



FEBS Letters 340 (1994) 104–108

FEBS 13706

**FEBS  
LETTERS**

# Deletion of highly conserved C-terminal sequences in the Kv1 K<sup>+</sup> channel sub-family does not prevent expression of currents with wild-type characteristics

V.N. Uebele<sup>c</sup>, S.W. Yeola<sup>c</sup>, D.J. Snyders<sup>b,c</sup>, M.M. Tamkun<sup>a,c,\*</sup>*Departments of <sup>a</sup>Molecular Physiology and Biophysics, <sup>b</sup>Medicine, and <sup>c</sup>Pharmacology, Vanderbilt Medical School, Nashville, TN 37232, USA*

Received 15 December 1993; revised version received 13 January 1994

## Abstract

The C-terminal regions of Kv1 K<sup>+</sup> channels show little conservation between isoforms except for the last four C-terminal residues, (E/L)TDV, which are well conserved from *Drosophila* to man. Deletions of the 4, 16, and 57 C-terminal residues of the human Kv1.5 channel did not affect whole cell current amplitude, midpoint of activation, degree of inactivation, or activation kinetics following expression in mouse L-cells. Similar results were obtained with the rat Kv1.1 channel. Therefore, the conserved (E/L)TDV motif, and most of the C-terminal amino acids, are not required for Kv1 channel expression.

**Key words:** Potassium channel; C-terminal sequence; Phosphorylation site; Kv1 sub-family

## 1. Introduction

Our understanding of voltage-gated K<sup>+</sup> channels has grown exponentially during the past decade, primarily due to the identification of the *Drosophila Shaker* channel [1–4]. To date, four *Shaker*-like K<sup>+</sup> channel gene sub-families have been defined in vertebrates, from which more than 25 K<sup>+</sup> channels have been cloned and functionally expressed in heterologous expression systems. The Kv1 sub-family is the largest and is homologous to the original *Drosophila Shaker* channel [5]. The Kv2, Kv3, and Kv4 mammalian families are homologous to the *Drosophila Shab*, *Shaw*, and *Shal* channels, respectively, and share approximately 50% amino acid identity with the Kv1 sub-family [6–8]. Within the Kv1 K<sup>+</sup> channel sub-family, members share high amino acid sequence identity within the six membrane spanning domains, pore forming sequence, and N-terminal regions involved in subunit assembly [1,9,10]. In contrast, the intracellular C-terminal region shows little conservation between isoforms and the sequence within these regions often defines the isoform. The exceptions are the last four C-terminal residues, (E/L)TDV, which are conserved within the Kv1 subfamily but absent from the Kv2, 3 and 4 subfamilies. This sequence motif is conserved in Kv1

family members from species as diverse as *Drosophila*, *Aplysia*, rat, and man. While extensive structure/function studies have identified channel domains involved in voltage-sensing [11], inactivation [12], ion permeation [13], subunit assembly [9], and drug/toxin block [14], little information exists as to the role of the C-terminal region. Interestingly, this region often contains consensus sites for protein kinase A and C phosphorylation [1].

To address the role of C-terminal amino acids in Kv1 channel function and biosynthesis, terminal truncations were performed on the rat Kv1.1 and human Kv1.5 channels and function assessed via voltage-clamp in transfected mouse L-cells. Control Kv1.5 constructs and constructs with deletions of the 4, 16, and 57 C-terminal amino acids were made, with the largest deletion also removing two putative protein kinase A phosphorylation sites. Maximum whole cell current amplitudes (at +60 mV) averaged 1.7 nA with no significant differences between constructs. The midpoint of activation (–14 to –18 mV), degree of inactivation at 250 ms (10 to 20%), and the activation kinetics ( $\tau = 1.8$  to 2.2 ms) were also unaffected by the truncations. Similar results were obtained with the 4 and 16 amino acid truncations of the rat Kv1.1 channel. Therefore, the conserved (E/L)TDV motif, and most of the C-terminal amino acids, are not required for the expression of wild-type Kv1 channels in fibroblasts. Perhaps these sequences function in cellular processes that are specific to electrically excitable cells.

\*Corresponding author. Fax: (1) (615) 322 7236.

