Mutations in the K^+ Channel Signature Sequence

Lise Heginbotham, Zhe Lu, Tatiana Abramson, and Roderick MacKinnon
Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Potassium channels share a highly conserved stretch of eight amino acids, a K^+ channel signature sequence. The conserved sequence falls within the previously defined P-region of voltage-activated K^+ channels. In this study we investigate the effect of mutations in the signature sequence of the *Shaker* channel on K^+ selectivity determined under bi-ionic conditions. Nonconservative substitutions of two threonine residues and the tyrosine residue leave selectivity intact. In contrast, mutations at some positions render the channel nonselective among monovalent cations. These findings are consistent with a proposal that the signature sequence contributes to a selectivity filter. Furthermore, the results illustrate that the hydroxyl groups at the third and fourth positions, and the aromatic group at position seven, are not essential in determining K^+ selectivity.

INTRODUCTION

The many different K^+ channels can be distinguished mainly by the way they gate. Some K^+ channels open in response to membrane voltage changes, some respond to varying levels of intracellular Ca^{2+}, and in other K^+ channels gating is tightly regulated by G-proteins or the binding of intracellular ligands (Hille, 1991). Ion conduction, on the other hand, is similar in all K^+ channels regardless of the gating mechanism. The selectivity sequence among permeant ions and the signatures of multi-ion conduction are essentially invariant features of conduction in K^+ channels.

Work in several laboratories, combining mutagenesis and biophysics, has demonstrated that pore-blocking scorpion toxins, ionic blockers, and conducting ions all interact with amino acids between S5 and S6 (commonly referred to as the P-region or H5 (MacKinnon and Miller, 1989; MacKinnon et al., 1990; MacKinnon and Yellen, 1990; Yellen et al., 1991; Hartmann et al., 1991; Yool and Schwarz, 1991; Kavanaugh et al., 1991; Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992; Kirsch et al., 1992; Heginbotham et al., 1992; De Biasi et al., 1993). Mutations of residues in the S4-S5 loop and within the S6 sequence have also been found to alter both single channel conductance and blockade (Choi et al., 1993; Slesinger et al., 1993; Lopez et al., 1994). It is conceivable that amino acid residues from this entire region (S5 through S6) may line an aqueous conduction pathway. However, the ion selective filter may be formed by a much smaller subset of amino acids. Given that ion selectivity is very similar in all the K^+ channels, it is reasonable to look for conservation at a primary sequence level that could underlie the common ion selectivity.

Fig. 1 shows a partial sequence alignment comparing K^+ channels from each of the major classes including voltage-activated, Ca^{2+}-activated, and inward rectifier K^+ channels. A complete alignment would reveal that the ROMK1, IRK1, and GIRK1 channels have almost no sequence similarity to the other channels outside of the region shown. But within this region, the channels are all highly homologous over a stretch of eight amino acids (TXXTXGYG) corresponding to the sequence TMTTVGYG in the *Shaker* voltage-activated K^+ channel. This homologous region, which we refer to as the signature sequence, is found in all K^+ channels so far cloned. Perhaps the signature sequence forms a catalytic domain that confers the common conduction properties characteristic of K^+ channels. Here we investigate the functional consequences of introducing point mutations into the signature sequence of a Shaker K^+ channel.

METHODS

Molecular biology and heterologous expression

The *Shaker* H4 K^+ channel (Kamb et al., 1987) in a Bluescript vector (Stratagene, La Jolla, CA) was modified to remove NH_2-terminal inactivation by deleting amino acid residues 6–46 (Hoshi et al., 1990; Yellen et al., 1991). For simplicity of presentation, we refer to residues 439–446 of the original, unmodified, *Shaker* H4 sequence as positions 1–8 (see Fig. 1). Oligonucleotide mutagenesis was performed using the dut^-ung^- selection scheme of Kunkel (1985) with the mutagene kit (Bio-Rad, Cambridge, MA), and mutations were confirmed by dideoxy sequencing (Sanger et al., 1977) of the region containing the mutation. RNA was synthesized using T7 RNA polymerase (Promega, Madison, WI) after a *Hind*III (New England Biolabs, Beverly, MA) linearization of the DNA. For most mutants (including T1A, T1S, T2A, and Y7V) two independent colonies of each mutation were characterized functionally to decrease the possibility that observed effects were the result of stray mutations.

Oocytes were isolated from *Xenopus laevis* (Xenopus One), defolliculated, injected with RNA, and incubated at 18°C until subsequent recording, as previously described (MacKinnon et al., 1988).

Electrophysiology

This study uses a single kind of measurement, the permeability ratio, to characterize ion selectivity in mutant *Shaker* K^+ channels. With K^+ present on the inside and univalent ion X^+ on the outside the permeability ratio P_X/P_K is found using the equation

$$\frac{P_X}{P_K} = \frac{[K]}{[X]} e \frac{V_{rev} F}{RT} \quad (1)$$

Received for publication 18 November 1993 and in final form 19 January 1994.

Address reprint requests to Lise Heginbotham, Department of Neurobiology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115

0006-3495/94/04/1061/07 $2.00

where [K] and [X] are the concentrations of K^+ and X^+, V_{rev} is the zero-current potential and R, T, and F have their usual meanings. Equation 1 is based on Nernst-Planck electrodiffusion (Hille, 1991) and is used as an operational definition of the permeability ratio in ion channel measurements.

In some channels, the reversal potential could be measured during depolarizing voltage steps. At voltages below the reversal potential activating current was inward, whereas at potentials above the reversal potential activating current was outward. In many cases, the reversal potential was at hyperpolarized potentials where the channels do not normally open. Therefore, channels were activated using an initial depolarizing pulse, and currents were measured after the membrane was subsequently stepped to different test potentials. The reversal potential was determined from the current measured during the tail potentials.

Because of limitations set by the low levels of expression or the unusual gating behavior of some mutant channels, several different experimental approaches were used to estimate the effect of mutations on permeability ratios.

