence was performed using a DNA Sequencer 377A (Applied Biosystems) at the Iowa State University Nucleic Acid Sequencing Facility, using commercially available universal and reverse sequencing primers (Stratagene) or specific primers synthesized by the facility based on known LKv1 sequences. The nucleotide and predicted amino acid sequences were analyzed using the Genetics Computer Group (Wisconsin package, version 9.1) suite of programs (Devereux et al., 1984). LKv1 sequences were compared with known and predicted proteins in the SwissProt and GenBank databases using the FASTA and TFASTA programs within the GCG package. In addition, BLAST searches were performed using the NCBI BLAST e-mail server (Altschul et al., 1990) comparing the LKv1 sequences with SwissProt, PIR, and GenPept databases. Transmembrane domains were determined both by align-
ment to other voltage-gated K channel protein sequences and by hydropathy analysis using the program TopPred II.

**Phylogenetic Analysis**

Alignments used to produce maximum parsimony trees were generated by GCG’s Pileup program and initially encompassed the entire amino acid sequence of the voltage-gated channels analyzed. However, any gaps in the resulting alignments were removed by deleting residues corresponding to the gaps. This method left mostly, but not exclusively, sequences from the T1 assembly domain, the six transmembrane domains, and the pore region in the analysis. Trees were constructed by maximum parsimony using the PAUP computer program, version 3.1.1 (Swofford, 1993) on a Power Macintosh 8100. All trees were generated by heuristic searches and the resulting tree topology was verified by PAUP’s branch and bound mode. Bootstrap values in percentages (Felsenstein, 1985) are indicated on the bootstrap consensus tree.

**Antibody Production and Immunocytochemistry**

The coding sequence for the first 75 residues of *Haemopis LKv1* was amplified using PCR with exact-match primers that had EcoRI and Sall restriction sites incorporated. The amplified fragment was isolated and subcloned into a Pharmacia PGEX4T-2 vector. The correct orientation and reading frame of the insert were verified by sequencing. The resulting fusion protein was expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column using standard protocols. The purified LKv1 fusion protein was used to generate polyclonal antibodies in rabbits. Two rabbits were injected with 200–400 µg of fusion protein and then boosted at 21-day intervals, as described in Harlow and Lane (1988). After the second boost, serum samples were collected 7 and 10 days after injection (Harlow and Lane, 1988). The sera were analyzed for specificity by comparing the staining obtained with the antisera and the preimmune sera on nitrocellulose filters spotted with purified fusion protein and the expressed PGEX4T-2 vector. The sera were titrated from undiluted to a 1:5000 dilution in immunoblot analysis. The final preparations were dehydrated in alcohol, cleared in xylene, and embedded as whole mounts in Depex mountant. Adult ganglia were fixed in 4% paraformaldehyde and the connective capsules opened with fine forceps before they were processed for antibody labeling in a similar way to the embryos. The labeled preparations were photographed on a Zeiss Axioskop using Ektachrome 64T film. The color positives were digitized using Adobe Photoshop and a Nikon Coolscan slide scanner. In Photoshop, the images were converted to black and white and image processed before being imported into Freehand (Macromedia) for composition and labeling.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979). For these experiments we used the BioRad minigel system, electroblotting to 0.2 µm nitrocellulose, and using HRP-conjugated secondary antibody (1:3000) for visualization of primary antibody diluted 1:2000 in Blotto (1:50 for affinity-purified antibody) in immunoblot analysis. The signal was developed with 3,3’-diaminobenzidine (0.03%) and H2O2 (0.01%) for 10 min. In some experiments, to test for the specificity of the polyclonal antisera, 30 µg of the LKv1 fusion protein was added to the diluted antisera before staining. The final preparations were dehydrated in alcohol, cleared in xylene, and embedded as whole mounts in Depex mountant. Adult ganglia were fixed in 4% paraformaldehyde and the connective capsules opened with fine forceps before they were processed for antibody labeling in a similar way to the embryos. The labeled preparations were photographed on a Zeiss Axioskop using Ektachrome 64T film. The color positives were digitized using Adobe Photoshop and a Nikon Coolscan slide scanner. In Photoshop, the images were converted to black and white and image processed before being imported into Freehand (Macromedia) for composition and labeling.