2. Experimental

2.1. Materials

Enzymes and buffers were from New England Biolabs (Beverly, MA) and Boehringer Mannheim (Indianapolis, IN). Rabbit antiserum raised against a synthetic peptide corresponding to the S1-S2 extracellular loop of human Kv1.5 was a generous gift from L. Philipson, University of Chicago. All other materials were reagent grade.

2.2. C-terminal truncation and expression

Kv1.5 deletions were generated by polymerase chain reaction (PCR) based mutagenesis using human Kv1.5 [15] in pGEM 7 as template for all deletions. The 5' oligonucleotide (TTCAAGCTCTCCCGCCA CTCC) annealed just upstream of a unique PstI site and the 3' oligonucleotides (-0 = CCGGTACCTCACAAATCTGTTTCCCGGC-T, -4 = CCGGTACCTCACCGCTGGTGTCCAGGACAGAG, -22 = CCGGTACCTCACTTGGCCTTGACGTTACTACTT, -57 = CCGGTACCTCAGACCTTCCGCTGGACTCCTCT), which included a 3' stop codon and KpnI restriction site, annealed at the desired point of truncation. The PCR conditions were 15 cycles of 5 minutes at 94°C, 1 min 94°C, 1 min 55°C, and 1.5 min 72°C. The PCR generated fragments were digested with PstI and KpnI, gel purified, ligated into the Bluescript vector, and sequenced to check for polymerase error. The PstI and KpnI 3' deletion fragments were then ligated into pGEM 7 containing the wild-type amino terminal portion of the channel. These constructs were then digested with SphI and KpnI (SphI removes all but 17 base pairs of the 5'-untranslated region) followed by T4 DNA polymerase blunting. The resulting Kv1.5 encoding fragment was subcloned into the Eco RV site of pMSVNeo and transfected into mouse L-cells as previously described in detail [15]. A similar strategy was used to generate the rat Kv1.1 truncations. The pMSVNeo vector contains a dexamethasone-inducible murine mammary tumor virus promoter controlling transcription of the inserted cDNA and a gene conferring neomycin resistance driven by the SV40 early promoter [16]. When channel expression was required, cultures were incubated with 4 μM dexamethasone (Dex) for 18 h as required. These conditions produced a near steady-state level of functional channel expression at the cell surface. Parallel transfections with pMSVNeo vector alone were performed in order to obtain sham-transfected negative controls. Western blot analysis of Kv1.5 antibody binding to L-cell membranes confirmed that the predicted truncations were made.

2.3. Electrical recording

The cell preparation and electrophysiological methods have been described in detail [15]. Micropipettes were pulled from Radnoti borosilicate glass and were heat polished. The pipettes were filled with 110 mM KCl, 5 mM K<sub>4</sub>BAPTA, 5 mM K<sub>2</sub>ATP, 1 mM MgCl<sub>2</sub> and 10 mM HEPES/KOH adjusted to pH 7.2, yielding a final intracellular K<sup>+</sup>

concentration of 145 mM. The bath solution contained 130 mM NaCl, 4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM glucose and 10 mM HEPES/NaOH adjusted to pH 7.35. All currents were recorded at room temperature using an Axopatch-1A patch-clamp amplifier. Criteria to ensure voltage-clamp quality were similar to those used previously and described in detail [15]. Pulse protocols are given in the figure legend. Activation curves were obtained from isochronal tail current measurements and fitted with a Boltzmann equation using a non-linear least squares procedure [15]. A one-way analysis of variance (ANOVA) was used to determine whether observed differences in the mean current peak amplitudes were significant.

3. Results

Fig. 1A compares the C-terminal amino acid sequence between the original *Drosophila Shaker A* protein and homologous channels cloned from *Aplysia*, *Xenopus*, rat, and man. The common motif in the *Drosophila*, *Aplysia*, *Xenopus*, and Kv1.1-1.6 sequences is the presence of the (E/L)TDV motif in the last four positions. Only in Kv1.5 is the valine substituted with a leucine and in Kv1.6 the aspartic acid is replaced by glutamic acid. These substitutions are conservative. In contrast to the conservation within the last three positions, the first position is either a neutral leucine or negatively charged glutamic acid, although in Kv1.3 a phenylalanine is present. This sequence conservation among such diverse species at the very C-terminus suggests that these amino acids play an essential role in either channel function or biosynthesis. The hypothesis at the outset of the studies described below was that truncations of the C-terminal domain should produce an effect which would help elucidate the role of this region.

