Biophysical Journal Volume 67 December 1994 2316–2325

Mutational Analysis of Ion Conduction and Drug Binding Sites in the Inner Mouth of Voltage-Gated K^+ Channels

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ABSTRACT Pore properties that distinguish two cloned, voltage-gated K^+ channels, Kv2.1 and Kv3.1, include single-channel conductance, block by external and internal tetraethylammonium, and block by 4-aminopyridine. To define the inner mouth of voltage-gated K^+ channels, segmental exchanges and point mutations of nonconserved residues were used. Transplanting the cytoplasmic half of either transmembrane segments S5 or S6 from Kv3.1 into Kv2.1 reduced sensitivity to block by internal tetraethylammonium, increased sensitivity to 4-aminopyridine, and reduced single-channel conductance. In S6, changes in single-channel conductance and internal tetraethylammonium sensitivity were associated with point mutations V400T and L403 M, respectively. Although individual residues in both S5 and S6 were found to affect 4-aminopyridine blockade, the most effective change was L327F in S5. Thus, both S5 and S6 contribute to the inner mouth of the pore but different residues regulate ion conduction and blockade by internal tetraethylammonium and 4-aminopyridine.

INTRODUCTION

Voltage-gated K^+ channels are thought to consist of four subunits, each of which contains six transmembrane segments, S1–S6, and a membrane-spanning linker between S5 and S6 (S5S6) (Tempel et al., 1988). Among the voltage-gated K^+ channels and inward rectifier K^+ channels, S5S6 is the region with the highest degree of sequence identity. Point mutations and chimeric constructs have shown that S5S6 affects ion selectivity, ion conductance, and tetraethylammonium (TEA) blockade from external and internal sites (Hartmann et al., 1991; Yellen et al., 1991; Yool and Schwarz, 1991; Heginbotham and MacKinnon, 1992). Thus, the 18–20 amino acid residues of S5S6, which has been modeled to span membrane twice (Hartmann et al., 1991; Yellen et al., 1991), might form part of the aqueous pore for transmembrane ion conduction.

Both TEA and 4-aminopyridine (4-AP) are thought to block the pore at binding sites within the internal mouth (Armstrong, 1971; Kirsch and Narahashi, 1983; Kirsch et al., 1986). Furthermore, quaternary ammonium ions have been shown to interact with 4-AP in a mutually exclusive manner (Kirsch et al., 1993), implying that the sites overlap. But unlike TEA, 4-AP block is not voltage-sensitive (Wagoner and Oxford, 1990; Kirsch et al., 1993), suggesting a more superficial location of the 4-AP site in the channel. Taking advantage of natural variations in 4-AP sensitivity between two different voltage-gated K^+ channels, we found that 4-AP block was not transferred with the transplantation of S5S6 from high affinity K^+ channel (Kv3.1) to low affinity K^+ channel (Kv2.1). Instead, both cytoplasmic halves of S5 and S6 were required (Kirsch et al., 1993). Thus, both cytoplasmic ends of S5 and S6 might be in the permeation pathway and form the inner mouth of the channel. These results agree with the importance of residues in S6 for specifying block by hydrophobic quaternary ammonium analogs (Choi et al., 1993). In addition, mutational analysis of *Shaker* K^+ channels has shown that single-channel conductance and ion selectivity are sensitive to mutations in the S4–S5 loop (Slesinger et al., 1993) and S6 (Lopez et al., 1994; Taglialatela et al., 1994). Taken together, these results indicate that the ion conduction pore might be a mosaic structure with contributions from the S5S6, as well as from transmembrane S5 and S6, and S4–S5 loop.

To specify regions other than S5S6 that contribute to the pore, we examined the ion conduction and drug sensitivities of six chimeric channels formed by transplanting segments of Kv3.1 (donor) into corresponding regions of Kv2.1 (host channel) and extended the analysis to point mutations of nonconserved residues in the critical regions of Kv2.1. We found that S4–S5 and post-S6 regions have little effect on ion conductance and blockade. In contrast, 5′S5 and 3′S6 significantly reduced single-channel conductance and conferred the internal TEA sensitivity of donor channel (Kv3.1). The changes in pore properties attributable to 5′S5 and 3′S6 regions were specified by different critical amino acid residues. A preliminary account of these results has been reported previously (Shieh et al., 1994).

Received for publication 6 July 1994 and in final form 29 August 1994.

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0006-3495/94/12/2316/10 $2.00

MATERIALS AND METHODS

Recombinant DNA techniques and mutagenesis

Standard methods of plasmid DNA preparation, site-directed mutagenesis, and DNA sequencing were used (Sambrook et al., 1989). The parent clone Kv2.1 (Frech et al., 1989), Kv3.1 (Yokoyama et al., 1989) as well as the site-directed mutants of the clones, were propagated in the transcription-

competent plasmid vector pBluescript SK(−) in the XL-1 blue MRF' *Escherichia coli* strain (Stratagene, La Jolla, CA).

Polymerase chain reaction mutagenesis

Chimera and site-directed mutations were introduced into the Kv2.1 channel cDNA in pBluescript SK(−) vector. For chimeric channels, mutagenesis oligonucleotides were designed to substitute segments of Kv3.1 for corresponding regions of Kv2.1. Oligonucleotides 38–59 bases in length duplicated the sequences of the S4–S5 linker, 5'S5 (N-terminal half of transmembrane segment), 3'S5 (C-terminal half of transmembrane segment S5), 5'S6, 3'S6, and post-S6 (see Kirsch et al., 1993). Oligonucleotide-directed mutagenesis was performed by polymerase chain reaction (PCR) using Taq DNA polymerase (Boehringer Mannheim Biochemical, Indianapolis, IN) as described previously (Kirsch et al., 1993). The mutated region of plasmid was verified by dideoxy sequencing using sequenase 2.0 (United States Biochemicals, Cleveland, OH).

In vitro transcription and oocyte injection

RNA was transcribed from *Not*I-linearized DNA using methylated cap analog and T7 RNA polymerase as described previously (Drewe et al., 1994).

Stage V or VI oocytes were defolliculated enzymatically (Drewe et al., 1994), injected with 75 nl of cRNA solutions at a concentration of 1–20 pg/nl, and used for recording 1–4 days after injection.