**Electrophysiological Analysis of LKv1 in HEK293 Cells**

A full-length *Hirudo* LKv1 cDNA, including 150 nt of upstream and 345 nt of downstream sequence, was subcloned into the EcoRI multiple cloning site of an eukaryotic
expression vector pCI-neo (Promega). Two micrograms of Hirudo LKv1-pCI-neo plasmid prepared by Qiagen Maxi-prec was transiently transected into a 35-mm tissue culture dish containing HEK293 cells using 10 µL Transfectamine (Gibco) following the manufacturer’s recommended protocol. In addition, 200 ng of green fluorescent protein cDNA subcloned into pCI-neo was cotransfected to allow detection of successfully transfected cells at the time of electrophysiological recording. The HEK293 cells were propagated in a high-glucose DMEM media (Gibco) supplemented with 10% fetal calf serum (Hyclone) and antibiotics. Electrophysiological recordings were obtained 48–72 h after transfection.

Whole-cell patch-clamp recordings were obtained from fluorescent cells visually identified under an inverted epi-fluorescent microscope. External solution consisted of (in millimoles): 140 NaCl, 5.8 KCl, 0.8 MgCl2, 3.0 CaCl2, 10 Hepes, 10 glucose, and pH was titrated to 7.4 with 1 N NaOH. In some experiments, 20 mM tetraethylammonium (TEA) or 4 mM 4-AP was added to the external solution. The patch electrodes fashioned from a 1.2-mm fiber-filled borosilicate glass capillary (WPI) were fire-polished and not shown). We were unable to identify a Hirudo cDNA library. From these screens, we obtained several partial Haemopis LKv1 cDNA clones containing 3’ coding region and the poly A’ tail, a 15-kb Haemopis genomic clone, in addition to a Hirudo cDNA clone containing a 1356-nucleotide open-reading frame terminated by a TGA stop codon. This open-reading frame is likely to be full length since it has a 5’ ATG initiation codon with a favorable A−/G+4 context of initiation (Kozak, 1986) just downstream from an in-frame TAA stop codon (data not shown). We were unable to identify a Haemopis cDNA containing the entire 5’ coding region; therefore, the complete sequence for Haemopis LKv1 is partly based on sequence derived from the 15-kb genomic clone (Fig. 1). The Haemopis genomic coding region contains only a single 0.7-kb intron that intervenes the sequence coding for the S2 transmembrane domain. The predicted amino acid sequence of the open-reading frame for LKv1 extends over 452 amino acids for Hirudo and 453 amino acids for Haemopis (Fig.1), with calculated molecular weights of 51.4 and 51.3 kD, respectively. The Haemopis and Hirudo LKv1 sequences share 92% identity on the amino acid level.

Figure 2 shows the sequence comparison of LKv1 (Hirudo) with the four most homologous sequences in the data bank, all of which belong to voltage-gated K channels of the Shaker subfamily found in mouse, Drosophila, Aplysia, and flatworm. While the N-terminal amino acid sequence of LKv1 appears unique, extensive conservation between LKv1 and these Shaker homologs is present in the T1 tetramerization domain (Li et al., 1992; Shen et al., 1993), in the six transmembrane domains (S1–S6), and in the K+ ion-selective pore region (P). However, there are also several significant differences. For example LKv1 has only five positively charged amino acids, compared to seven for the other Shaker homologs in the S4 transmembrane domain, a domain that has been implicated in voltage sensing (Papazian et al., 1991). This is partly because LKv1 is missing a three–amino acid turn of the α-helix containing one of the positive charges. There is also a three–amino acid deletion in the S5 transmembrane α-helix, which in this case is compensated for by a three–amino acid insert (CML)