1. Channels that expressed to high levels

These channels were studied using excised membrane patches, in either the outside-out or inside-out configuration (Hamill et al., 1981). Unless otherwise noted, the internal (intracellular) solution contained K^+, whereas extracellular solution contained the test monovalent cation.

2. Channels that expressed only to low levels

These channels were studied using a two-electrode voltage clamp, and the different test ions were washed into the bath solution. In each case, a measurement was made when the bath solution contained K^+ to allow an estimation of the internal K^+ concentration.

3. Channels that conducted only in the presence of bath K^+

Several mutant channels require K^+ in the extracellular solution to display measurable ionic current. Typically, currents are large in the presence of extracellular K^+ but disappear when only Na^+ is present. The same "K^+ titration" phenomenon has been described for a naturally occurring K^+ channel, RCK4, where low K^+ decreases the number of channels that open during a depolarizing pulse (Pardo et al., 1992).

Because these mutants did not express to high enough levels for macroscopic patch recordings, they were studied using two-electrode voltage clamp. However, the requirement for external K^+ did not allow the experiments described in section 2 of Electrophysiology above. Instead, to estimate selectivity, the reversal potential was measured as a function of external K^+ concentration, as Na^+ was gradually substituted for external K^+. If a channel is perfectly selective for K^+ over Na^+, then the reversal potential is fixed thermodynamically according to the Nernst equation (Eq. 1, when X = K^+). To quantify the permeability ratio, P_{Na}/P_K, in these mutants, the measured reversal potentials were compared with reversal potentials predicted by the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949),

$$V_{rev} = \frac{RT}{F} \ln \left[\frac{P_{Na}[Na]_o + P_K[K]_o}{P_K[K]_i} \right]. \quad (2)$$

The selectivity of titrating channels could not be determined with the same precision as in nontitrating channels. Deviations from Eq. 1 are most noticeable when K^+ concentrations are very different on the two sides of the membrane. Using two-electrode voltage clamp, K_i^+ is fixed; thus the measurements will be most informative at low K_o^+ concentrations. However, it is at low K_o^+ concentrations that the ionic current in these mutant channels diminishes and disappears.

Reversal potentials were determined using the standard tail protocol described above. To isolate ionic current from gating current, measurements made with either Na^+ or N-methyl-D-glucamine (NMDG) in the bath (recordings in these two ions were equivalent and contained insignificant levels of ionic current) were subtracted from measurements recorded with K^+ in the bath.

For the Y7F mutant channel, we used the cut-open oocyte voltage clamp (Taglialatela et al., 1992) to measure the reversal potential with K^+ in the extracellular solution and the test ion in the intracellular solution. In these cases, the bath and guard solutions contained 100 mM [K^+], while the internal bath and internal perfusion solutions contained 100 mM test ion.

For all electrophysiological experiments, voltage protocols were computer-controlled using an Everex (Fremont, CA) 386 computer, coupled to an Indec (Sunnyvale, CA) AD/DA interface. Currents were measured using either an Axopatch 2B patch clamp amplifier (Axon Instruments, Foster City, CA), an OC-725 oocyte clamp for two-electrode voltage clamp (Warner Instruments, Hamden, CT), or a cut-open oocyte voltage clamp (kindly provided by Drs. E. Stefani and A. Brown). Patch clamp electrodes were coated with beeswax to decrease capacitance, and typically had resistances from 0.5–1.5 MΩ. Electrodes for the two-electrode voltage clamp and cut-open voltage clamp were filled with 3 M KCl and had resistances of 0.5–1.8 MΩ and 0.5–1.0 MΩ, respectively. Data were filtered at 1–5 kHz and sampled at 0.25–10 kHz.

Solutions

In each of these experiments, the external solution contained 100 mM XCl (where X was either Li^+, Na^+, K^+, Rb^+, Cs^+, or NH_4^+), 0.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM Hepes-XOH (pH 7.1). External solution containing NMDG contained 100 mM NMDG, 0.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM Hepes-HCl (pH 7.1). For experiments using patch-clamp or cut-open oocyte recording, the internal solution contained 100 mM XCl (where X was either K^+, Rb^+, Cs^+, or NH_4^+), 1.0 mM $MgCl_2$, 5 mM EGTA, and 10 mM Hepes-XOH (pH 7.1).

RESULTS

In this study we used a single type of measurement to survey selectivity in the mutant *Shaker* K^+ channels. The reversal potential under bi-ionic conditions was determined with K^+ on one side of the membrane and various test ions (Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+) on the other side. The permeability ratio for the test ion, X, against K^+, P_X/P_K, was calculated using Eq. 1. The equation applies specifically to electrodiffusion, but we use it here as an operational definition of the permeability ratio. We began by focusing on residues containing side-chain oxygens or aromatic rings to test whether these could form essential K^+ selective binding sites.

Mutations at positions 1, 3, and 4

Mutations of the threonine residues within the signature sequence were designed to test the necessity of having a hydroxyl-containing amino acid at these positions for intact K^+ selectivity (positions 1, 3, and 4 as shown in Fig. 1). Fig. 2 shows currents carried by wild-type *Shaker* K^+ channels and mutant channels T3A and T4A. The recordings were made in the presence of internal K^+ and external Cs^+ (Fig. 2 *A*) or Na^+ (Fig. 2 *B*). The mutant channels with a substituted Ala residue, which lacks a side-chain hydroxyl, selected strongly for K^+ over both the smaller Na ion and larger Cs ion. With Cs^+ on the outside,