Whole-cell recording

Whole-cell currents were recorded in oocytes using a two micro-electrode voltage clamp as described previously (Drewe et al., 1994). Oocytes were impaled with two 3 M KCl-filled micropipettes (tip resistance, 0.1–0.5 MΩ) that served as voltage-recording and current-injecting intracellular electrodes. A grounded shield was placed between the micropipettes to reduce capacitative coupling. Membrane currents were recorded through a virtual ground circuit connected to the bath via agar bridges and Ag:AgCl reference electrodes. The response time of the voltage clamp in uninjected oocytes for 30 mV steps was approximately 1.5 ms (10–90% subsidence of the capacitative transient current). Analysis of the decay phase of the capacitative current gave a series resistance of approximately 1.2 kΩ in uninjected oocytes. Precautions taken to avoid voltage errors from uncompensated series resistance included placement of electrodes for the virtual ground current-measuring amplifier within 0.5 mm of the oocyte and adjustment of cRNA concentrations to reduce the level of current expression to less than 20 μA at +40 mV. Linear capacitative and leakage currents were subtracted on-line by a P/4 pulse protocol. Internal TEA injection was performed using a pneumatic pressure ejection system (Picospritzer II; General Valve Corp.) connected to the voltage electrode as described previously (Taglialatela et al., 1991). Bath solution for whole-cell recording consisted of (mM): 50 NaOH; 50 KOH; 100 methanesulfonic acid; 2 $CaCl_2$; 10 HEPES (pH 7.3). Bathing solution flowed continuously at a rate of 3 ml/min through a recording chamber of 0.5 ml total volume. Experiments were performed at room temperature (21–23°C). 4-AP (Sigma Chemical Co., St. Louis, MO) stock solution was made by dissolving 4-AP in bathing solution to a concentration of 200 mM (pH adjusted to 7.3 with HCl). Data were expressed as means ± SD where appropriate. A two-tailed Student's t-test was used to evaluate the significance of the difference between means ($p < 0.01$).

Patch-recordings and data analysis

Single-channel cell-attached and inside-out recording in oocytes were performed after manual removal of the vitelline envelope as described previously (Kirsch et al., 1992). Isotonic KCl bathing solution was used to zero the resting potential. Patch pipettes were filled with normal Na^+-Ringer's solution, which consisted of (mM): 120 NaCl; 2.5 KCl; 2 $CaCl_2$; 10 HEPES, pH 7.2. Depolarizing bathing solution consisted of (mM): 100 KCl; 10 EGTA; 10 HEPES, pH 7.2. Data were low-pass-filtered at 0.5–1 kHz. (−3 dB, 4-pole Bessel filter) before digitization at 4 kHz. Channels were activated with rectangular test pulses from negative holding potentials. Holding potentials were adjusted to minimize simultaneous openings of multiple channels. Open and closed transitions were detected using a half-amplitude threshold criterion as described previously (Kirsch et al., 1992).

β-Function analysis of internal TEA blockade followed the method of Yellen (1984). Single-channel recordings in inside-out patches were made at a 1-kHz bandwidth and digitally filtered (Gaussian) at 100–500 Hz to bring the open channel variance in TEA into the appropriate range (Yellen, 1984). β-Functions were fit to all-points histograms of open channel current, and the on/off rates were obtained from the fitted curves. Rate constants at +40 mV are obtained.

RESULTS

Pore properties of chimeric channels

Pore properties that distinguish Kv2.1 from Kv3.1 include single-channel conductance, block by external and internal TEA, and block by 4-AP (Hartmann et al., 1991; Kirsch and Drewe, 1993). These differences were used to identify potential pore-lining regions. Consistent with the notion that S5S6 contains the external mouth of the pore, we found that blockade by external TEA was unchanged in the six chimeric channels examined (Table 1). In contrast, single-channel conductance, G_s, which is thought to be specified by additional regions was altered in the chimeric channels. Outward current amplitudes were measured at 0 to +60 mV in cell-attached patches, and S4S5, 3'S5, 5'S6, and post-S6 chimeras showed an increase in G_s of 20–40% compared with that of Kv2.1 (Fig. 1 and Table 1). It should be noted, however, that none of these changes in conductance approached that of the S5S6 chimera, in which case the conductance of the chimera closely mimicked that of the donor Kv3.1 channel (Hartmann et al., 1991). In contrast to these effects, we found that 5'S5 and 3'S6 chimeras that previously were identified as putative inner mouth regions reduced single-channel conductance by factors of 1.8 and 3.4, respectively (Table 1, Fig. 3). These results, which could not be predicted from the properties of the two parent channels, suggest that although the S5S6 region plays a dominant role in determining ion conduction, the 5'S5 and 3'S6 regions can play a role particularly when "mismatched" with an inappropriate S5S6.

A second line of evidence for the critical role of 5'S5 and 3'S6 comes from determination of sensitivity of outward currents to block by internal TEA. Fig. 2 shows that S4S5, 5'S6, and post-S6 chimeric channels have little influence on TEA block; the sensitivity in the chimeras falls intermediate to the two parent channels. By contrast, both 5'S5 and 3'S6 chimeric channels show large reductions in TEA sensitivity similar to that of Kv3.1 (Fig. 4).

We have shown previously that in S5S6, mutations of residues affect both internal TEA block and ionic conductance (Kirsch et al., 1992; Taglialatela et al., 1993). Because 5'S5 and 3'S6 regions show changes in conductance, internal TEA, and 4-AP blockade, we next examined the role of individual residues located in these putative inner mouth regions for specifying pore properties. The properties of the

TABLE 1 Single-channel characteristics and TEA blockade in Kv2.1, Kv3.1, and chimeric channels

	Conductance* pS	Open Time‡ (ms)	[TEA]i§ IC_{50} (mM)	[TEA]e¶ IC_{50} (mM)
Kv2.1 wt	10.2 ± 0.4 (4)‖	13.5 ± 1.4 (4)	0.14 ± 0.03 (2)	5.7 ± 1.4 (4)
Kv3.1 wt	20.5 ± 1.6 (4)	17.8 ± 1.6 (3)	8.1 ± 2.0 (5)	0.21 ± 0.16 (3)
S4-S5	13.6 ± 1.6 (3)	20 ± 4 (3)	0.66 ± 0.22 (6)	6.1 ± 0.6 (4)
5′S5	5.8 ± 0.6 (3)	3.7 ± 0.5 (3)	5.9 ± 3.1 (7)	4.2 ± 0.3 (4)
3′S5	12.7 ± 0.7 (5)	16.9 ± 2.3 (5)	0.15 ± 0.07 (6)	4.3 ± 1.0 (4)
5′S6	14.0 ± 0.8 (3)	20.4 ± 2.3 (3)	0.62 ± 0.15 (3)	2.6 ± 0.3 (5)
3′S6	3.0 ± 0.9 (4)	0.5 ± 0.2 (3)	8.4 ± 2.7** (6)	5.9 ± 0.2 (3)
post 5′S6	12.3 ± 1.0 (4)	14.9 ± 4.8 (3)	1.06 ± 0.34 (4)	6.5 ± 1.2 (4)

* Single-channel conductance is obtained by fitting outward current amplitude at 0, +20, +40, and +60 mV in cell-attached patch.

‡ Open time is measured at +40 mV in cell-attached single-channel recording.

§ IC_{50} is the concentration required to reduce 50% current at activating potential +40 mV. IC_{50} values were obtained by microinjection of TEA to voltage-clamped oocytes and whole cell current measurements. Internal TEA blockade is voltage-dependent with blocking site located at an electrical distance equivalent to 32 ± 9% (from 19 to 42% among 2 wild types and 6 chimeric channels) of voltage drop within the channel when it is measured using equation described by Woodhull (1973).