RESULTS

Cloning and Characterization of LKv1 in Haemopis and Hirudo

Degenerate universal primers to regions conserved in the four voltage-gated K channel subfamilies, Shaker, Shal, Shab, and Shaw in Drosophila (Butler et al., 1989), were used for PCR amplification of Haemopis genomic DNA. The resulting PCR products were subcloned into M13 vector and screened at low stringency with mixed K channel probe amplified by PCR with the universal primers of cDNAs from the four Drosophila K channel subfamilies. From this screen, a single partial 1.5-kb genomic LKv1 clone was isolated and sequenced. Probes based on the LKv1 sequence were subsequently used to screen a Haemopis oligo(dT)-primed cDNA library, a Haemopis genomic library, as well as a CNS-specific random primed Hirudo cDNA library. From these screens, we obtained several partial Haemopis LKv1 cDNA clones containing 3’ coding region and the poly A’ tail, a 15-kb Haemopis genomic clone, in addition to a Hirudo cDNA clone containing a 1356-nucleotide open-reading frame terminated by a TGA stop codon. This open-reading frame is likely to be full length since it has a 5’ ATG initiation codon with a favorable A−/G+4 context of initiation (Kozak, 1986) just downstream from an in-frame TAA stop codon (data not shown). We were unable to identify a Haemopis cDNA containing the entire 5’ coding region; therefore, the complete sequence for Haemopis LKv1 is partly based on sequence derived from the 15-kb genomic clone (Fig. 1). The Haemopis genomic coding region contains only a single 0.7-kb intron that intervenes the sequence coding for the S2 transmembrane domain. The predicted amino acid sequence of the open-reading frame for LKv1 extends over 452 amino acids for Hirudo and 453 amino acids for Haemopis (Fig.1), with calculated molecular weights of 51.4 and 51.3 kD, respectively. The Haemopis and Hirudo LKv1 sequences share 92% identity on the amino acid level.

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**Shaker, Shab, Shal, and Shaw** K channel core sequences from different organisms. The tree is rooted using sequences from the first homology domain of rat Na channel II (Noda et al., 1986) as an outgroup; however, the same topology was obtained for unrooted trees. LKv1 is clearly grouped together with the Shaker-related K channels, which, in addition, all share what may be the Shaker subfamily signature sequence of SxxxLRV at the beginning of the S4 domain. Interestingly, however, in nearly all the trees we generated, LKv1 occupied a separate branch from that of the Shaker homologs from flatworms, *Aplysia*, *Drosophila*, and mouse which form a monophyletic clade that is supported by a bootstrap value of 99%. Flatworms are acoelomats and clearly less evolutionarily advanced than leeches which, like *Aplysia*, *Drosophila*, and mouse, belong to the coelomats. Thus, these results suggest that LKv1 diverged from a common ancestor prior to the origin of the group of monophyletic Shaker homologs. Furthermore, the two jellyfish K channels, *jShak1* and *jShak2*, are also placed separately, indicating that the Shaker-related group of voltage-gated K channels contains several evolutionarily divergent lineages.

**Immunocytochemical Localization of LKv1**

For studying the tissue expression and localization of LKv1, we generated polyclonal antibodies in rabbits to a GST-fusion protein containing the first 75 residues of the N-terminus of *Haemopis* LKv1 sequence. On immunoblots of *Haemopis* CNS extracts LKv1 antiserum, as well as affinity-purified antiserum, recognizes a single 54-kD band not present in the preimmune serum (Fig. 4). Furthermore, preabsorption of the LKv1 antiserum with LKv1-GST fusion protein abolishes staining by the LKv1 antiserum of the 54-kD protein (Fig. 4). The molecular weight of this band is in close correspondence to the predicted molecular weight of LKv1 of 51 kD. In addition to *Haemopis*, the LKv1 antiserum recognizes an identical 54-kD band in CNS extracts from *Hirudo* and *Macrobdella* (data not shown). Figure 5(A) shows an adult midbody ganglion from *Haemopis* labeled with LKv1 antiserum. All central neuronal somata are antibody positive in contrast to glial and muscle cells which are not labeled by the antibody. In control experiments, preimmune serum as well as LKv1 antiserum preabsorbed with LKv1-GST fusion protein did not show immunocytochemical labeling (data not shown). To address whether LKv1 is expressed by all neurons and whether expression is truly specific to the nervous system, we used LKv1 antiserum to label-staged leech.
embryos. In embryos, it is possible to assay all components of the nervous system including the CNS, PNS, and all other tissues. Figure 5(C) shows the labeling of peripheral neurons located in the body wall of a *Macrobdella* E12 embryo hemisegment. The results show that all known peripheral neurons are
Table 1 Percent Conservation of Amino Acids between LKv1 and Four Shaker K Channel Homologs

<table>
<thead>
<tr>
<th>Shaker B</th>
<th>AK01a</th>
<th>SKv1.1</th>
<th>LKv1</th>
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<tr>
<td>88%</td>
<td>87%</td>
<td>84%</td>
<td>80%</td>
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<td>78%</td>
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<td>80%</td>
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<td>50%</td>
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Percent amino acid identities are shown for pairwise alignments between LKv1 and Shaker homologs from flatworm (SKv1.1), Aplysia (AK01a), Drosophila (Shaker B), and mouse (Kv1.1). Only the identities in the core sequences of the T1 assembly domain, the S1–S6 transmembrane domains, and the pore region were measured.

