Anomalous Mole Fraction Effect Induced by Mutation of the H5 Pore Region in the *Shaker* K^+ Channel

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ABSTRACT Mutagenesis of the H5 region of the *Shaker* K^+ channel has provided strong evidence that these amino acids form a major portion of the ionic pore. We have previously observed that a single-site mutation (T441S) in this region increased the apparent relative permeability of the channel to NH_4^+. We now report that this increased relative permeability to NH_4^+ is sensitive to small changes in external K^+ in a pattern consistent with an anomalous mole fraction effect. The effect is not apparent in the wild-type channel. These findings, in combination with other studies showing effects of this particular mutation on the binding of tetraethylammonium and hydroxylamine, support the hypothesis that T441S alters the affinity of a putative ion binding site for NH_4^+ and ammonium derivatives. The mutation T441S alters ionic selectivity and reveals the multi-ion nature of the mutant *Shaker* K^+ channel.

INTRODUCTION

The anomalous mole fraction effect is a phenomenon that is observed in several types of ion channels, and is characterized by the ability of a mixture of two permeant ions to depress overall permeability of a channel to a value that is less than the permeabilities measured with either ionic species alone (review by Hagiwara, 1983). The occurrence of this phenomenon has been interpreted as evidence that channels form long multi-ion pores in which different species of permeant ions experience different binding site affinities and energy barriers; when more than one type of ion is present in the pore, a mutual interference during translocation is thought to result in the observed depression of overall permeability (Hille and Schwarz, 1978).

Decreases in permeability that characterize a mole fraction effect have been measured using permeability ratios as well as conductance (Hagiwara et al., 1977; Eisenman et al., 1986; Wagoner and Oxford, 1987; Shapiro and DeCoursey, 1991). Originally described in inward rectifiers (review by Hagiwara, 1983), this effect has also been observed in voltage-dependent Ca^{2+} channels (Hess and Tsien, 1984; Almers and McCleskey, 1984; Friel and Tsien, 1989), K^+ channels (Ashcroft and Stanfield, 1983; Plant, 1986; Eisenman et al., 1986; Wagoner and Oxford, 1987; Shapiro and DeCoursey, 1991; Heginbotham and MacKinnon, 1993; Perez-Cornejo and Begenisich, 1994), and other channel types. The present study describes a mole fraction effect on the apparent permeability ratio for NH_4^+ and K^+ that is dependent on the pore sequence of the *Shaker* K^+ channel.

The superfamily of voltage-gated channels is characterized by repeated structural motifs of six membrane-spanning regions (S1–S6) that assemble in tetrameric complexes to form an ion-selective pore across the membrane (review, MacKinnon, 1991). Evidence from all three of the principal classes of channels (Na^+, K^+, and Ca^{2+}) shows that the linker region between S5 and S6 (known as the H5, SS1-SS2, or P region) contributes to the pore lining sequence (review by Catterall, 1993). Mutations in the H5 region of the K^+ channel, or the equivalent regions of the other ion channels, have been demonstrated to alter a number of properties including conduction, selectivity, and sensitivity to pore-blocking compounds (review by Sather et al., 1994). Regions adjacent to H5 also contribute to the pore and its vestibules at either end, and from many studies it is clear that a number of amino acid sites can influence permeation properties in the K^+ channel (Kirsch et al., 1992; Slesinger et al., 1993; Taglialatela et al., 1994; Lopez et al., 1994).

Our initial studies of permeation focused on the mutation T441S in the H5 region and estimated that the reversal potential for NH_4^+ was shifted to a value substantially more positive than that for the wild-type channel, a finding indicative of a large increase in NH_4^+ permeability (Yool and Schwarz, 1991). This result was based on interpolation of the current-voltage plots generated from measurements of single-channel amplitudes in bi-ionic recording conditions that used 100 mM NH_4^+ in the external saline, and K^+ in the internal saline. Although the essential observation of enhanced relative permeability to NH_4^+ in the mutant T441S was correct, we have found that our original determination of the magnitude of the increase in permeability was an overestimate. Results reported here confirm that the mutation T441S in the H5 pore region of the *Shaker* channel increases the relative permeability of the channel to NH_4^+. The new data define the magnitude of the increase and provide evidence that the mutation T441S allows the expression of an anomalous mole fraction effect.