¶ TEA at concentration of 5 mM was bath applied and the blocking effect was monitored at +40 mV. The IC_{50} for external TEA blockade was obtained according to the equation (assuming 1:1 binding) residual current = $IC_{50}/([TEA] + IC_{50})$, where [TEA] = TEA concentration (mM).

‖ Number of oocytes tested.

** Student's t-test, $p < 0.01$.

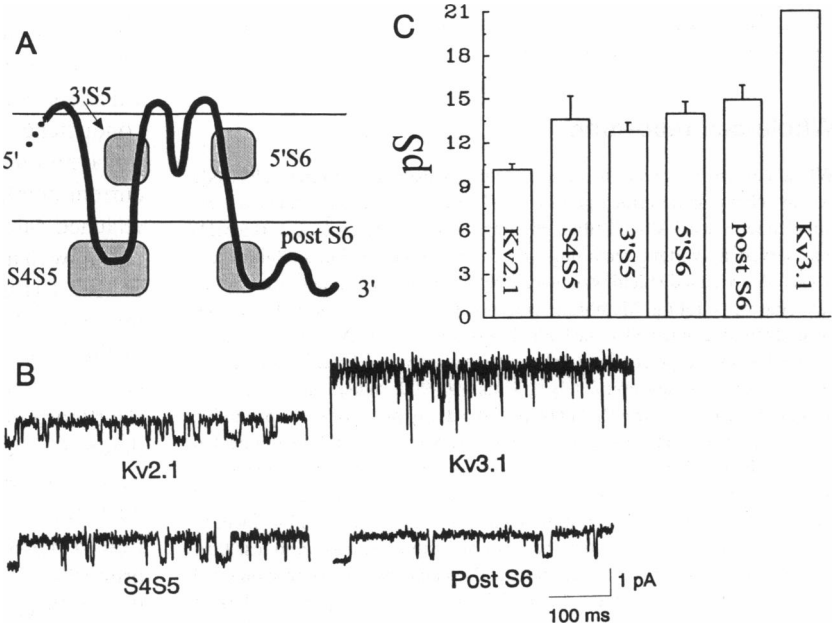

FIGURE 1 Changes in single-channel conductance in S4S5, 3′S5, 5′S6, and post-S6 chimeric channels. (*A*) This cartoon shows four different chimeric channel constructs. Chimeras were made by substituting segments of Kv3.1 (donor) for corresponding regions of Kv2.1 (host) and the mutated regions were indicated in the boxes. The nomenclature and the amino acid sequences of substitution were described previously (Kirsch et al., 1993). (*B*) Representative single-channel records of Kv2.1, Kv3.1, and S4S5, post-S6 chimeric channels. Single-channel currents were recorded from cell-attached patches, pulsed to +40 mV from holding potential of −90 mV for 500 ms. Pipette solution was normal 120 mM Na^+-Ringer, and the oocytes were bathed in a depolarizing isotonic KCl solution. (*C*) Four chimeric channels showed modest increase in single-channel conductance to level between those of Kv2.1 and Kv3.1. Conductance was obtained in cell-attached patches by fitting outward current amplitude at 0, +20, +40, and +60 mV from holding potentials of −80 to −60 mV. The conductance values are the average of 3–5 patches.

mutant channels are compared with the wild-type (WT) Kv2.1 channel.

V400T selectively reduces slope conductance

The 5′S5 and 3′S6 regions of Kv2.1 contain seven amino acids that are different from Kv3.1. Therefore, we substituted each of the nonconserved amino acids in Kv2.1 with the corresponding Kv3.1 residue and then examined pore properties of each mutation. Fig. 5 summarizes the conductance properties of each mutant. Substitution of Thr for Val 400 (V400T) reduced G_s to 50% of WT, which is similar to that observed in the 3′S6 chimeric channel. Mutations in the remaining positions did not affect conductance (Table 2).

L332I and L403 M specify resistance to block by internal TEA

With two exceptions, mutations in the 5′S5 and 3′S6 regions gave only minor reductions in the apparent internal TEA binding affinity (Fig. 6 and Table 2). The two exceptions were substitution of L332I in 5′S5 and substitution of L403 M in 3′S6. In each case, TEA sensitivity was reduced about 20-fold, compared with WT, to approach that observed in the 5′S5 and 3′S6 chimeric channels, respectively. To study the mechanism by which these mutations alter blockade, we applied TEA to inside-out patches and recorded single-channel currents in Kv2.1, L403 M, and Kv3.1 (Fig. 7 *A*). At a test potential of +40 mV, Kv2.1 channels have a mean open time

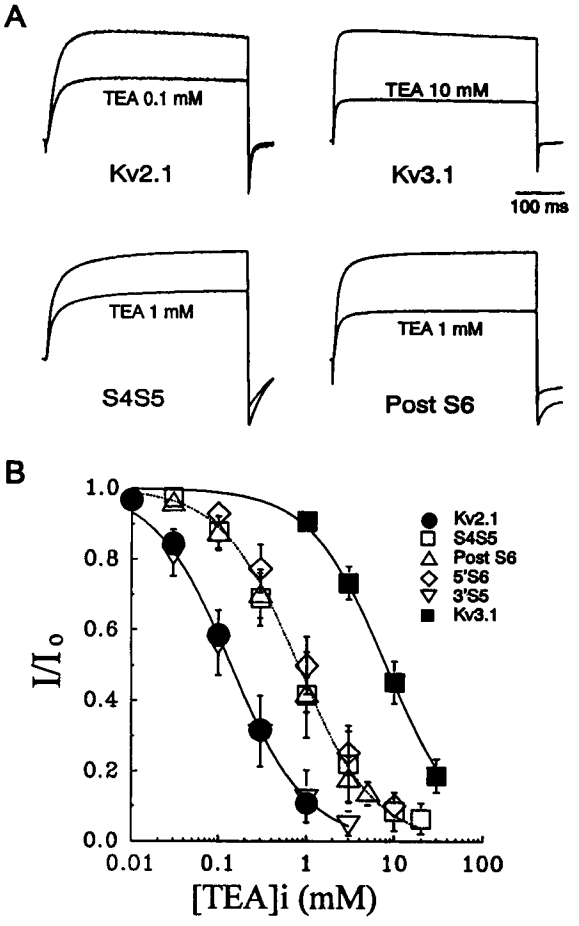

FIGURE 2 Changes in internal TEA sensitivity in S4S5, 5′S6, and post-S6 chimeric channels. (*A*) Sensitivity to internal TEA. Whole cell outward ionic currents were recorded at +40 mV from holding potential of −80 mV for 500 ms before and after internal TEA application. Currents were normalized to the peak current at +40 mV in the absence of internal TEA. (*B*) Concentration-response curves for internal TEA blockade were obtained by measuring whole cell steady-state outward currents at the end of test pulses of 500 ms duration. The data points represent fractional current (I/I_o, I_o: control current) after TEA block at +40 mV. The smooth curves were fitted to a 1:1 binding model.