labeled by the antibody including sensillar neurons (Johansen et al., 1992), the HO cells which are putative stretch receptors (Blackshaw et al., 1982), and the NNC cell which innervates the nephridium (Wenning, 1983). In addition, all the axons constituting the peripheral nerves express LKv1. In contrast, muscle cells and other nonneuronal tissues do not show labeling with LKv1 antiserum. Thus, LKv1 appears to be a neuron-specific K conductance that is expressed in somata and axons by all known neurons in both the CNS and PNS. The earliest detection of LKv1 by antibody labeling is in E8–E9 embryos [Fig. 5(B)], suggesting that the channel is expressed shortly after the neurons differentiate.

Functional Expression of LKv1 in Transiently Transfected HEK293 Cells

To characterize the electrophysiological properties of LKv1, we transiently expressed the current in HEK293 cells. Untransfected HEK293 control cells did not have detectable endogenous voltage-gated K currents. A recombinant plasmid containing the entire open-reading frame of the Hirudo LKv1 cDNA, including 150 nt of upstream and 345 nt of downstream sequence, was used for transfection. Figure 6(A) shows an example of whole-cell voltage-clamp currents obtained in response to depolarizing steps from a holding potential of −80 mV in successfully LKv1-transfected HEK293 cells. The biophysical and pharmacological properties of these currents are summarized in Table 2. LKv1 currents expressed in HEK293 cells have the properties of a delayed rectifier [Fig. 6(A)] and do not inactivate fully even during prolonged depolarizations (data not shown). However, LKv1 does exhibit a limited degree of voltage-dependent inactivation relative to the peak current at positive potentials [Fig. 6(C)]. These properties of LKv1 are in contrast to the previously described currents of Shaker-related invertebrate K channels which are all transient A-type currents with rapid activation and inactivation kinetics (Papazian et al., 1987; Pfaffinger et al., 1991; Kim et al., 1994; Jegla et al., 1995). The LKv1 current activated at relatively positive membrane potentials [Fig. 6(B)] with a $V_{50}$ of 8.3 mV, as determined from Boltzman fits of g(V) (Table 2). Analysis of the reversal potential for tail currents of LKv1 [Fig. 6(C,D)] shows that it is close to the calculated $E_K$ of −80 mV under these recording conditions, suggesting that LKv1 is selective mainly for K$^+$ ions. Pharmacologically, LKv1 is sensitive to TEA with more than 90% of the current blocked by 20 mM, whereas 4 mM 4-AP reduces the current by only 10–15% [Fig. 6(E,F); Table 2]. The sensitivity of LKv1 to TEA correlates well with the presence of a phenylalanine at position 406 in the pore region. It has previously been reported that the presence of an aromatic amino acid at this position is responsible for conferring sensitivity to external TEA (Heginbotham and MacKinnon, 1992).

In previous studies using a two-electrode voltage-clamp, it was demonstrated that two voltage-dependent K currents, a delayed rectifier type current $I_K$ and a transient A-type current $I_A$, could be identified in isolated soma of leech neurons (Johansen and Kleinhaus, 1986, 1990). A comparison of the properties of these two currents with LKv1 (Fig. 7 and Table 2) suggests that LKv1 may correspond to $I_K$ in these cells. Both LKv1 and $I_K$ do not inactivate; they have similar half-maximal activation voltages $V_{50}$ of 8.3 and 5.2 mV, respectively; and they are both relatively insensitive to 4-AP, whereas they are more than 90% reduced by 20–25 mM TEA (Table 2). This is in contrast to $I_A$, which rapidly inactivates with a time constant of 90 ms, which has a $V_{50}$ of −17 mV, and which is completely blocked by 3 mM 4-AP and has no demonstrable sensitivity to TEA (Table 2). However, the properties of $I_K$ and LKv1 are not completely congruent, as the activation kinetics of LKv1 expressed in HEK293 cells are more than twice as slow as those determined for $I_K$ at comparable voltages.