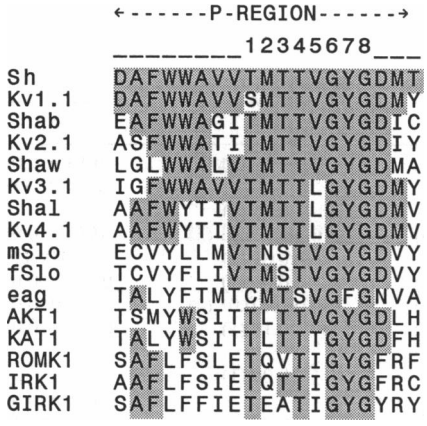

FIGURE 1 Sequence alignment of the P-region from different cloned K^+ channels. Residues identical to those in the Shaker (Sh) channel are highlighted. Four independent genes from *Drosophila*, *Shaker* (Tempel et al., 1987; Pongs et al., 1988; Kamb et al., 1988), *Shab*, *Shaw*, and *Shal* (Butler et al., 1989; Wei et al., 1990), and their mammalian homologues Kv1.1 (Baumann et al., 1988), Kv2.1 (Frech et al., 1989), Kv3.1 (Yokoyama et al., 1989), and Kv4.1 (Pak et al., 1991), respectively, encode voltage-activated K^+ channels. fSlo and mSlo are Ca^{2+}-activated K^+ channels cloned from *Drosophila* and mouse (Atkinson et al., 1991; Butler et al., 1993), respectively, and eag is a cyclic nucleotide-regulated K^+ channel (Warmke et al., 1991; Bruggemann et al., 1993). AKT1 and KAT1 are inward rectifying K^+ channels cloned from the plant *Arabidopsis thaliana* (Anderson et al., 1992; Sentenac et al., 1992; Schachtman et al., 1992). ROM1 and IRK1 are two inward rectifying channels cloned from rat outer medulla and mouse cardiac tissue (Ho et al., 1993; Kubo et al., 1993a). GIRK1 is a G-protein modulated K^+ channel (Kubo et al., 1993b).

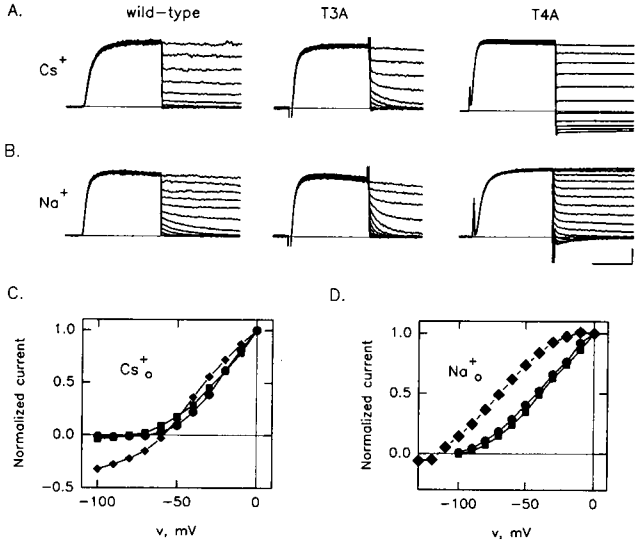

FIGURE 2 The wild-type Shaker channel and the T3A and T4A mutant channels display similar selectivity. Raw current traces from wild-type, T3A, or T4A channels, with either 100 mM Cs^+ (*A*) or Na^+ (*B*) in the extracellular solution. Currents from wild-type (Cs^+ and Na^+) and T4A (Cs^+) channels were recorded from excised, inside-out membrane patches, with 100 mM K^+ in the internal solution. The cut-open oocyte voltage clamp was used to record currents from T4A with 100 mM Na^+ in both the bath and guard chambers, and 100 K^+ in the internal solution. The T3A mutant (Cs^+ and Na^+) was examined using a two-electrode voltage clamp. In each case, current was activated by a step to 0 mV, followed by hyperpolarizations ranging from 0 to −100 mV (0 to −130 mV for T4A in Na^+), in 10-mV increments. The scale bar, shown under the T4A Na^+ trace, corresponds to 25 ms and the following levels of current: wild-type Cs^+, 105 pA; T3A Cs^+, 261 nA; T4A Cs^+, 209 pA; wild-type Na^+, 200 pA; T3A Na^+, 261 nA; T4A Na, 532 nA. Leak and capacitive currents have been subtracted from the wild-type and T3A traces using a P/4 subtraction protocol (Bezanilla and Armstrong, 1977). The T4A traces show raw current, without leak subtraction. Isochronal I-V curves, from the records shown in (*A*) and (*B*), in the presence of external Cs^+ (*C*) or Na^+ (*D*). The data were normalized to the amount of current at 0 mV (wild-type, ●; T3A, ■ ; T4A, ◆).

the reversal potentials were −72 and −59 mV (Fig. 2 *C*) for the T3A and T4A mutant channels, respectively; these channels were at least as selective as the wild-type channel. The inward currents were more prominent in the recording from the T4A mutant channel, but the reversal potential was essentially identical to the wild-type channel (Fig. 2 *A*).

These mutant channels were also highly selective for K^+ over Na^+ (Fig. 2, *B* and *D*). Both the wild-type and T3A mutant channels showed no inward Na^+ current. In the T4A mutant, inward current was observed only at potentials below −110 mV, therefore the channel is selective for K^+ over Na^+ by a ratio of 75:1. These experiments showed that mutant channels devoid of the hydroxyl group contributed by either Thr 3 or 4 remained highly selective for K^+ over Na^+ and Cs^+. The same mutant channels also displayed wild-type selectivity for K^+ over NH_4^+ and Rb^+ (Table 1).

Several other amino acid substitutions at positions 3 and 4 were tolerated and produced channels that were selective like the wild-type (Table 1). Some mutations at position 3 failed to produce current in oocytes and were not studied further (see Fig. 5).

The third threonine residue within the signature sequence is located at position 1. Of the four substitutions made at position 1, only serine, which conserves the hydroxyl functional group, yielded channels with wild-type selectivity (Table 1). T1A and T1V mutant channels did not express current (see Fig. 5), whereas the T1G mutant gave a nonselective channel (see below).

