The P-Region and S6 of Kv3.1 Contribute to the Formation of the Ion Conduction Pathway

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ABSTRACT The loop between transmembrane regions S5 and S6 (P-region) of voltage-gated K^+ channels has been proposed to form the ion-conducting pore, and the internal part of this segment is reported to be responsible for ion permeation and internal tetraethylammonium (TEA) binding. The two T-cell K^+ channels, Kv3.1 and Kv1.3, with widely divergent pore properties, differ by a single residue in this internal P-region, leucine 401 in Kv3.1 corresponding to valine 398 in Kv1.3. The L401V mutation in Kv3.1 was created with the anticipation that the mutant channel would exhibit Kv1.3-like deep-pore properties. Surprisingly, this mutation did not alter single channel conductance and only moderately enhanced internal TEA sensitivity, indicating that residues outside the P-region influence these properties. Our search for additional residues was guided by the model of Durell and Guy, which predicted that the C-terminal end of S6 formed part of the K^+ conduction pathway. In this segment, the two channels diverge at only one position, Kv3.1 containing M430 in place of leucine in Kv1.3. The M430L mutant of Kv3.1 exhibited permeant ion- and voltage-dependent flickery outward single channel currents, with no obvious changes in other pore properties. Modification of one or more ion-binding sites located in the electric field and possibly within the channel pore could give rise to this type of channel flicker.

INTRODUCTION

The type-*l* K^+ channel in T cells, which is encoded by the *Shaw*-related Kv3.1 gene (Grissmer et al., 1992b), has a single channel K^+ conductance (γ) of 27 pS, and is relatively insensitive to block by internal tetraethylammonium (TEA_i; Kirsch et al., 1991; 1992a; Grissmer et al., 1992a; Taglialatela et al., 1994). The γ of Kv3.1 is reduced from 27 to 14 pS when the outward current is carried by Rb^+ rather than K^+ (Kirsch et al., 1992a, b; Grissmer et al., 1992a). The second K^+ channel in T cells, type *n*, a product of the *Shaker*-related Kv1.3 gene (Grissmer et al., 1990), has a γ of 14 pS, a K^+/Rb^+ conductance ratio of 1, and is half blocked by 0.3 mM TEA_i (Grissmer et al., 1992a). Our search for residues that may be responsible for these differences in internal pore properties was based on a heuristic model of the *Shaker* K^+ channel proposed by Durell and Guy (1992).

In this model, the ion conduction pathway consists of outer and inner vestibules connected by a narrow stem. The P-region, a stretch of about 20 amino acids in the highly conserved linker between predicted transmembrane segments S5 and S6, lines part of the K^+ conduction pathway. The model also predicts that the inner vestibule is formed by the S4/S5 loop and the C-terminal end of the S6 transmembrane segment. Experimental evidence for this model comes from studies of chimeric channels (Hartmann et al., 1991; Kirsch et al., 1993; Taglialatela et al., 1993, 1994; Lopez et al., 1994), and of channels that have been mutated at specific sites within these regions (MacKinnon and Yellen, 1990; Yool and Schwarz, 1991; Yellen et al., 1991; Heginbotham et al., 1992; Slesinger et al., 1993; Choi et al., 1993). The goal of the present study was to determine whether residues in the P-region and in the C-terminal half of the S6 segment contribute to the distinct electrophysiological properties of Kv3.1. Fig. 1 indicates the locations of residues mutated in this study.

MATERIALS AND METHODS

Mutagenesis

Site-directed mutagenesis was performed by a two-step polymerase chain reaction method using sense and antisense mutant primers, and confirmed by dideoxy sequencing (Sanger et al., 1977). The mutated mouse Kv3.1 gene was cloned into the *pBluescript* vector driven by the T3 promoter. cRNA was in vitro transcribed using an mCAP kit (Stratagene, La Jolla, CA) and injected into *Xenopus* oocytes (Grissmer et al., 1990, 1992a; Soreq and Seidman, 1992).