**DISCUSSION**

In this study, we have cloned a novel voltage-gated K channel, LKv1, in two species of leech. Phylogenetic analysis shows that while LKv1 is clearly related to the Shaker subfamily of K channels and contains what may be this subfamily’s signature sequence of SxxxxLVR at the beginning of the S4 domain: It occupies a separate branch from that of Shaker homologs from flatworm, Aplysia, Drosophila, and vertebrates, which form a distinct monophyletic clade. LKv1 is also placed separately from two recently cloned jellyfish K
channels, jShak1 and jShak2 (Jegla et al., 1995). From this phylogenetic analysis, it is clear that LKv1 and jShak1 and jShak2 cannot be true homologs of the K channels comprising the monophyletic Shaker clade, but rather are orthologs. Thus, the Shaker subfamily as defined by containing the SxxxLRV motif contains several divergent lineages of voltage-gated K channels, underscoring the evolutionary diversity of K channels (Salkoff and Jegla, 1995). However, whether LKv1 and the jellyfish Shakers are unique to their respective organisms and fulfill a specialized function in these species, or whether they define new subgroups with representatives in other organisms remains to be determined.

The functional properties of LKv1 when expressed in HEK293 cells are that of a noninactivating delayed rectifier current. This is the first example of a Shaker-related α-subunit in invertebrates with these properties. All previously described invertebrate Shakers have been of the rapidly activating and inactivating A-type currents; however, in mammals Shaker genes have been found to code for proteins giving rise to both types of conductances (Stuhmer et al., 1989). LKv1 is activated at relatively positive potentials with a $V_{50}$ of 8.3 mV. This is more positive than that for the higher metazoan Shakers, which typically have $V_{50}$ values around −15 mV, but not as extreme as the positive shift in voltage dependence of the Shaker-related jellyfish K channels, which have $V_{50}$ values at approximately 20 mV (Jegla et al., 1995). Although LKv1 has only five positively charged amino acids in the S4 transmembrane domain, its reduced voltage sensitivity may not necessarily reflect a difference in

![Figure 3](image_url) Consensus maximum parsimony tree derived from Hirudo LKv1 and 13 other voltage-gated K channels representing the Shaker, Shal, Shab, and Shaw subfamilies. The tree is rooted using sequences from the first homology domain of rat Na channel II as an outgroup (RatNaII, X03639) (Noda et al., 1986). The strict consensus of 100 maximum parsimony trees is depicted with associated bootstrap support values. The consistency index for each of the individual most parsimonious trees used to construct the consensus tree was 0.78. The following voltage-gated K channel sequences were used: Drosophila Shaker (DroShak, P08512) (Papazian et al., 1987); mouse Shaker (MShak, P16388) (Tempel et al., 1988); Aplysia Shaker (AplShak, M95914) (Pfaffinger et al., 1991); flatworm Shaker (FwormShak, L26968) (Kim et al., 1994); jellyfish Shaker 1 and 2 (JShak1/JShak2, U32922/U32923) (Jegla et al., 1995); Drosophila Shab, Shal, and Shaw (DroShab/DroShal/DroShaw, M32659/M32660/M32661) (Butler et al., 1989); mouse Shaw (MShaw, S69381) (Goldman-Wohl et al., 1994); mouse Shal (MShal, M64226) (Pak et al., 1991a); mouse Shab (MShab, M64228) (Pak et al., 1991b); and Aplysia Shab (AplShab, S68356) (Quattrocki et al., 1994).

![Figure 4](image_url) Polyclonal antiserum identifies LKv1 as a 54-kD protein on immunoblots of Haemopis CNS extracts. Lane 2 shows labeling of a 54-kD band which is not present in the preimmune serum (lane 1). Lane 3 demonstrates that preabsorption of LKv1 antiserum with LKv1 fusion protein abolishes staining of the 54-kD protein. Lane 4 shows labeling of the 54-kD protein by affinity-purified LKv1 antibody.
gating charges. The jellyfish K channel, jShak2, while being shifted to even more positive activation potentials than LKv1, still has seven positive charges in the S4 region, as do all higher metazoan Shakers. Apart from fewer positive charges in the S4 domain, LKv1 has several other significant differences in its amino acid sequence from that of the other Shaker-related channels. For example, there is a three–amino acid deletion in the S5 domain which is compensated for by a three–amino acid insert, CML, unique to LKv1 and found in both the Haemopis and Hirudo sequence. In addition, LKv1 has a very short C-terminal domain consisting of only 17 amino acids. As for Shakers in mammals (Chandy et al., 1990) and for the Shaker-related K channels in the jellyfish Polyorchis (Jegla et al., 1995), alternative splicing is not likely to play a major role in generating functional diversity because only a single intron is present in the coding region for LKv1 of Haemopis genomic DNA. This differs from Drosophila, in which extensive alternative splicing from a single Shaker gene gives rise to a wide range of functional properties (Jan and Jan, 1990).