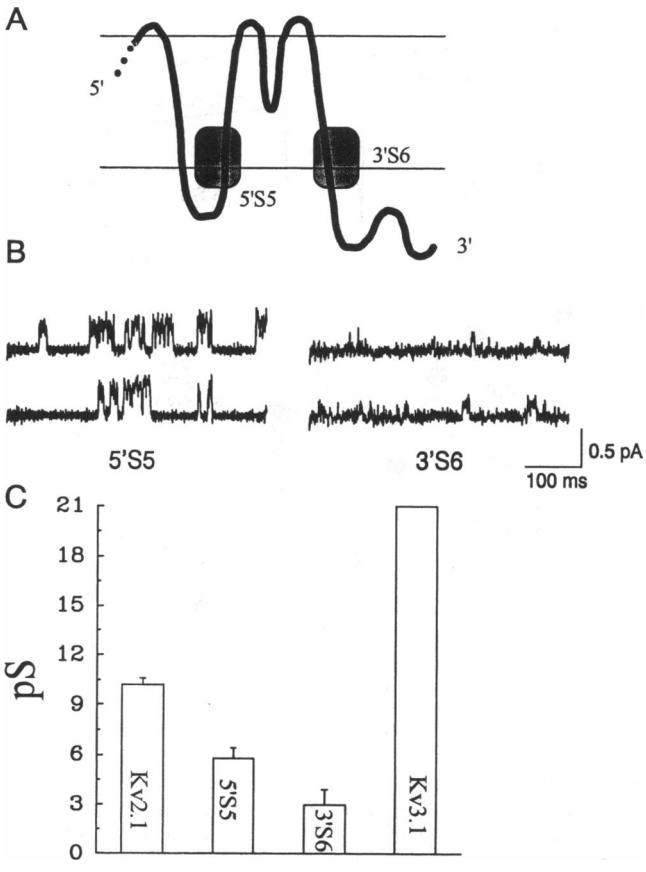

FIGURE 3 Changes in conductance in 5′S5 and 3′S6 chimeras. The cartoon in *A* shows two chimeric channels with the mutated regions indicated in boxes. *B* shows representative single-channel records of 5′S5 and 3′S6 chimeras. Both 5′S5 and 3′S6 chimeric channels reduced single-channel conductance compared with Kv2.1 and Kv3.1 (*C*). The measurements were described in Fig. 1.

of 10 ms. After application of 0.1 mM TEA, the open time was shortened to such an extent that unitary amplitudes were slightly truncated and the number of brief closed intervals during the bursts was markedly increased. In contrast, the mutant L403 M channel had a mean open time similar to that of Kv2.1, but application of 0.1 mM TEA produced no obvious change in the single-channel openings. At 3 mM, however, TEA reduced amplitude by 20% without changing open time. Closed intervals that corresponded to blocked states were too fast to resolve. These results are consistent with the notion that L403 M caused an acceleration in the TEA dissociation rate from the open channel such that TEA block was altered from "flickery, intermediate" to "non-flickery, fast" blockade (Yellen, 1984). Similarly, in Kv3.1, TEA at 10 mM reduced single-channel amplitude by 50% without producing resolvable blocked intervals. By fitting the amplitude histograms of the raw currents records to a β-functions as described by Yellen (1984), we measured the association and dissociation rates. We found that the on-rate for TEA to block in all three channels was unchanged but the off-rates were markedly different. L403 M increased TEA off-rate from 0.5 to 12 ms^{-1}, which is twofold less than 30 ms^{-1} obtained in Kv3.1. The IC_{50} values estimated by this method were 0.25, 4.9, and 10 mM for Kv2.1, L403 M, and Kv3.1, respectively. These values were comparable with 0.14, 3, and 8 mM obtained by microinjection of TEA and measurement of whole-cell currents. This result suggests that position 403 is directly involved in TEA blockade and that the effect of L403 M is to destabilize TEA binding rather than to prevent access of the drug to its binding site. By contrast, the mutant L332I at 5′S5 simply reduced mean open time from 10 to 3 ms (Fig. 7 *B*) and open probability from 0.34 to 0.05 (Table 2). These changes in gating increase resistance to TEA blockade because of reduced access to the binding site. We suggest, therefore, that the effect of mutation at this position on internal TEA blockade is indirect.

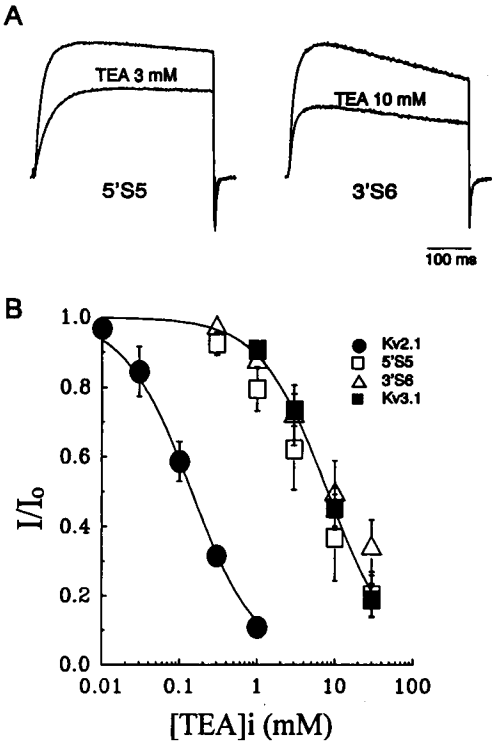

FIGURE 4 Decreases in internal TEA sensitivity in 5′S5 and 3′S6 chimeric channels. *A* shows representative outward currents of 5′S5 and 3′S6 chimeras recorded at +40 mV before and after internal TEA application. Both 5′S5 and 3′S6 have internal TEA sensitivity similar to that of donor channel, Kv3.1 (*B*).

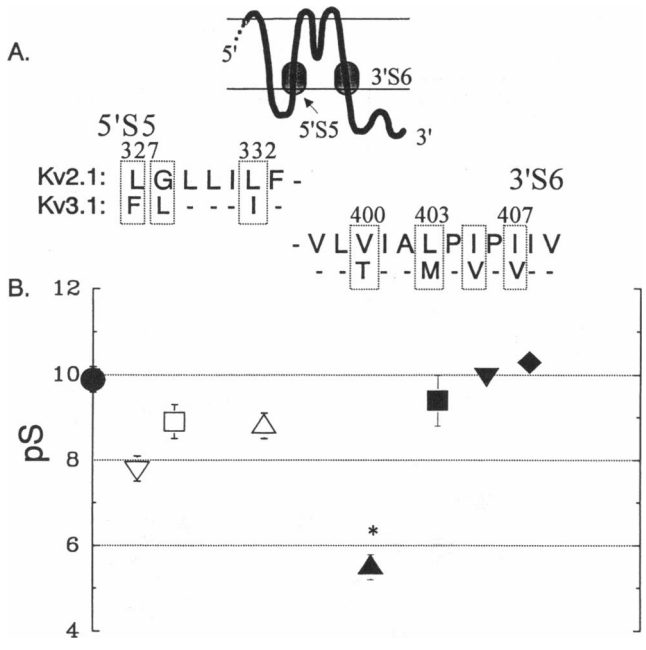

FIGURE 5 V400T decreases single-channel conductance. (*A*) Point mutations were made in 5′S5 and 3′S6 regions shown in shaded boxes by substituting nonidentical amino acid residues in Kv2.1 with corresponding amino acid residues in Kv3.1. Sequences are aligned in the bottom panel. Conserved residues are indicated by dashes. The numbers refer to the Kv2.1 amino acid sequence. *B* shows changes in single-channel conductances observed in seven point mutations. Each symbol was lined up to each amino acid substitution shown in *A*. The solid circle indicates single-channel conductance of Kv2.1 wt. Only V400T shows reduction in outward conductance to the level observed from 3′S6 chimeric channel.