Mutations at tyrosine 7

The aromatic ring of an amino acid could interact favorably with conducting ions (Heginbotham and MacKinnon, 1992; Kumpf and Dougherty, 1993). Every cloned K^+ channel contains a Tyr or Phe at position 7 (Fig. 1), and in *Shaker* the Y7F mutant channel is K^+ selective (Table 1). To test whether an aromatic residue at this site is required for K^+ selectivity we studied the effect of mutations at position 7. One of these, Y7V, expressed well enough to study using two-electrode voltage clamp of whole oocytes and was clearly selective for K^+ over Na^+ (Fig. 3). The currents from this mutant exhibited an unusual time course; the currents rapidly decayed during the depolarizing pulse, and a large hooked tail was present upon repolarization. Like some other previously described K^+ channels, the Y7V mutant required that there be some K^+ present in the external medium to

TABLE 1 Selectivity of mutant *Shaker* channels

Mutant	A. Selective Mutants*			
	Na^+	Rb^+	Cs^+	NH_4^+
Wild-type	< 0.02 ± 0.01 (3)	0.82 ± 0.07 (4)	0.08 ± 0.01 (5)	0.13 ± 0.02 (7)
T1S	< 0.01 (1)	0.79 (1)		0.13 (1)
M2G‡	< 0.01 ± 0.01 (2)		< 0.06 (1)	
M2I‡	< 0.01 ± 0.00 (6)	0.86 ± 0.01 (6)	< 0.08 ± 0.01 (3)	0.13 ± 0.01 (6)
M2N‡	< 0.14 (1)		< 0.09 (1)	< 0.06 (1)
T3A	< 0.03‡ ± 0.01 (2)	0.51§ ± 0.08 (2)	0.06 (1)	0.08§ ± 0.02 (2)
T3S‡	< 0.02 ± 0.00 (6)	0.80 ± 0.03 (6)	< 0.09 ± 0.01 (4)	0.13 ± 0.01 (5)
T4A	< 0.01‡ ± 0.03 (2)	0.77§ ± 0.06 (5)	0.10 (1)	0.14§ ± 0.00 (2)
T4G‡	< 0.02 (1)			
T4N‡	< 0.02 (1)		0.09 (1)	
T4S	< 0.01‡ ± 0.00 (2)	0.74 (1)		0.11 ± 0.01 (2)
V5C‡	< 0.06 (1)		< 0.09 (1)	< 0.09 (1)
V5L	< 0.03 ± 0.00 (2)	0.47 ± 0.14 (3)	0.16 (1)	0.10 ± 0.00 (2)
V5T‡	< 0.04 (1)		0.07 ± 0.01 (3)	0.09 ± 0.02 (2)
Y7F	< 0.13¶ (3)		< 0.19‖ (1)	0.16‖ ± 0.02 (3)
Y7V	< 0.20¶ (3)			

Mutant	B. Nonselective Mutants**				
	Li^+	Na^+	Rb^+	Cs^+	NH_4^+
T1G	0.32 ± 0.04 (3)	0.33 ± 0.21 (3)	0.94 ± 0.21 (3)	0.94 ± 0.21 (3)	1.00 ± 0.17 (3)
V5A	0.86 ± 0.05 (4)	0.91 ± 0.09 (4)	1.05 ± 0.10 (4)	1.05 ± 0.10 (4)	1.81 ± 0.31 (4)
V5G	0.64 ± 0.06 (4)	0.74 ± 0.00 (4)	1.00 ± 0.10 (4)	1.00 ± 0.10 (4)	1.49 ± 0.15 (4)
V5N	0.63 ± 0.04 (3)	0.67 ± 0.00 (4)	0.94 ± 0.06 (3)	0.82 ± 0.09 (3)	1.22 ± 0.14 (3)
V5Q	0.57 ± 0.06 (2)	0.70 ± 0.04 (4)	0.88 ± 0.06 (3)	1.00 ± 0.07 (3)	1.48 ± 0.21 (3)
G6A	0.55 (1)	0.84 ± 0.06 (7)	1.00 ± 0.00 (3)	1.00 ± 0.09 (7)	1.10 ± 0.11 (4)
G6C	0.61 ± 0.12 (2)	1.03 ± 0.08 (5)	0.82 ± 0.05 (4)	0.98 ± 0.12 (5)	1.42 ± 0.21 (4)
G6P	0.09 ± 0.02 (3)	0.22 ± 0.03 (5)	0.99 ± 0.17 (3)	1.05 ± 0.14 (3)	1.05 ± 0.14 (3)
G6Q	0.68 ± 0.34 (2)	0.90 ± 0.07 (9)	1.06 ± 0.10 (7)	0.95 ± 0.06 (6)	1.24 ± 0.12 (4)
G6S	0.48 (1)	0.92 ± 0.05 (14)	0.95 ± 0.09 (4)	0.98 ± 0.10 (8)	1.06 ± 0.15 (4)
G8A	0.72 (1)	1.08 ± 0.08 (4)	0.94 ± 0.06 (3)	1.08 ± 0.08 (4)	1.01 ± 0.07 (3)
G8C	0.75 (1)	1.05 ± 0.04 (7)	0.81 ± 0.06 (5)	0.91 ± 0.10 (4)	1.65 ± 0.13 (4)
G8P	0.68 ± 0.13 (2)	1.01 ± 0.14 (4)	1.32 ± 0.35 (3)	0.95 ± 0.06 (3)	1.15 ± 0.20 (3)
G8Q	0.56 ± 0.06 (2)	0.99 ± 0.06 (6)	1.01 ± 0.10 (4)	0.96 ± 0.06 (5)	1.29 ± 0.13 (4)
G8S	0.64 (1)	1.09 ± 0.15 (6)	1.06 ± 0.06 (4)	1.21 ± 0.19 (5)	1.42 ± 0.23 (4)

The channels are grouped with respect to Na^+ permeability. Each value represents the P_x/P_K ± SE (or range of mean for $n = 2$), with the number of independent determinations shown in parentheses.

* Unless otherwise indicated, measurements were made in macroscopic patches, as described in the Methods section.

‡ Assayed using two-electrode voltage clamp, when the test ion was the only monovalent cation in the bath.

§ Reversal potential determined using excised patches, with 105 mM K^+ in the internal solution and 95 mM test ion in the external solution.

¶ Selectivity was determined by titrating bath $[K^+]$. Relative permeability was estimated as illustrated in Fig. 3 *C* for Y7V.

‖ Measurements were made using the cut-open oocyte technique.