Electrophysiology

Patch-clamp experiments

Patch-clamp experiments were carried out in the outside-out configuration (Grissmer et al., 1990, 1992b). The external solution was based on mammalian Ringer containing in mM: 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, pH 7.4. In other external solutions, NaCl was replaced by either K^+ or Rb^+. The internal pipette solution contained in mM: 140 KF or RbF, 2 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, and 11 K_2-EGTA. In other experiments, internal solutions with 1 mM EDTA and no added Mg^{2+} or Ca^{2+} were used. TEA was applied to outside-out patches, either in the bath (TEA_o), or to the inner surface (TEA_i) by changing the internal solution with a pressure-perfused quartz pipette placed inside the patch pipette. The patch-clamp amplifier (List L/M-EPC 7, Adams and List Associates, Ltd., Great Neck, NY) was used in the voltage-clamp mode. In all patch-clamp experiments, the command input of the amplifier was controlled by a computer (PDP 11/73) via a digital-to-analog converter (Indec, Sunnyvale, CA), and membrane currents were recorded at a bandwidth of 2 or 5 kHz. The holding potential was adjusted to $E = -80$ mV. Correction for capacitive currents was achieved by analog subtraction.

Analysis of burst duration and flicker frequency

Single-channel openings were elicited by 32 repetitive voltage steps (1 Hz) to different potentials from a holding potential of −80 mV. The current

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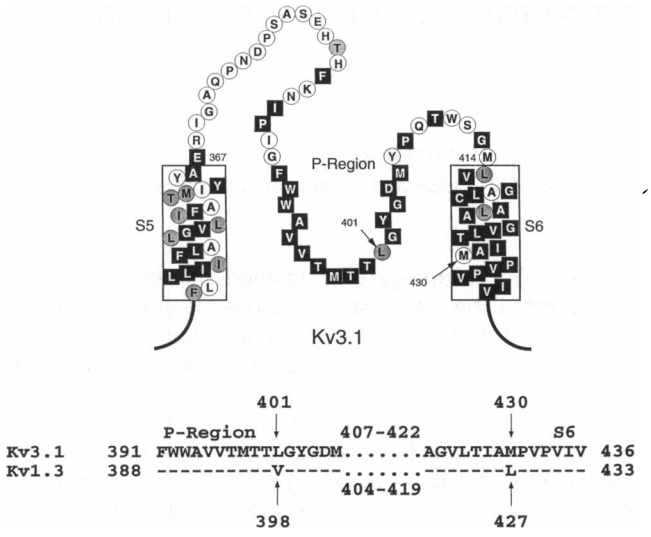

FIGURE 1 (*A*). The amino acid sequence of Kv3.1 extending from S5 through S6 segments. Black squares: amino acids in Kv3.1 that are identical to those in Kv1.3. Gray circles: residues in Kv3.1 that are replaced by conservative substitutions in Kv1.3. Open circles: residues in Kv3.1 that are dissimilar to those in Kv1.3. (*B*) Amino acid alignment of the P-region and S6 segment of Kv3.1 and Kv1.3. Dashed lines represent residues in Kv1.3 that are identical to those in Kv3.1. Leucine 401 and methionine 430, the two residues that were mutated, are indicated by arrows.

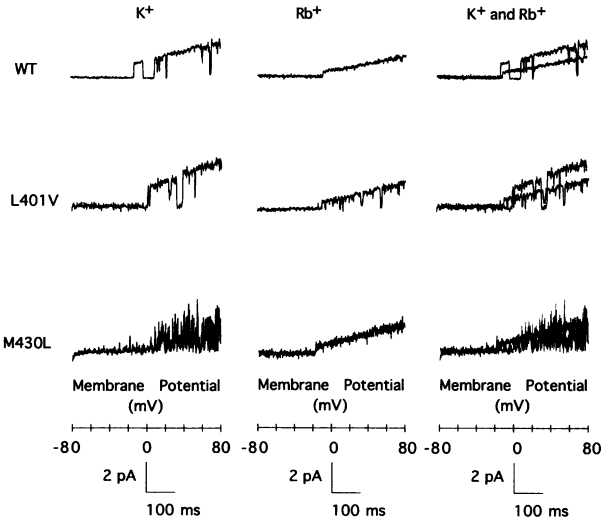

FIGURE 2 Single K^+ (left column) and Rb^+ currents (middle column) carried by WT Kv3.1 (top row), L401V (middle row) and M430L (bottom row). The column on the right shows superimposed traces of K^+ and Rb^+ currents. Single K^+ and Rb^+ conductances were characterized using voltage ramps from −100 to +80 mV. For these experiments, outside-out patches isolated from oocytes expressing WT or mutant channels were bathed in mammalian Ringer, while internal solutions contained either 140 mM KF or RbF (see Materials and Methods).

responses were stored on a PCM recorder (Toshiba, Unitrade Inc., PA), and later analyzed on an IBM-compatible computer using pClamp programs (Axon Instruments, Burlingame, CA) via the TL-1 DMA Labmaster interface. To quantify channel openings and closings, we determined the open time (or burst duration, τ_b) during the 32 voltage steps by excluding closed times shorter than 500 μs. Since this procedure excludes short flickery openings, we quantified channel flicker by measuring the open (τ_f) and closed (τ_c) times during a burst. In the wild-type (WT) channel, τ_b would be equivalent to τ_f.