L327F, I405V, and I407V enhance 4-AP binding affinity

4-AP blocks channels in a gating-dependent manner such that the drug accumulates in closed channels and dissociates slowly from open channels (Choquet and Korn, 1992; Kirsch and Drewe, 1993). Therefore, the apparent 4-AP sensitivity of different K^+ channels can be indirectly influenced by differences in gating. Activation gating is perturbed in many pore-mutant K^+ channels and, as we have shown previously, an empirical, linear relationship exists between deactivation time constants and 4-AP IC_{50} (Kirsch et al., 1993). We asked whether mutation of any of the individual, nonconserved residues in 5′S5 and 3′S6 could selectively alter 4-AP sensitivity without changing gating. A residue that meets this criterion would be a good candidate for the 4-AP binding site. Mutation-induced changes in 4-AP binding affinity, compared with WT Kv2.1, are shown in Fig. 8 *A*. Three residues stand out as potential contributors to 4-AP binding but, as shown in Fig. 8 *B* where 4-AP IC_{50} was plotted as a function of deactivation, time constant L327F has the greatest influence on 4-AP block and the least effect on gating. Thus, in Fig. 8 *B*, Kv3.1 has a fast deactivation time constant 1 ms and high 4-AP affinity (IC_{50} 0.1 mM), compared with Kv2.1, which has a deactivation time constant of 7 ms and low 4-AP affinity (17 mM 4-AP, IC_{50}). Mutants G328L, L332I, V400T, and L403 M also show good correlation between 4-AP IC_{50} and deactivation constant. By contrast, L327F, I405V, and I407V do not follow this relationship, although their IC_{50} decreased from 17 mM to the range of 0.5–2 mM. These results suggest that the increase in 4-AP binding affinity in L327F, I405V, and I407V is independent of gating and that the most effective mutation is L327F. Thus, one amino acid residue (L327) in 5′S5 and two amino acid residues (I405, and I407) in 3′S6 are critical for 4-AP binding, and these results may account for our previous observation that a 5′S5 and 3′S6 double chimera transfers Kv2.1 from low 4-AP-sensitive to high 4-AP-sensitive channel.

DISCUSSION

Mosaic structure of the pore

The cytoplasmic end of the K^+ channel pore has been modeled to have a wide vestibule narrowing to a tunnel (Armstrong 1971; Hille, 1992) or deep pore (Kirsch et al., 1992). The vestibule can accommodate either a hydrated K^+ or a TEA ion because both have a radius of about 4 Å, but only K^+ can become dehydrated and pass through the deep pore (Armstrong 1971). Internal TEA and 4-AP have easy access to and from their binding sites only after the channels have been activated by depolarization (Armstrong and Hille, 1972; Choquet and Korn, 1992; Kirsch and Drewe, 1993);

TABLE 2 Single-channel characteristics, gating, TEA, and 4-AP blockade in point mutations in 5'S5 and 3'S6 regions

Channel	Conductance (pS)	Open time (ms)	P_{open}*	Activation‡ $V_{0.5}$ (mV)	Inactivation§ τ (s)	Deactivation¶ τ (ms)	$[TEA]_i$‖ IC_{50} (mM)	$[TEA]_e$ IC_{50} (mM)	4-AP** IC_{50} (mM)
A. In 5'S5									
L327F	7.8 ± 0.3 (5)	10.7 ± 1.8 (5)	0.16 ± 0.06 (5)	13 ± 2 (7)	3.9 ± 0.5 (6)	5.3 ± 0.8 (17)	0.21 ± 0.16 (3)	6.0 ± 2.5 (6)	0.58 ± 0.21 (6)
G328L	8.9 ± 0.4 (6)	5.9 ± 1.2 (6)	0.13 ± 0.03 (6)	29 ± 3 (7)	1.7 ± 0.4 (10)	2.3 ± 0.3 (10)	0.75 ± 0.23 (5)	4.8 ± 0.7 (4)	0.97 ± 0.23 (6)
L332I	8.8 ± 0.3 (6)	3 ± 1 (6)	0.05 ± 0.02 (6)	19 ± 2 (2)	1.8 ± 0.3 (7)	2.8 ± 0.5 (10)	2.5 ± 1.1 (7)	5.5 ± 2.0 (5)	3.8 ± 1.2 (5)
B. In 3'S6									
V400T	5.5 ± 0.3 (5)	9.8 ± 2.6 (3)	0.07 ± 0.04 (4)	10 ± 3 (7)	0.9 ± 0.1 (6)	3.7 ± 0.4 (6)	0.77 ± 0.34 (4)	5.5 ± 1.2 (4)	5.3 ± 1.8 (5)
L403M	9.4 ± 0.6 (4)	9.7 ± 3.9 (7)	0.13 ± 0.03 (7)	14 ± 3 (12)	1.0 ± 0.1 (8)	4.5 ± 0.3 (12)	3.1 ± 0.7*** (5)	5.8 ± 0.6 (4)	9.8 ± 2.6 (3)
I450V	10.0 ± 0.1 (3)	8.0 ± 0.3 (3)	0.16 ± 0.03 (3)	−1 ± 2 (6)	2.1 ± 0.4 (6)	3.3 ± 0.5 (7)	0.51 ± 0.22 (2)	7.2 (2)	1.5 ± 0.8 (4)
I407V	10.3 ± 0.1 (4)	11.2 ± 1.6 (4)	0.17 ± 0.03 (4)	9 ± 1 (8)	2.3 ± 0.3 (7)	4.7 ± 0.7 (8)	0.59 ± 0.24 (6)	6.2 ± 0.2 (7)	3.8 ± 1.9 (4)

* Open probability (P_{open}) was measured at +40 mV for the period of duration of 500 ms. The values were the average of 40–60 nonzero opening traces.
‡ $V_{0.5}$ is the midpoint of the steady-state conductance versus test pulse potential relationship obtained from fitting a Boltzmann equation. Test potentials range from −30 to +55 mV (5 mV increments).
§ Inactivation time constant obtained from decay of test pulse current at +40 mV. Currents were fitted with a single exponential.
¶ Deactivation time constant obtained from the decay of tail currents at a test potential of −80 mV. Tail currents were fitted with a single exponential.
‖ Internal TEA blockade is voltage-dependent with blocking site located at an electrical distance equivalent to 31 ± 8% (from 22 to 42% among 7 mutants) of voltage drop within the channel.
** 4-AP was bath-applied to voltage-clamped oocytes, and IC_{50} values were obtained at +40 mV.
Note: $V_{0.5}$, inactivation time constant, and deactivation time constant for Kv2.1 and Kv3.1 are: −1 ± 1, 12 ± 1 mV; 5.3 ± 0.7, 7.5 ± 1.5 s; and 7.6 ± 0.7, 1.3 ± 0.1 ms, respectively (Kirsch et al., 1993).
*** Student's t test, $p < 0.01$.