** Mutants that displayed dramatically altered Na^+ permeability were studied using two-electrode voltage clamp.

produce ionic currents (Fig. 3) (Pardo et al., 1992). The disappearance of current at low external K^+ concentrations limited our ability to tell whether the mutant channel was as selective for K^+ over Na^+ as wild-type. The permeability ratio for Na^+ against K^+ was assessed by varying the concentration of bath K^+ and allowed us to place a limit on P_{Na}/P_K of 0.2 or less (Fig. 3 *C*, see Methods). Also, because of the requirement for K^+ in the external medium, we were unable to test the channel's ability to discriminate between K^+ and other usually permeant ions such as Cs^+, Rb^+ and NH_4^+. Nevertheless, we can conclude that the conserved aromatic residue at position 7 is not required for the channel to discriminate between Na^+ and K^+. Two other nonconservative position 7 mutants, Y7A and Y7P, when expressed in oocytes produced only gating currents and no ionic currents, similar to what was previously described for the mutant channel W434F (Perozo et al., 1993).

Mutations resulting in loss of K^+ selectivity

Mutations at four positions resulted in loss of K^+ selectivity. Fig. 4 *A* shows representative current traces from channels with a mutation at each position. The currents from mutants T1G, V5A, G6C, and G8Q were recorded in the presence of Na^+ as the only extracellular monovalent cation. The reversal potential near zero indicates a permeability ratio $P_{Na}/P_K \approx 1$ in each case. The channels also allowed Li^+, Rb^+, Cs^+, and NH_4^+ to conduct with permeability ratios (against K^+) close to unity (Fig. 4 *B*, Table 1). An example is shown in Fig. 4 *B*, where the current traces show the V5G mutant channel recorded in the presence of several different test cations in the bath. All test cations were about equally permeant except for the organic cation NMDG, which was impermeant.

At position 1, the conservative T1S mutation showed wild-type selectivity (see above), whereas the nonconservative

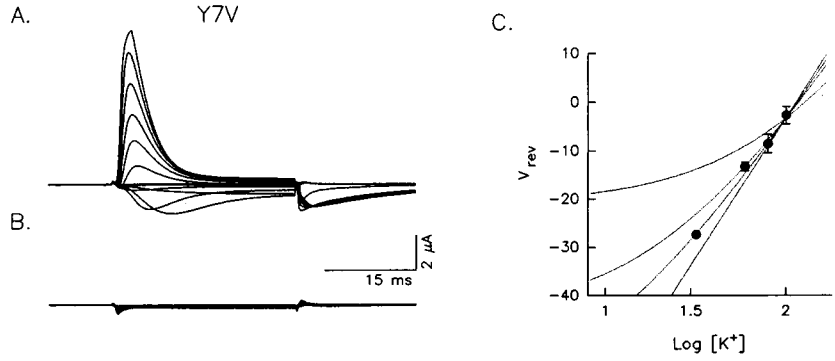

FIGURE 3 Selectivity of the Y7V mutant channel. (*A*) Ionic current from the Y7V channel when the bath solution contained 100 mM KCl. (*B*) Currents from the same oocyte when K^+ in the bath solution was replaced with Na^+. In both (*A*) and (*B*), currents were recorded using two electrode-voltage clamp and were elicited by stepping to membrane potentials between −70 and +50 mV (10-mV increments) from a holding potential of −80 mV, with a tail potential of −80 mV. Leak and capacitive currents were subtracted by measuring currents generated by the same protocol when NMDG was the only cation in the bath. The current remaining in (*B*) is the result of a slight change in the capacitive transient during the time course of the experiment. (*C*) Dependence of the reversal potential on the bath K^+ concentration. Reversal potentials were measured at the indicated K^+ concentrations. The dotted lines are drawn according to Eq. 2, with $P_{Na}:P_K$ = 1:2, 1:5, 1:10, and 0:1, where $[K]_o+[Na]_o$ = 100 mM, and assuming $[K^+]_i$ = 114 mM. Each point and error bar represents the mean ± SE of 3–5 measurements in separate oocytes.

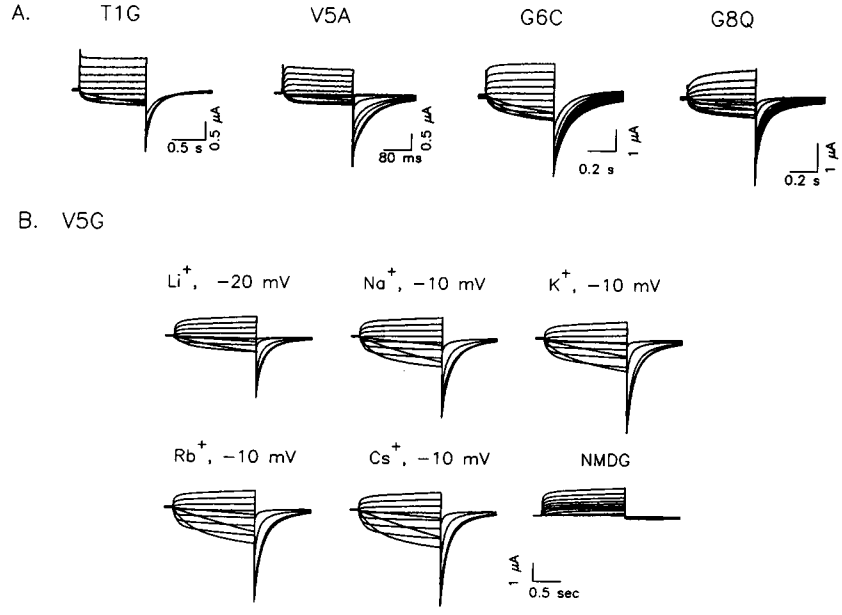

FIGURE 4 Recordings from oocytes expressing nonselective mutant channels using two electrode voltage-clamp. (*A*) Currents were recorded from oocytes expressing either the T1G, V5A, G6C, or G8Q channels, with 100 mM Na^+ in the bath solution. (*B*) Selectivity of the V5G mutant channel. Currents were recorded in bath solutions containing 100 mM of the indicated monovalent cation, and the reversal potential is shown in each case. Currents were generated in response to steps from −70 to +40 mV (−70 to +20 mV for T1G and V5G), in 10-mV increments, from an initial holding potential of −90 mV. The subsequent tail potential was −110 mV. A linear leak, measured between −90 and −100 mV, was scaled and subtracted from these traces.