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MATERIALS AND METHODS

Wild-type and mutant T441S cDNA constructs of ShB spliced to the 5′ untranslated region of the *Xenopus* β-globin gene were linearized with *Eco*R1 and transcribed in vitro with T3 polymerase (Yool and Schwarz, 1991). Mutant T441S channels lacking N-type inactivation were prepared by deletion of the N-terminal region from amino acids 6 to 81, using existing restriction sites for *Sfi*I and *Rsr*II. Hybrid channels were created by linking polypeptides for the wild-type and T441S channels together; the cDNA for the hybrid channel was prepared by splicing the NH_2-terminal region of the T441S sequence (at the *Nco*I site, corresponding to the initiation methionine) to the COOH-terminal region of the wild-type sequence (at the *Pvu*I site, corresponding to amino acid 572). Oocytes prepared from *Xenopus laevis* were injected with 1–25 ng of mRNA in 50 nl of sterile water, and incubated at 18°C for at least 2 days before recording. Macropatch currents were recorded at 22–23°C, primarily in cell-attached patches. Inside-out patches also were used in some of the macropatch recordings, as specified in the Results. Electrodes (1–3 MΩ) were filled with external test salines. Test saline compositions: 100 mM of NH_4^+ or K^+ or a combination of both as chloride salts, 4.3 mM $MgCl_2$, 5.0 mM HEPES, pH 7.3. The bath saline was high K^+ (100 mM KCl, 4.3 mM $MgCl_2$, and 5 mM HEPES, pH 7.3) to provide a compatible internal milieu for inside-out patches and to hold the resting potential of the egg near 0 mV for cell-attached patches. The actual resting potential for control oocytes in bath saline with 100 mM K^+ was -7.7 ± 2.9 mV (mean ± SD, $n = 55$). This nonzero value indicates that the intracellular concentration of K^+ in the oocytes is ~134 mM; thus these cells are expected to give rise to reversal potentials near −8 mV in "symmetrical" K^+ recording conditions in the cell-attached configuration. Channels were activated with tail or ramp voltage protocols from holding potentials of −80 or −100 mV. Ramp protocols were initiated with an activation step to +40 mV, followed by a 35-ms ramp either from +40 to −160 mV, or from −160 to +40 mV. Reversal potentials measured by both ramp protocols in the same patches showed no difference. Longer ramp durations were impractical because of time-dependent channel inactivation and deactivation processes. Data were filtered at 2 kHz, and sampled at 10 kHz. Recordings were analyzed with pClamp software (Axon Instruments, Foster City, CA). Leak and capacitance were subtracted using P/2 or P/4 techniques.

RESULTS

Permeation of NH_4^+ and K^+ in wild-type and T441S mutant channels was assessed by examining reversal potentials in macropatches with two methods: ramped voltage protocols and tail current protocols. As shown below, both lines of evidence showed that T441S has an enhanced relative permeability to NH_4^+ that is influenced by a mole fraction effect in the presence of low concentrations of external K^+.

Ramped voltage analyses

Macroscopic currents that represented the combined activity of several hundred channels in a single patch could be recorded from the plasma membrane of *Xenopus* oocytes. These macropatches offered rapid control of voltage and a better signal-to-noise ratio than single-channel recordings.