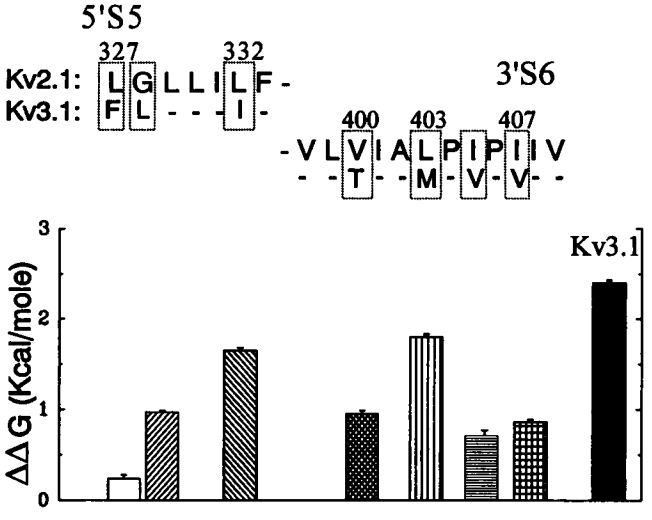

FIGURE 6 Internal TEA binding affinity. The changes in internal TEA blockade were converted to the incremental change in the apparent free energy of binding from the formula, $\Delta\Delta G = -RT \ln(Kd_{wt}/Kd_{mut})$, in which Kd_{wt} and Kd_{mut} are the IC_{50} values for Kv2.1 wild-type and mutant channels, respectively. R is the gas constant, and T is absolute temperature. Only L332I and L403 M reduced TEA binding affinity to the level similar to that of Kv3.1 (*solid bar*).

however, the blocking mechanisms are different. Quaternary ammonium ions physically occlude the inner mouth of the pore as evidenced by the ability of permeant ions entering the external mouth to dislodge internal TEA from its binding site, and by the inability of TEA to reside in the closed channel (Armstrong, 1975). In contrast, 4-AP is readily trapped in the closed channel (Kirsch and Drewe, 1993), where it can selectively immobilize a component of gating charge movement that corresponds to a slow step near the end of the activation process (McCormack et al., 1994). Thus, 4-AP may act by entering the inner mouth to reach a gating-sensitive site that may not necessarily be in the conduction pathway itself. We favor the idea that 4-AP site is in the ion conduction pathway; however, based on several lines of evidence: (1) 4-AP is active in the cationic form as indicated by the ability of a quaternary analog to mimic 4-AP when applied internally (Kirsch and Narahashi, 1983; Kirsch and Drewe, 1993); (2) 4-AP block is inhibited by tetrapentylammonium in a mutually exclusive manner, indicative of overlapping sites in the conduction pathway (Kirsch et al., 1993); and (3) the onset of 4-AP block was markedly slowed by externally applied blocking ions, Cs^+, Rb^+, and $(NH_4)^+$, that enter the pore via the extracellular mouth (Kirsch et al., 1986). A further distinction between TEA and 4-AP is that TEA blockade is voltage-dependent, suggesting that the binding site is within membrane electric field (Armstrong and Hille, 1972; Swenson 1981), whereas 4-AP blockade is voltage-insensitive, suggesting that the binding site is outside the membrane electric field (Wagoner and Oxford, 1990; Kirsch et al., 1993). Therefore, both TEA and 4-AP are useful as probes of the cytoplasmic end of the pore, but must interact with distinct sites within the pore.

Indeed, the alterations in pore properties observed in the 5'S5 and 3'S6 chimeric channels indicate that different regions affect K^+ conduction and blockade by internal TEA and 4-AP. Point mutations further specify different critical amino acid residues in these two regions responsible for changes in pore properties. Also, it should be noted that our observation that external TEA block was unaffected is consistent with the notion that the extracellular end of the pore and a portion of the ion-conducting path is contributed by the S5S6 loop (Hartmann et al., 1991). In comparison with results obtained in 5'S5 and 3'S6, the S4S5 loop and post-S6 flanking regions had small effects on conductance and

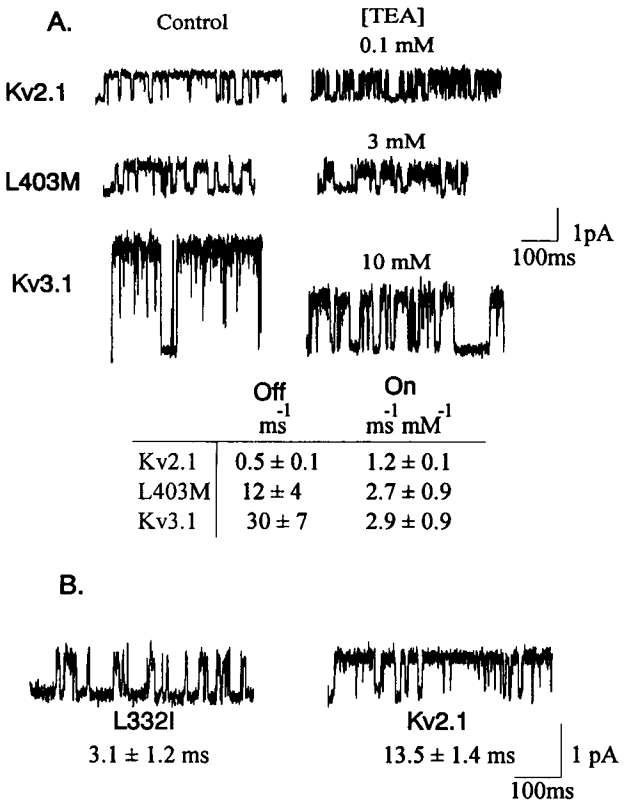

FIGURE 7 Mechanisms by which changes in internal TEA sensitivity in L403 M and L332I. *A* shows TEA blockade from intracellular side in Kv2.1, L403 M, and Kv3.1. Single-channel events were recorded in inside-out patches exposed intracellularly to 120 mM KCl (*left-hand* column) and 120 mM KCl with 0.1, 3, and 10 mM TEA added for Kv2.1, L403 M, and Kv3.1, respectively (*right-hand* column). Currents were evoked by test pulses to +40 mV from holding potential −80 to −60 mV, and were low-pass-filtered at 1 kHz. (−3 dB, 4-pole Bessel filter) before digitization at 4 kHz. TEA blockade was quantified by fitting amplitude histograms to β-functions as described by Yellen (1984). The on- and off-rates are shown in the inset. L403 M increases TEA off rate by 20 fold with no changes in on rate. Each value is the average from 2, Kv2.1; 7, L403 M; and 5 patches, Kv3.1. *B* shows single-channel recording from cell-attached patches. Pipette solution was normal 120 mM Na^+-Ringer, and the oocytes were bathed in a depolarizing isotonic KCl solution. At +40 mV, L332 single-channel mean open time was reduced from 13 (wild-type Kv2.1) to 3 ms.