T1G mutant was not selective. At position 5 (Val), some nonconservative mutations supported wild-type ion selectivity (Cys, Leu, Thr), whereas others gave rise to nonselective channels (Ala, Gly, Asn, Gln) (Table 1). Interestingly, the nonconservative mutations at position 5 produced a more or less binary outcome of either selective or nonselective channels; none displayed partial selectivity. No mutation at position 6 or 8 produced a selective channel; replacement of either Gly residue with Ala, Cys, Pro, Gln, or Ser resulted in channels that were nonselective.

Many of the mutant channels had gating properties that were different than those of wild-type. In particular, the nonselective channels deactivated with slow kinetics regardless of the position of the mutation. The time scales for the different traces in Fig. 4 *A* are not the same; in general, deactivation is slow compared with the wild-type. But slow deactivation is not necessarily linked to nonselectivity; the T4A mutant channel deactivated at an extremely slow rate and was still highly selective (Fig. 2 *A*). We did not pursue the study of gating in these channels further. In general, channel gating was very sensitive to mutations made in the P-region whether or not the mutation altered ion selectivity.

DISCUSSION

Previous mutagenesis studies of voltage-activated K^+ channels have shown that P-region amino acids interact with pore-blocking toxins, ionic blockers, and permeant ions. The amino acid sequences of the many distantly related K^+ channels reveal a highly conserved stretch of eight residues within the P-region. In the present study we ask how mutations within this signature sequence affect ion selectivity. The study is incomplete, because not all possible substitutions were made at every position. Furthermore, the approach we have taken to identify "essential residues" introduces an unavoidable bias, inasmuch as mutations at different positions

cannot be compared in terms of their severity. For example, there are no substitutions for Gly that can be considered conservative. Regardless of these limitations, our results allow us to draw several new conclusions.

First, we have found that the channel will tolerate nonconservative mutations at three positions within the signature sequence. Channels without a hydroxyl-containing amino acid at positions T3 and T4 are still selective for K^+ (Table 1). (These results are in direct contrast with a previous report that mutations at T3 in a *Shaker* K^+ channel substantially increased P_{NH4+}/P_{K+} (Yool and Schwarz, 1991)). Similarly, a nonconservative substitution of the aromatic amino acid at position 7 left the channel selective for K^+ over Na^+. Because Y7 is the only aromatic residue within the highly conserved eight amino acid stretch, we suspect that K^+ selectivity is not based on aromatic-cation interactions. Our results do not exclude the possibility that the T3, T4, or Y7 side chains interact directly with permeant ions. But we can conclude that K^+ channels, however they discriminate between K^+ and Na^+, can do it in the absence of these specific functional groups when they are altered one at a time.

In contrast to the mutations described above, some mutations at residues 1, 5, 6, and 8 affected the channel's selectivity (Fig. 5). These findings are consistent with the notion that the signature sequence is an important determinant of K^+ selectivity, but we are unable to draw any mechanistic conclusions about why these mutant channels are nonselective. It is interesting to note that at position 1, the conservative T1S mutation gave a K^+ selective channel, while the T1G mutation rendered the channel nonselective. However, these data alone are insufficient to conclude that position 1 hydroxyl groups interact directly with K^+ to form part of a selectivity filter.

The high conservation and the results of this study argue strongly that the signature sequence underlies ion selectivity in K^+ channels. Until there is a high resolution structure of a K^+ channel we can only speculate on how the signature sequence might form a selectivity filter. However, structural information about K^+ selective binding sites found in compounds such as valinomycin, nonactin, crown ethers, and the enzyme dialkylglycine decarboxylase may provide us with some insight (Ovchinnikov et al., 1974; Toney et al., 1993). In general, the binding sites coordinate a K^+ ion through interactions with oxygen atoms. In fact, interactions between K^+ ions and oxygen atoms were proposed more than 20 years ago to underlie the selectivity of K^+ channels (Bezanilla and Armstrong, 1972; Hille, 1973). The peptide formed by the signal sequence could provide these oxygen atoms through carbonyl oxygens (from the backbone) and hydroxyl oxygens (for instance, from position 1). In some proteins, such as alpha-lactalbumen and a rhinovirus coat protein, the sequence GYG (so highly conserved in the signature sequence) occurs within a turn (Acharya et al., 1989; Kim et al., 1989). If it occurred within a K^+ pore, such a turn might free backbone carbonyls from hydrogen bonding and allow them to interact with a K^+ ion.

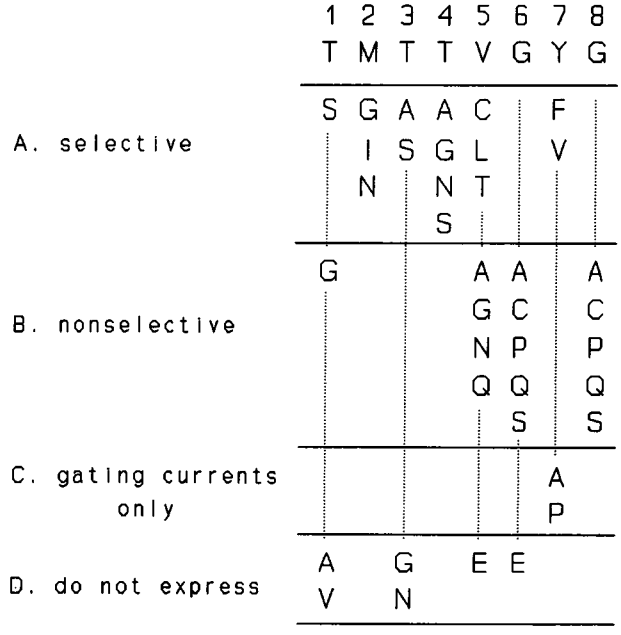

FIGURE 5 Chart illustrating the phenotype of different mutant channels. Each channel is grouped according to its selectivity for K^+ over Na^+. (*A*) The K^+/Na^+ selectivity of mutants labeled "selective" was indistinguishable from that of the wild-type channel. (*B*) Each of the nonselective channels is clearly permeable to Na^+. (*C*) Oocytes injected with cRNA from these mutant channels express gating current but immeasurable ionic current. (*D*) These mutant channels show no functional expression, based on electrophysiological recordings (< 50 nA of current in the entire oocyte would be indistinguishable from the background).