Two channels were chosen for the work presented here, the human Kv1.5 channel [17] and the rat Kv1.1 channel [1,7]. The results obtained with both channels were qualitatively identical and only the data obtained with the Kv1.5 channel are presented in detail below.

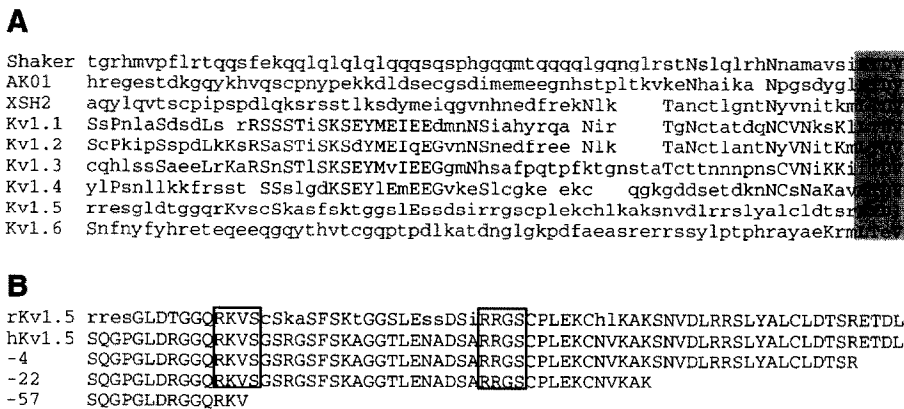


Fig. 1. Comparison of C-terminal amino acid sequences within the Kv1 K<sup>+</sup> channel subfamily. (A) The sequences for six rat Shaker-like channels are compared to each other and to the original *Drosophila Shaker A* channel and the *Aplysia* (AK01) and *Xenopus* (XSH2) Shaker-like channels. Note the striking conservation within the last four amino acids (shaded amino acids in boldface type). The *Shaker* sequence is from [19], Kv1.3 from [20], Kv1.6 from [21], AK01 and XSH1 from [10], and the remaining sequences from [1]. (B) Comparison of the C-terminal sequences of the rat and human Kv1.5 channels and truncated Kv1.5 mutations. Amino acids common to three or more channels are in capital letters. Two putative protein kinase A phosphorylation sites in the Kv1.5 sequence are boxed. The human Kv1.5 sequence is from GenBank accession no. M60451.