Antibody localization studies show that LKv1 is

Figure 5  Expression of LKv1 in leech nervous system. (A) LKv1 antiserum labels central neuronal somata (n) in adult Haemopis ganglia, in contrast to glia and muscle cells, which are not labeled by the antibody. Anterior is at the top; scale bar = 150 μm. (B) Labeling of all developing CNS neurons by LKv1 antiserum (three representative neurons are indicated by arrows) in a whole-mount E9 embryonic ganglion. Anterior is at the top; scale bar = 70 μm. (C) Labeling by LKv1 antiserum of peripheral neurons and nerves in the bodywall of a E12 Macrobodella embryo hemisegment. Among the identified peripheral neurons labeled are the nephridial nerve cell (NNC), the putative stretch receptor HO cells (arrows), and slightly out-of-focus four groups of sensillar neurons (S2–S5). The axons of peripheral and central neurons project to and from the CNS (G) through the AA, MA, DP, and PP nerves. Anterior is to the left and dorsal is at the top; scale bar = 75 μm.
expressed by all neuronal somata and axons in both the CNS and PNS. Thus, LKv1 may be the major delayed rectifier current modulating excitability in leech neurons. This is in contrast to Drosophila neurons and muscle, where the main delayed rectifier current is encoded by Shab (Tsunoda and Salkoff, 1995a), and where Shaker currents appear to be expressed mainly in muscles and photoreceptors (Tsunoda and Salkoff, 1995b). In leech, LKv1 is not only expressed in mature neurons but also in the newly differentiated neurons in ganglia of E8–E9 embryos, suggesting a developmental role for LKv1 as excitability of the neurons is gradually being established.

Comparison of the biophysical and pharmacological properties of LKv1 as expressed in HEK293 cells with those of I\textsubscript{K} and I\textsubscript{A}, two previously identified native voltage-gated K channel currents in the soma of leech neurons (Johansen and Kleinhaus, 1986, 1990), suggests that LKv1 corresponds to I\textsubscript{K}. LKv1 and I\textsubscript{K} are both delayed rectifier-type currents which do not inactivate; they have similar voltage dependence for activation; and they are both relatively insensitive to 4-AP and are more than 90% blocked by 20–25 mM TEA. Furthermore, I\textsubscript{K} has been demonstrated to be present in every central neuron examined in several leech species including medial nociceptive neurons and touch cells, which do not possess the transient inactivating A-type K current, I\textsubscript{A} (Johansen and Kleinhaus, 1990). Thus, the known distribution of I\textsubscript{K} correlates well with the finding from the antibody localization studies that LKv1 is present in the soma of all neurons. The only difference in the properties measured for both I\textsubscript{K} and LKv1 is that the activation kinetics of LKv1 in HEK293 cells are more than twice as slow as those determined for I\textsubscript{K} in situ at comparable membrane potentials. However, several cell-specific factors due to the use of a heterologous expression system may account for the observed differences in activation and/or inactivation kinetics of native and expressed channels (Baro et al., 1996). First, distinct cellular milieus between HEK293 cells and leech neurons could lead to cell-specific differences in posttranslational modifications that could alter the properties of the currents. Second, the gating properties of channels can be affected by their phosphorylation state owing to the action of cell specific protein kinases or phosphatases (Levitan, 1994; Drain et al., 1994). Third, the presence or absence of β-sub-