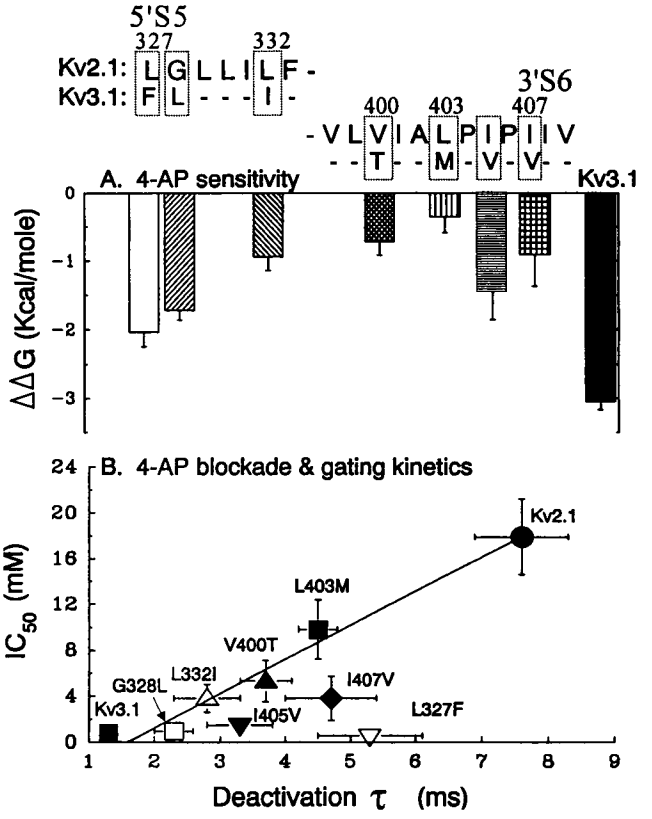

FIGURE 8 4-AP binding affinity and correlation between 4-AP blockade and deactivation time constant. *A* shows changes in 4-AP binding affinity observed from point mutations in 5′S5 and 3′S6 regions relative to that of Kv2.1. Mutations in these regions enhance 4-AP binding to different degrees. 4-AP binding affinity for Kv3.1 is shown as solid bar. (*B*) 4-AP IC_{50} obtained in Kv2.1 (●), Kv3.1 (*cross-hatched square*), and mutants are plotted as a function of deactivation time constant (ms) measured at −80 mV. L327F (▽), I405V (▼) and I407V (♦) do not fit this relationship.

internal TEA blockade. Also, mutations in S4S5 and post-S6, unlike 5′S5 and 3′S6, did not alter 4-AP binding (Kirsch et al., 1993). Thus, the cytoplasmic half of S5 and S6 transmembrane segment might form the internal mouth of voltage-dependent K^+ channels.

S4S5 loop and S6 regions have been reported to affect conductance, Rb^+ selectivity, and blockade by TEA, Ba^{2+}, and Mg^{2+} in *Shaker* channel (Isacoff et al., 1991; Slesinger et al., 1993; Lopez et al., 1994; Taglialatela et al., 1994). In Kv2.1, a mutation in S4S5 loop stabilizes binding with a synthetic peptide corresponding to the first 20 amino acids of *Shaker* channels (Isacoff et al., 1991) and enhances channel inactivation. Taken together, S4S5 and post-S6 are near the permeation pathway, and the results are consistent with the notion that these two flanking regions may form part of the channel vestibule.

The observations that the conductance of 5′S5 and 3′S6 chimeric channels were reduced upon transplanting segments from the high conductance channel Kv3.1 are unexpected. However, a single substitution of V400T in 3′S6 was sufficient to make such a change. This implies that the geometry of the conduction pore or the major components determining ion conduction varies among different K channels. For instance, in *Shaker* B, transplantation of the S6 transmembrane segment of Kv3.1 confers a large conductance when transplanted into *Shaker* B, whereas S5S6 (P-region) of Kv3.1 only confers the external TEA binding site (Lopez et al., 1994; Taglialatela et al., 1994). Unlike *Shaker* B, S5S6 of Kv3.1 confers large conductance, high affinity external TEA binding, and low affinity internal TEA binding when transplanted into Kv2.1 (Hartmann et al., 1991). These results support the notion that the pore is a mosaic structure with contributions from S5, S6, and flanking regions. At this point, we cannot exclude the possibility that some pore properties, particularly ion conductance, are strongly dependent on packing of these different components of the channel protein. In fact, the influence of mismatched packing between the inner mouth components and the P-region may be re-

sponsible for the low conductance observed in the 5′S5 and 3′S6 chimeras.

Internal TEA site

The interaction of the impermeant TEA^+ ion with the inner mouth of the K^+ channel mimics that of the permeant K^+ ion because: (1) TEA's radius approximates that of a fully hydrated K^+ ion; (2) like K^+, TEA has access only to the open channel; (3) also like K^+, TEA^+ interacts competitively with ions that enter the pore from the outside; (4) even before it was measured experimentally, the single-channel K^+ conductance was accurately estimated on the basis of the block rate for quaternary ammonium ions (Armstrong, 1975). The fact that TEA blocks the pore whereas K^+ is a permeant ion has been attributed to the ability of K^+ ion to shed part of its hydration shell as it passes through the narrow regions of the pore. But because TEA binds at sub-millimolar concentrations, in K^+ channels such as *Shaker* and Kv2.1, additional forces must act to stabilize preferentially the interaction of TEA over that of K^+ in these channels. The TEA binding site therefore should include at least some residues that confer selectivity such that substitutions at these critical positions yield altered TEA block without affecting K^+ conductance, whereas other residues affect both. Examples of the former include two P-region substitutions, T441S (Yellen et al., 1991) and M440I (Choi et al, 1993), that selectively reduced TEA block without altering K^+ conductance. The chemical nature of the stabilizing forces at these positions remains to be determined. An example of the latter would be residue V374 in Kv2.1 because a variety of substitutions at this locus altered both TEA block and K^+/Rb^+ conductance. The two effects were closely linked because TEA block was enhanced with Rb^+ and reduced with K^+ as the current carrier (Taglialatela et al., 1993) and, therefore, the residue may be more closely associated with K^+/Rb^+ binding than with TEA. In the present work, we have identified a residue L403 located outside the P region, which selectively affected TEA block without affecting K^+ conductance. Our observation that the mutation L403 M selectively increased the dissociation rate may be related to decreased side-chain hydrophobicity (~3 kcal/mol) introduced at this site by the Leu→Met substitution (Creighton, 1992). However, this point remains to be established. Our result is consistent with previous work in which the same mutation in *Shaker* also reduced TEA block (Lopez et al., 1994), and we now show that an enhanced dissociation rate is responsible. Our results also support the hypothesis of Choi et al. (1993) that the S6 region contains a hydrophobic pocket for TEA binding, based on the observation that substitution of hydrophobic Val at position T469 in *Shaker* selectively enhance the binding of long chain TEA analogs without changing K^+ conductance. These results suggest that residues in the P-region and the S6 segment combine to form a TEA selective site that can discriminate between TEA and K^+ in part, on the basis of hydrophobic interactions that keep TEA tightly bound.