We would like to thank G. Yellen for helpful discussions during the course of this work and for providing us with the V5C, G6C, and G8C clones, and P. Hidalgo, M. Root, and K. Swartz for critical readings of the manuscript. This work was supported by NIH grant GM47400. ZL is a fellow of the Harvard Mahoney Neuroscience Institute

REFERENCES

Acharya, K. R., D. I. Stuart, N. P. C. Walker, and M. Lewis. 1989. Refined structure of baboon alpha-lactalbumen at 1:7 angstroms resolution. *J. Mol. Biol.* 208:99–127.

Anderson, J. A., S. S. Huprikar, L. V. Kochian, W. J. Lucas, and R. F. Gaber. 1992. Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cervisiae. Proc. Natl. Acad. Sci. USA.* 89:3736–3740.

Atkinson, N. S., G. A. Robertson, and B. Ganetzky. 1991. A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. *Science (Wash. DC).* 253:551–555.

Baumann, A., A. Grupe, A. Ackermann, and O. Pongs. 1988. Structure of the voltage-dependent potassium channel is highly conserved from *Drosophila* to vertebrate central nervous systems. EMBO J. 7:2457–2463.

Bezanilla, F., and C. M. Armstrong. 1972. Negative conductance caused by entry of sodium and cesium ions into the potassium channels of squid axons. *J. Gen. Physiol.* 60:588–608.

Bezanilla, F., and C. M. Armstrong. 1977. Inactivation of the sodium channel. I. Sodium current experiments. *J. Gen. Physiol.* 70:549–566.

Bruggemann, A., L. A. Pardo, W. Stühmer, and O. Pongs. 1993. Ether-á-go-go encodes a voltage-gated channel permeable to K^+ and Ca^{2+} and modulated by cAMP. *Nature (Lond.).* 365:445–448.

Butler, A., A. Wei, K. Baker, and L. Salkoff. 1989. A family of putative potassium channel genes in *Drosophila. Science (Wash. DC).* 243: 943–947.

Butler, A., S. Tsunoda, D. P. McCobb, A. Wei, and L. Salkoff. 1993. *mSlo*, a complex mouse gene encoding "Maxi" calcium-activated potassium channels. *Science (Washington DC).* 261:221–224.

Choi, K. L., C. Mossman, J. Aube, and G. Yellen. 1993. The internal quaternary ammonium receptor site of Shaker potassium channels. *Neuron.* 10:533–541.

De Biasi, M., G. E. Kirsch, J. A. Drewe, H. A. Hartmann, and A. M. Brown. 1993. Cesium selectivity conferred by histidine substitution in the pore of the potassium channel Kv2.1. *Biophys. J.* 64:341*a*. (Abstr.)

Frech, G. C., A. M. J. VanDongen, G. Schuster, A. M. Brown, and R. H. Joho. 1989. A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning. *Nature (Lond.).* 340: 642–645.

Goldman, D. E. 1943. Potential, impedance, and rectification in membranes. *J. Gen. Physiol.* 27:37–60.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch. Eur. J. Physiol.* 391:85–100.

Hartmann, H. A., G. E. Kirsch, J. A. Drewe, M. Taglialatela, R. H. Joho, and A. M. Brown. 1991. Exchange of conduction pathways between two related K^+ channels. *Science (Wash. DC).* 251:942–944.

Heginbotham, L., T. Abramson, and R. MacKinnon. 1992. A functional connection between the pores of distantly related ion channel as revealed by mutant K^+ channels. *Science (Wash. DC).* 258:1152–1155.

Heginbotham, L., and R. MacKinnon. 1992. The aromatic binding site for tetraethylammonium ion on potassium channels. *Neuron.* 8:483–491.

Hille, B. 1973. Potassium channels in myelinated nerve. Selective permeability to small cations. *J. Gen. Physiol.* 61:669–686.

Hille, B. 1991. Ionic Channels of Excitable Membranes. Sinauer Associates, Inc., Sunderland, MA.

Ho, K., C. G. Nichols, W. J. Lederer, J. Lytton, P. M. Vassilev, M. V. Kanazirska, and S. C. Hebert. 1993. Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature (Lond.).* 362: 31–38.

Hodgkin, A. L., and B. Katz. 1949. The effect of Na ions on the electrical activity of the giant axon of the squid. *J. Physiol. (Lond.).* 108:37–77.

Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1990. Biophysical and molecular mechanisms of Shaker potassium channel inactivation. *Science (Wash. DC).* 250:533–538.

Kamb, A., L. E. Iverson, and M. A. Tanouye. 1987. Molecular characterization of *Shaker*, a *drosophila* gene that encodes a potassium channel. *Cell.* 50:405–413.

Kamb, A., J. Tseng-Crank, and M. Tanouye. 1988. Muliple products of the *Drosophila Shaker* gene may contribute to potassium channel diversity. *Neuron.* 1:421–430.

Kavanaugh, M. P., M. D. Varnum, P. B. Osborne, M. J. Christie, A. E. Busch, J. P. Adelman, and R. A. North. 1991. Interaction between tetraethylammonium and amino acid residues in the pore of cloned voltage-dependent potassium channels. *J. Biol. Chem.* 266:7583–7587.

Kavanaugh, M. P., R. S. Hurst, J. Yakel, M. D. Varnum, J. P. Adelman, and R. A. North. 1992. Multiple subunits of a voltage-dependent potassium channel contribute to the binding site for tetraethylammonium. *Neuron.* 8:493–497.