RESULTS AND DISCUSSION

As shown in Fig. 1, the P-region of Kv3.1, extending from proline 388 to proline 408, differs from Kv1.3 at four positions (I389, G390, L401, and Y407). Only L401 is located in the deep P-region (extending from F391 to M406), which is thought to be responsible for ion permeation and TEA_i binding (Kirsch et al., 1992a, b; Taglialatela et al., 1993). We created the L401V mutation to make the deep P-region of Kv3.1 identical to that of Kv1.3 anticipating that the mutant channel would exhibit Kv1.3-like deep-pore properties. Contrary to our expectation, WT Kv3.1 and L401V channels had identical single-channel K^+ and Rb^+ conductances of 27 pS and 14 pS, respectively, and a Rb^+:K^+ conductance ratio of ~0.5 (top and middle rows in Fig. 2). The TEA_i-sensitivity of the L401V mutant (Table 1 and Fig. 3) was intermediate between that of Kv3.1 and Kv1.3 channels, suggesting that L401 is part of, or is in close proximity to, the TEA_i binding site in Kv3.1, but additional residues outside the deep P-region probably participate in formation of the binding pocket. We conclude that the differences in internal pore properties between the two channels are not solely determined by residues between F391 and M406 in the P-region.

In contrast to our results, an identical mutation (L374V) in a Kv2.1/Kv3.1 chimera containing the Kv3.1-P-region was reported to alter single K^+ conductance and K^+:Rb^+ selectivity without affecting TEA_i blockade (Kirsch et al., 1992b; Taglialatela et al., 1993), and the reverse mutation in the native Kv2.1 channel (V374L) had similar effects (Kirsch et al., 1992a). The inconsistencies between our data and published results could stem from differences in the interactions of residues in the P-region with those in the remainder of the protein.

In the Durrell and Guy model (1992), the C-terminal half of the S6 region has been proposed to contribute to the formation of the ion conduction pathway. Experimental evidence supports this model (Choi et al., 1993; Kirsch et al., 1993; Lopez et al., 1994; Taglialatela et al., 1994). Kv3.1 and Kv1.3 differ by only one residue in this region, which extends from A423 to V436 (see Fig. 1), a methionine in Kv3.1 (M430) corresponding to a leucine in Kv1.3. We generated the M430L mutation in Kv3.1 with the hope that the mutant channel would exhibit Kv1.3-like pore properties. Although the mutant channel did not behave as expected, it produced the interesting phenotype described below.

Outward K^+ currents through the M430L mutant channel were flickery, preventing accurate measurement of single K^+ channel conductance (Fig. 2, bottom row). This noisy behavior was observed as a series of short openings and closings followed by longer closings, and was not associated with any obvious change in other channel properties (Table 1). It persisted when measurements were made in Mg^{2+}-free and Ca^{2+}-free internal or external solutions, suggesting that flicker was not due to block by divalent ions. Outward Rb^+ currents, in contrast, did not exhibit this noisy behavior, and

TABLE 1 Biophysical and pharmacological properties of Kv3.1 and mutant channels

	WT Kv3.1	M430L Kv3.1	L401V Kv3.1	WT Kv1.3
Activation*				
$V_{1/2}$ (mV)	7.2 ± 3.2 (n = 12)	8.3 ± 3.9 (n = 3)	12, −14 (n = 2)	−36 ± 2.2 (n = 4)
K_n (mV)	12.8 ± 0.8 (n = 12)	11.1 ± 1.5 (n = 6)	13, 6.6 (n = 2)	5.3 ± 0.84 (n = 4)
Inactivation				
τ_h @ 40 mV (ms)	396 ± 80 (n = 9)	412 ± 94 (n = 6)	295 ± 36 (n = 6)	233 ± 33 (n = 8)
Use-dependence‡	No	No	No	Yes
Deactivation§				
τ_{tail} @ −50 mV (ms) (in NR)	1.8 ± 0.37 (n = 5)	2, 2.13 (n = 2)	2.33 ± 0.12 (n = 4)	73.5 ± 6.18 (n = 4)
τ_{tail} @ −50 mV (ms) (in KR)	3.5 ± 0.69 (n = 4)	3.17, 5.8 (n = 2)	2.4, 3.06 (n = 2)	27.1 ± 2.4 (n = 4)
Single-channel conductance (pS)				
K^+	27	Flicker	27	14
Rb^+	14	14	14	14
Pharmacology				
TEA_o (mM)	0.22 ± 0.05 (n = 6)	0.30, 0.03 (n = 2)	0.29, 0.06 (n = 2)	12.8 ± 1.4 (n = 6)
TEA_i (mM)	13, 15 (n = 2)	18.3 ± 3.1 (n = 3)	6.6 ± 1.85 (n = 3)	0.63 ± 0.18 (n = 3)