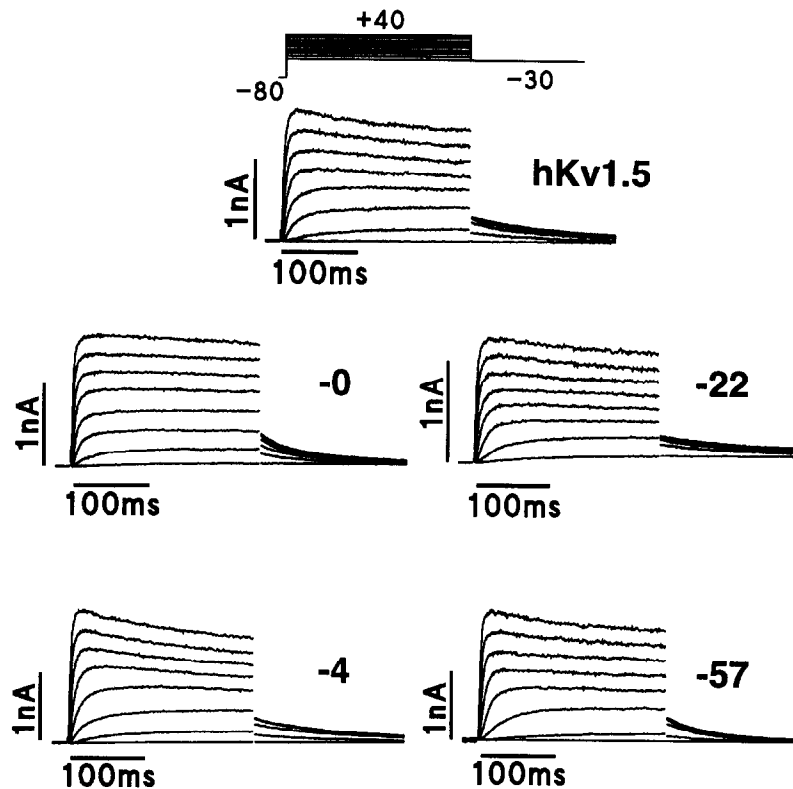


Fig. 2. Whole-cell voltage-clamp analysis of the wild-type and truncated Kv1.5 channels following expression in L-cells. Currents activated by 250 msec duration voltage steps in 10 mV increments in cells expressing wild-type hKv1.5, or the -0, -4, -22, and -57 mutant channels are shown. The hKv1.5 and -0 constructs differed only in the amount of 3'-untranslated channel nucleotide sequence. Construction of and expression of hKv1.5 is described in [15].

Four Kv1.5 constructs were prepared to examine the effect of deleting increasing amounts of the channel C-terminus. One construct included the complete coding sequence but omitted any 3'-untranslated sequence while the other three involved deletions of 4, 22, and 57 C-terminal amino acids as indicated in Fig. 1B. The Kv1.1 deletions removed the 4 and 16 most C-terminal amino acids.

Following stable expression in transfected mouse L-cells, membrane fractions were prepared and analyzed for Kv1.5 protein by Western blot analysis to confirm that the Kv1.5 protein was synthesized with the predicted truncations (data not shown). Once confirming that C-terminal truncation did not prevent Kv1.5 channel protein synthesis, voltage-clamp analysis was undertaken to characterize channel function and expression levels. Representative current tracings (scaled to match peak currents) in response to depolarizing stimuli are shown in Fig. 2. C-terminal truncation had no effect on channel activation rate, whole cell current amplitude, or percent inactivation at the end of the 250 ms pulse. There were minor variations in all parameters both between and within clonal lines. For example, note the difference in inactivation between the two sets of tracings with wild-type amino acid sequence (Fig. 2, hKv1.5 and -0). Fig. 3 shows the voltage activation curves for the different con-

structs. Again no significant differences were observed. Similar results in terms of these parameters were obtained with the two C-terminal truncations of the rat Kv1.1 channel (data not shown).

Quantitative comparisons between the wild-type and truncated Kv1.5 channels are presented in Table 1. The data presented here represent averages from both multiple cells and at least two distinct clonal lines. Maximum whole cell current amplitude (at +60 mV) ranged from 0.1 to 20.0 nA with no significant differences or trends in terms of current amplitude between constructs. The midpoint of activation (-14 to -20 mV), degree of inactivation at 250 ms (10 to 20%), and the activation kinetics ( $\tau = 1.8$  to 2.2 ms) were also unaffected by the truncations.

#### 4. Discussion

The data presented above indicate that the C-terminal amino acids of the Kv1.5 channel are not required for the expression of functional channels in the L-cell expression system. If these C-terminal amino acids were critical for biosynthesis, subunit assembly, or nominal function, no voltage-activated current would have been detected. The 57 amino acid deletion included two conserved consen-

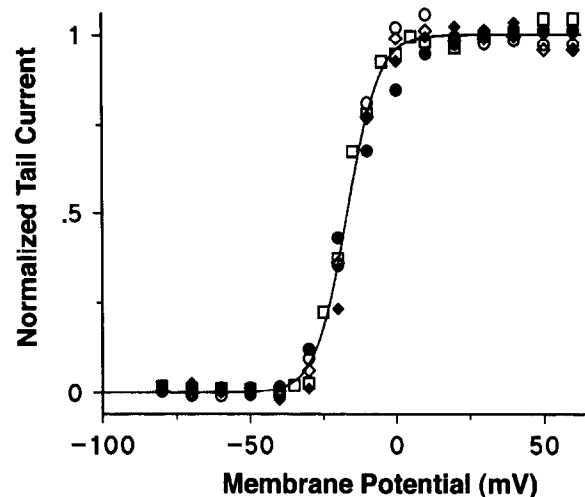


Fig. 3. Voltage-activation curves of the wild-type and truncated Kv1.5 channels. Activation curves were fit by a single Boltzmann function as previously described [14]. hKv1.5,  $\square$ ; -0 deletion,  $\bullet$ ; -4 deletion,  $\diamond$ ; -22 deletion,  $\circ$ ; -57 deletion,  $\blacklozenge$ . Data are from individual, representative experiments summarized in Table 1. The continuous line is the average activation curve for all but the hKv1.5 control data.