Kim, S., T. J. Smith, M. S. Shcpman, M. G. Rossmann, D. Pevear, F. J. Dutko, P. J. Felock, F. D. Diana, and M. A. McKinlay. 1989. Crystal structure of human rhinovirus serotype. *J. Mol. Biol.* 210:91–111.

Kirsch, G. E., J. A. Drewe, H. A. Hartmann, M. Taglialatela, J. de Biasi, A. M. Brown, and R. H. Joho. 1992. Differences between the deep pores of K^+ channels determined by an interacting pair of nonpolar amino acids. *Neuron.* 8:499–505.

Kubo, Y., T. J. Baldwin, Y. N. Yan, and L. Y. Jan. 1993a. Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature (Lond.).* 362:127–132.

Kubo, Y., E. Reuveny, P. A. Slesinger, Y. N. Jan, and L. Y. Jan. 1993b. Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature (Lond.).* 364:802–806.

Kumpf, R. A., and D. A. Dougherty. 1993. A mechanism for ion selectivity in potassium channels: computational studies of cation-π interactions. *Science (Wash. DC).* 261:1708–1710.

Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* 82:488–492.

Lopez, G. A., Y. N. Jan, and L. Y. Jan. 1994. Evidence that the S6 segment of the *Shaker* voltage-gated K^+ channel comprises part of the pore. *Nature (Lond.).* 367:179–182.

MacKinnon, R., P. H. Reinhart, and M. M. White. 1988. Charybdotoxin block of *Shaker* K^+ channels suggests that different types of K^+ channels share common structural features. *Neuron.* 1:997–1001.

MacKinnon, R., L. Heginbotham, and T. Abramson. 1990. Mapping the receptor site for charybdotoxin, a pore-blocking potassium channel inhibitor. *Neuron.* 5:767–771.

MacKinnon, R., and C. Miller. 1989. Mutant potassium channels with altered binding of charybdotoxin, a pore-blocking peptide inhibitor. *Science (Wash. DC).* 245:1382–1385.

MacKinnon, R., and G. Yellen. 1990. Mutations affecting TEA blockade and ion permeation in voltage-activated K^+ channels. *Science (Wash. DC).* 250:276–279.

Ovchinnikov, Yu. A., V. T. Ivanov, and A. M. Shkrob. 1974. Membrane-active complexones. Elsevier Scientific Publishing Co., New York. 93 pp.

Pak, M. D., K. Baker, M. Covarrubias, A. Butler, A. Ratcliffe, and L. Salkoff. 1991. mShal, a subfamily of A-type K^+ channel cloned from mammalian brain. *Proc. Natl. Acad. Sci. USA.* 88:4386–4390.

Pardo, L. A., S. H. Heinemann, H. Terlau, U. Ludewig, C. Lorra, O. Pongs, and W. Stuhmer. 1992. Extracellular K^+ specifically modulates a rat brain K^+ channel. *Proc. Natl. Acad. Sci. USA.* 89:2466–2470.

Perozo, E., R. MacKinnon, F. Bezanilla, and E. Stefani. 1993. Gating currents from a nonconducting mutant reveal open-closed conformations in Shaker K+ channels. *Neuron.* 11:353–358.

Pongs, O., N. Kecskemethy, R. Müller, I. Krah-Jentgens, A. Baumann, H. H. Kiltz, I. Canal, S. Llamazares, and A. Ferrus. 1988. *Shaker* encodes a family of putative potassium channel proteins in the nervous system of *Drosophila. EMBO J.* 7:1087–1096.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Schachtman, D. P, J. I. Schroeder, W. J. Lucas, J. A. Anderson, and R. F. Gaber. 1992. Expression of an inward-rectifying potassium channel by the *Arabidopsis* KAT1 cDNA. *Science (Wash. DC).* 258:1654–1658.

Sentenac, H., N. Bonneaud, M. Minet, F. Lacroute, J.-M. Salmon, F. Gaymard, and C. Gignon. 1992. Cloning and expression in yeast of a plant potassium ion transport system. *Science (Wash. DC).* 256:663–665.

Slesinger, P. A., Y. N. Jan, and L. Y. Jan. 1993. The S4-S5 loop contributes to the ion-selective pore of potassium channels. *Neuron.* 11:739–749.

Taglialatela, M., L. Toro, and E. Stefani. 1992. Novel voltage clamp to record small, fast currents from ion channels expressed in *Xenopus oocytes. Biophys. J.* 61:78–82.

Tempel, B. L., D. M. Papazian, T. L. Schwarz, Y. N. Jan, and L. Y. Jan. 1987. Sequence of a probable potassium channel component encoded at *Shaker* locus of *Drosophila. Science (Wash. DC).* 237:770–775.

Toney, M. D., E. Hohenester, S. W. Cowan, and J. N. Jansonius. 1993. Dialkylglycine decarboxylase structure: bifunctional active site and alkali metal sites. *Science (Wash. DC).* 261:756–759.

Warmke, J., R. Drysdale, and B. Ganetzky. 1991. A distinct potassium channel polypeptide encoded by the *Drosophila eag* locus. *Science (Wash. DC).* 252:1560–1562.

Wei, A., M. Covarrubias, A. Butler, K. Baker, M. Pak, and L. Salkoff. 1990. K^+ current diversity is produced by an extended gene family conserved in *Drosophila* and mouse. *Science.* 248:599–603.

Yellen, G., M. Jurman, T. Abramson, and R. MacKinnon. 1991. Mutations affecting internal TEA blockade identify the probable pore-forming region of a K^+ channel. *Science (Wash. DC).* 251:939–941.

Yokoyama, S., K. Imoto, T. Kawamura, H. Higashida, N. Iwabe, T. Miyata, and S. Numa. 1989. Potassium channels from NG108–15 neuroblastoma-glioma hybrid cells: primary structure and functional expression from cDNAs. *FEBS Lett.* 259:37–42.

Yool, A. J., and T. L. Schwarz. 1991. Alteration of ionic selectivity of a K^+ channel by mutation of the H5 region. *Nature (Lond.).* 349:700–704.