Figure 6 Biophysical and pharmacological properties of Hirudo LKv1 expressed in HEK293 cells. (A) Family of outward currents in a HEK293 cell transiently transfected with LKv1 cDNA. The traces were obtained by whole-cell patch-clamp recording from a holding potential of −80 mV with voltage steps to 34 mV in 12-mV increments. (B) Peak conductance–voltage relationship of the current traces shown in (A). The solid line represents a Boltzmann fit of the data (see Materials and Methods). For this current family, \( V_{50} \) was 9.9 mV and the slope factor \( a \) was 9.1 mV/e. (C) Tail-current reversal of LKv1 currents. The currents were elicited by a depolarizing step to 34 mV from a holding potential of −80 mV. After the depolarization, the holding potential were varied in 30-mV increments from −120 to −30 mV. The four traces are superimposed. (D) Plot of the relationship between peak tail-current amplitude and voltage. The reversal potential of the tail currents is close to −80 mV which is the calculated \( E_{K} \). (E) LKv1 current is more than 90% blocked by 20 mM TEA. The left panel shows a control current, the middle panel shows the current in the presence of 20 mM TEA, and the right panel is after wash, showing that the effect of TEA is reversible. The currents were elicited from a holding potential of −80 mV by a depolarizing step to 34 mV. (F) The response of LKv1 current to 4 mM 4-AP. The left panel shows the control current, the middle panel is the current in the presence of 4 mM 4-AP, and the right panel is the current after wash. The currents were elicited from a holding potential of −80 mV by a depolarizing step to 34 mV.
units interacting with the α-subunit of the channel (Pongs, 1995) may also account for differences in the biophysical properties of α-subunits expressed in heterologous cells. Thus, the majority of the evidence, and especially the pharmacological data, strongly suggest that LKv1 is the α-subunit responsible for the $I_K$ current in leech neurons.

The further cloning and molecular and biophysical characterization of different leech conductances in combination with the particularly advantageous features of the leech CNS promise to make possible a more comprehensive understanding of how neuronal networks underlying specific behaviors are organized, how functionally different cells within these networks express different combinations of ion conductances, and how the specific properties of the conductances correlate with a neuron’s particular role in behavior.

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<table>
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<tr>
<th></th>
<th>LKv1 in HEK293 Cells</th>
<th>$I_K$ in Leech Retzius Cells*</th>
<th>$I_A$ in Leech Retzius Cells*</th>
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<tr>
<td><strong>Activation</strong></td>
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<td>$V_{50}$ (mV)</td>
<td>8.3 ± 0.3 (n = 8)</td>
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<tr>
<td>Slope $a$ (mV/°)</td>
<td>11.2 ± 0.4 (n = 8)</td>
<td>7.8</td>
<td>9.0</td>
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<tr>
<td>Time constant at 10 mV (ms)</td>
<td>18.5 ± 0.9 (n = 6)</td>
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<td>2</td>
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<tr>
<td><strong>Inactivation</strong></td>
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<tr>
<td>Time constant at 10 mV</td>
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<td>&gt;1 s</td>
<td>90 ms</td>
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<td><strong>Pharmacology</strong></td>
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<tr>
<td>Sensitivity to TEA†</td>
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<td>&gt;95% (25 mM)</td>
<td>Not sensitive</td>
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<tr>
<td>Sensitivity to 4-AP†</td>
<td>10–15% (4 mM; n = 4)</td>
<td>10–15% (3 mM)</td>
<td>100% (3 mM)</td>
</tr>
</tbody>
</table>

* The biophysical and pharmacological data for $I_K$ and $I_A$ were obtained from Johansen and Kleinhaus (1986).
† The sensitivity to TEA and 4-AP is given as the reduction in percentage of peak current in the presence of the drug.

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**Table 2 Biophysical and Pharmacological Properties of LKv1 and Comparison with Two Native Voltage-Gated K Currents, $I_A$ and $I_K$, Found in Leech Neuronal Somata**

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**Figure 7** Comparison of LKv1 expressed in HEK293 cells with the two native voltage-gated K currents, $I_K$ and $I_A$, in the soma of leech neurons. (A) Family of outward currents in a HEK293 cell transiently transfected with LKv1 cRNA. The traces were obtained by whole-cell patch-clamp recording from a holding potential of −80 mV with voltage steps to 34 mV in 12-mV increments. (B) Family of delayed rectifier currents, $I_K$, obtained by two-electrode voltage clamp in an acutely isolated leech Retzius cell. The currents were elicited from a holding potential of −40 mV in response to depolarizing steps to 30 mV in 10-mV increments (modified from Fig. 3 in Johansen and Kleinhaus, 1986). (C) Family of transient K currents, $I_A$, obtained by two-electrode voltage clamp in an acutely isolated leech lateral nociceptive cell. The currents were elicited from a holding potential of −70 mV in response to depolarizing steps to 10 mV in 10-mV increments (modified from Fig. 1 in Johansen and Kleinhaus, 1990). All three families of K currents are shown on the same time scale of 100 ms.
REFERENCES