Data show mean ± SEM with number of experiments in parenthesis. *Activation was measured as described (Grissmer et al., 1992a).
‡Cumulative inactivation elicited by a train of depolarizing steps to +40 mV once every second from a holding potential of −80 mV. The test pulse duration was 200 ms. The K^+ current amplitude through Kv1.3 currents decreased during this train while Kv3.1 currents did not change.
§Tail currents were measured as the rate of channel closing upon repolarization in 4.5 mM or 160 mM external K^+.
NR = Normal ringer; KR = K-ringer.

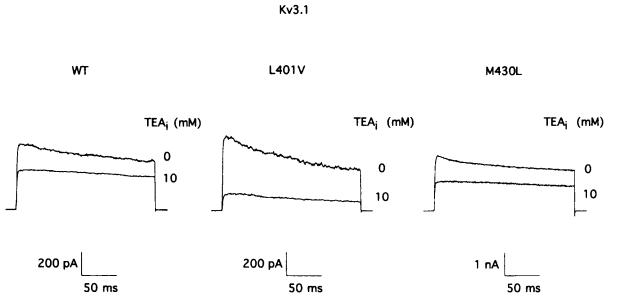

FIGURE 3 Sensitivity of WT Kv3.1, and the L401V and M430L mutant channels to TEA_i. Outside-out patches were held at −80 mV and stepped to +40 mV for 200 ms. TEA (mM) was perfused through the patch pipette (see Materials and Methods).

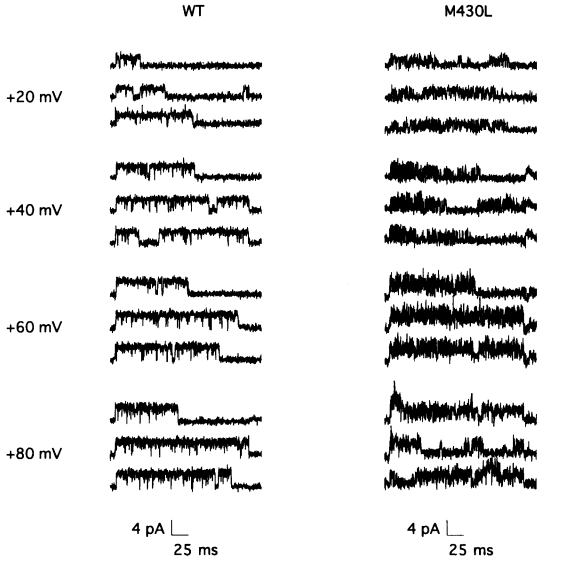

FIGURE 4 Single K^+ currents recorded from Kv3.1 (left column) and the M430L mutant (right column) as a function of voltage. Outside-out patches from oocytes expressing Kv3.1 and M430L mutant channels were depolarized to different voltages from a holding potential of −80 mV.

the single-channel Rb^+ conductance was 14 pS, like WT Kv3.1 channels (Fig. 2, bottom row). One explanation for this permeant ion-dependent flicker is that the mutant channel enters a short-lived closed state, e.g., by the selective alteration of ion-binding sites within the channel, resulting in flicker only when K^+, but not Rb^+, is traversing the pore.