sus sites for protein kinase A phosphorylation, hence these two sites are not required for Kv1.5 channel expression. If the C-terminal sequence is involved in channel synthesis or function an effect on one of the parameters examined would be expected. However, if this sequence plays a subtle role such as controlling the rate of subunit assembly, the effect of its deletion could be missed in the present study. The results shown here are not specific to the Kv1.5 channel in that similar deletions of 4 and 16 amino acids from the Kv1.1 channel also had no effects on the parameters examined. Similar C-terminal truncations have been performed with the Kv2.1 channel with no major effect on function [18]. However, the Kv2.1 subfamily does not possess the C-terminal TDV motif. Since the Kv3 and 4 subfamilies also lack this sequence it is likely the motif functions in a role specific to the Kv1 subfamily.

These data are surprising given the conservation of the TDV motif both within the mammalian Kv1 sub-family and between these family members and the *Shaker B*,

*Aplysia* and *Xenopus* channels (Fig. 1). Even with *Shaker* splice variants within the C-terminus the ETDV motif is maintained [19]. One explanation for the results reported here is that this motif interacts with cellular machinery absent from the L-cell. For example, if the motif is involved in cytoskeletal interactions specific to a neuron or myocyte, the effect of its deletion may not be detected in the L-cell system. Interestingly, three members of the Kv1 sub-family have been localized to axons and perhaps nerve terminals while the Kv2 and Kv4 subfamilies, which lack the TDV motif, are found on the dendrites and cell bodies [22,23,24]. Expression of the deletion mutations presented above in cells capable of targeting channels to a polarized surface will address the possibility that this motif is involved in channel targeting.

**Acknowledgements:** This research was supported by NIH grants HL 49330 (MMT), HL 47599 (DJS), and HL 46681 (MMT, DJS). MMT is an Established Investigator of the American Heart Association. VNU was supported by NIH training grant GM 07628. We thank Dr. L. Philipson for providing the human Kv1.5 antiserum.

Table 1  
Comparison of wild-type and truncated hKv1.5 function

	<i>n</i>	$I_{+60\text{mV}}$ (nA)	% Inactivation (at 250 ms)	$\tau$ (ms)	$E_{1/2}$ (mV)
hKv1.5	40	$1.6 \pm 1.2$ (0.5–20.0)	$20.0 \pm 2.0$	$1.8 \pm 3.0$	$-14.0 \pm 5.0$
-0 deletion	4	$1.4 \pm 1.2$ (0.2–2.0)	$10.5 \pm 4.2$	$1.8 \pm 0.2$	$-18.1 \pm 1.7$
-4 deletion	5	$1.7 \pm 2.9$ (0.1–6.0)	$18.0 \pm 2.5$	$2.1 \pm 0.6$	$-18.8 \pm 1.5$
-22 deletion	6	$1.3 \pm 1.7$ (0.7–3.0)	$15.7 \pm 3.2$	$2.2 \pm 0.6$	$-20.2 \pm 3.4$
-57 deletion	11	$2.6 \pm 1.5$ (1.0–6.1)	$19.4 \pm 7.1$	$1.8 \pm 0.2$	$-14.8 \pm 0.2$

The peak current at +60 mV ( $I_{+60\text{mV}}$ ), % inactivation at 250 ms, time constant of activation ( $\tau$ ), and midpoint of the voltage activation curve ( $E_{1/2}$ ) are represented as the mean  $\pm$  standard deviation from the indicated number of cells. A minimum of two separate clonal cell lines were examined in each case. The  $\tau$  values are averaged from determinations at +50 and +60 mV, a range over which there was little voltage dependence with respect to this value. The hKv1.5 values are from cell lines expressing wild type channels as described in [14]. Differences among mean current peak amplitudes, are not significant (one-way ANOVA,  $P = 0.31$ ).

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