4-AP binding site

4-AP reaches its blocking site from the cytoplasmic side of the channel (Kirsch and Narahashi, 1983; Choquet and Korn, 1992), and its access to the site is strongly enhanced by depolarization-induced activation gating (Kirsch and Drewe, 1993; McCormack et al., 1994). Thus, change in both gating and the critical residues within the binding site affect 4-AP blockade. In particular, channels with fast deactivation rates were associated with high 4-AP affinity (Kirsch et al., 1993). Although substitutions of short pieces from both 5′S5 and 3′S6 are required to transfer high affinity 4-AP block from Kv3.1 to Kv2.1 (Kirsch et al., 1993), only 3′S6 affected 4-AP block without changing deactivation rates. Therefore, we speculated that the 4-AP binding site may be in 3′S6, in which case the role of 5′S5 may be to control gating and indirectly affect 4-AP access. Our present results, however, support the notion that both 5′S5 and 3′S6 may be directly involved in 4-AP binding because point mutations of residues in both regions (Fig. 8 and Table 2) were found to enhance 4-AP block without affecting deactivation. Thus, 5′S5 mutations L327F, selectively enhanced 4-AP affinity, whereas G328L and L332I accelerated deactivation with lesser effects on 4-AP block. We previously speculated that in 3′S6 V400 might be a critical residue for 4-AP block, because the corresponding residue in *Shaker* was shown to influence block by quaternary ammonium (QA) ions (Choi et al., 1993) that compete with 4-AP (Kirsch et al., 1993). Instead, our present results show that I405 and I407 are more important for 4-AP block. Our interpretation, therefore, is that QA and 4-AP have overlapping but not identical attachment points within the internal mouth. Electrostatic repulsion may account for the mutually exclusive interactions between the two blockers. This notion is also consistent with the observation that both blockers compete with the inactivation particle (Grissmer and Cahalan, 1989; Wagoner and Oxford, 1990) that binds to the internal mouth of the pore (Isacoff et al., 1991).

Our results indicate that the wide range of 4-AP sensitivity among different K^+ channels may be related to both gating differences as well as three residues (L-327, I-405, and I-407 in Kv2.1). Thus, among slowly inactivating, cloned K^+ channels, the combination of Leu-Ile-Ile found at these three positions in Kv2.1 and other members of the *Shab* subfamily (Pak et al., 1991) is associated with low 4-AP sensitivity (IC50 > 10 mM). By contrast the combination Phe-Val-Val found in members of the *Shaw* subfamily (Yokoyama et al., 1989; McCormack et al., 1990) and Leu-Val-Val found in *Shaker* subfamily (Stuhmer et al., 1989; Christie et al., 1989; Grupe et al., 1990; Stocker et al., 1990; Swanson et al., 1990; Tseng-Crank et al., 1990; Wei et al., 1990; Baldwin et al., 1991; Hart et al., 1993) are associated with high 4-AP sensitivity (IC_{50} < 1 mM). It should be noted however that the L-V-V combination does not confer high 4-AP sensitivity in rapidly inactivating channels such as Kv1.4 and members of the *Shal* subfamily. Therefore, differences in gating are also relevant to 4-AP block.

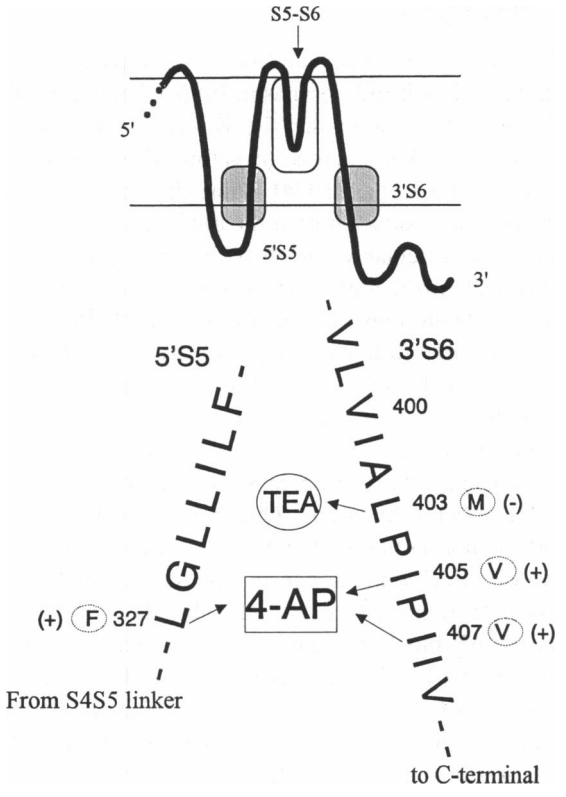

FIGURE 9 This cartoon summarizes the sequences and critical amino acid residues affecting TEA and 4-AP binding in putative inner mouth (*shaded boxes*). The numbers reference the Kv2.1 amino acid sequence. The amino acid residues of the substitutions are shown in dashed circles, which either increase (+) or decrease (−) drug bindings.

Our results suggest that different potencies of 4-AP block might be caused by different combinations of amino acid residues that form the 4-AP binding site rather than single residues as is the case for external TEA blockade (Heginbotham and MacKinnon, 1992; Kirsch et al., 1992). Because all four of the substitutions among the three critical residues are nonpolar, we have no ready explanation for why the F-V-V and L-V-V combinations are more favorable for 4-AP block than the L-I-I combination. We speculate that the residues may influence the geometry of the binding site and that possibly the higher total volume of the side chains of the unfavorable combination, compared with the two favorable combinations, may impede 4-AP block by steric factors.

In conclusion (see Fig. 9), both putative cytoplasmic sides of S5 and S6 segments have effects on single-channel conductance, internal TEA, and 4-AP blockade. L327F in 5′S5 and I405V, I407V in 3′S6 region enhance 4-AP binding affinity. In 3′S6 chimera, changes in single-channel conductance, internal TEA sensitivity were associated with V400T and L403 M, respectively. Thus, 5′S5 and 3′S6 form the inner mouth of pore, and contain different residues that regulate 4-AP and internal TEA blockade.

We thank Dr. A. M. Brown for his support and encouragement throughout this work. We thank G. Schuster and W.-Q. Dong for expert oocyte injection and culture, M. Champagne and M. Lindsey for molecular biology support, and G. Cantrell and C.-D. Zuo for technical assistance.

This work was supported in part by National Institutes of Health grants NS29473 to G. E. Kirsch and NS23877 and HL37044 to A. M. Brown. Additional support was provided by the Texas Advanced Technology Program (grant number 4949–032) to A. M. Brown.

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