The location of such ion binding sites in relation to the electrical field can be determined by a measurement of the voltage dependence of channel flicker. Three parameters, τ_b (burst duration), τ_f (open time during a burst), and τ_c (closed time), were used to quantify current flicker as a function of voltage (see Materials and Methods); in the WT channel, τ_b would be equivalent to τ_f. For these experiments, outside-out patches were held at −80 mV and stepped to various depolarizing potentials. In experiments with 160 mM K^+ inside and 4.5 mM K^+ outside, channel flicker was apparent at all voltages (Fig. 4) and the markedly shortened τ_f (0.7 ms) was voltage-independent; the total burst duration of the mutant channel (τ_b = 40 ms) was identical to that of WT Kv3.1 channels and was constant at potentials more positive than +10 mV. One interpretation of the data is that the putative ion-binding sites are positioned outside the membrane electrical field. Alternatively, the high concentration of internal K^+ ions may saturate ion-binding sites within the pore and thereby mask any voltage dependence of flicker.

To distinguish between the two possibilities, additional experiments were performed with 160 mM Rb^+ on the inside, and either 4.5 or 160 mM K^+ on the outside (Fig. 5 and Table 2). Under these conditions, Rb^+ ions would enter the pore from the inside and K^+ ions from the outside. As the driving force for K^+ increases with membrane depolarization, external K^+ ions would enter the pore and compete with Rb^+ for binding sites, thereby causing flicker in a predictable

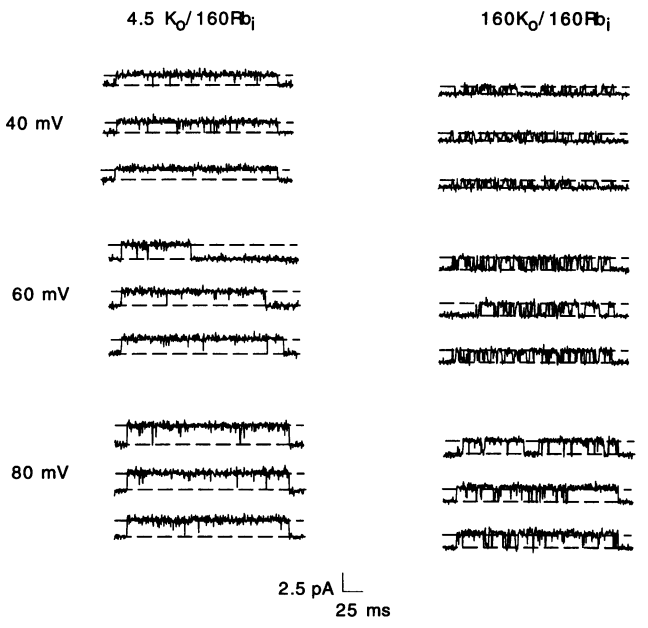

FIGURE 5 Single K^+ currents in outside-out oocyte patches recorded from the M430L mutant at +40, +60, and +80 mV (holding potential of −80 mV) under different ionic conditions. Left: 160 Rb_i^+/4.5 K_o^+. Right: 160 Rb_i^+/160 K_o^+.

TABLE 2 Closed and open time distributions of WT and M430L Kv3.1 channels

	4.5 K^+_o/160 Rb^+_i		160 K^+_o/160 Rb^+_i	
	WT	Mutant	WT	Mutant
Open time (ms)				
+40 mV	18.7	25.3	38.7	0.9
+60 mV	17.2	20.7	21.7	7.6
+80 mV	24.7	22.2	33.6	20.4
Closed time (ms)				
+40 mV	1.5	1.3	1.3	1.3
+60 mV	1.3	1.1	1.3	0.7
+80 mV	1.1	1.5	1.0	0.5

voltage-dependent manner. In support of this hypothesis, outward Rb^+ currents through the mutant channel demonstrated voltage-dependent flicker when the external K^+ concentration was 160 mM (Fig. 5, Table 2). τ_f of the mutant channel was reduced in a voltage-dependent manner compared with WT, being most pronounced at +40 mV and gradually approaching WT values at +80 mV where the driving force for inwardly migrating K^+ would be small (Table 2); τ_b and τ_c did not change (Table 2). With 4.5 mM external K^+, the outward Rb^+ current through the mutant channel did not demonstrate flickery behavior at any voltage, as expected (Fig. 5; compare with Fig. 2, also see Table 2). Collectively, these results imply that one or more ion-binding sites must be located within the ion conduction pathway (or sufficiently close to the pore), to sense the increased rate of external K^+ ions traversing the pore.

In conclusion, the L401V mutation altered sensitivity to TEA_i without changing K^+/Rb^+ permeation, while the M430L mutation in the C-terminal half of S6 produced permeant-ion and voltage-dependent channel flicker. The data presented in this report support the notion that L401 in the P-region and M430 in the C-terminal half of S6 contribute to the formation of the ion conduction pathway of Kv3.1.

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