Fig. 1 shows the current traces recorded from voltage ramps in macropatches of wild-type, T441S mutant, and hybrid channels from different oocytes. With NH_4^+ saline externally, the zero-current point is shifted to the left for the T441S mutant (Fig. 1, *C–E*), demonstrating a positive shift in the reversal potential as compared with the wild type (Fig. 1, *A* and *B*), which indicates a greater relative perme-

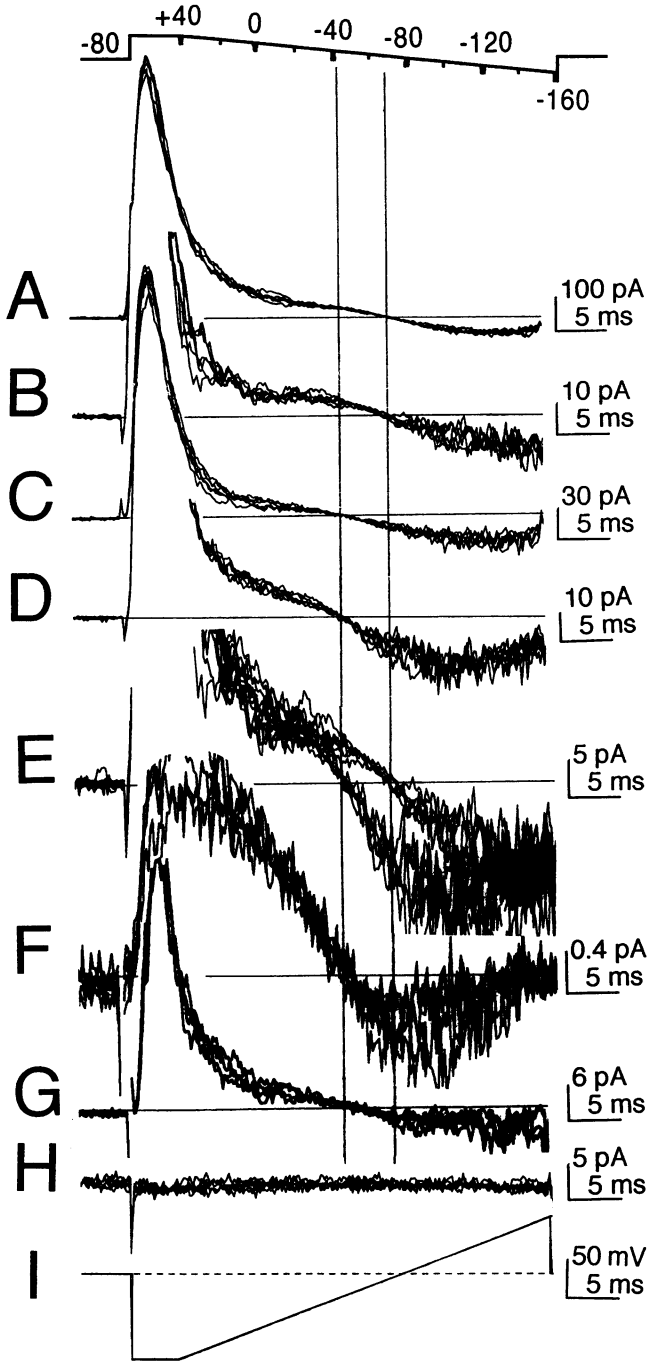

FIGURE 1 Voltage-ramp analyses of reversal potentials in wild-type and T441S mutant channels recorded in external NH_4^+ saline without added K^+. From a holding potential of −80 mV, the command potential was stepped to +40 mV for 5 ms to activate channels and then ramped from +40 to −160 mV in 35 ms (diagram at *top*). Superimposed traces are shown for wild-type (*A* and *B*), T441S (*C* and *D*), N-terminal-deleted T441S (*F*), and hybrid wild-type T441S (*G*). Traces from *B* and *D* are overlaid (*E*) at an expanded vertical scale. Peak outward currents in *B*, *D*, and *E* were truncated in constructing the figure. A sham-injected oocyte (*H*) recorded in the same conditions shows the negligible contribution of endogenous oocyte currents. Leak and capacitance were subtracted with a P/−2 protocol (holding potential −90 mV, step −150 mV for 5 ms, ramp from −150 to −50 mV for 35 ms). The voltage recorded during a ramp response with a large current is superimposed on the command potential to illustrate the close agreement (*I*).

ability to NH_4^+. Although inactivation and deactivation processes contribute differentially throughout the duration of the current ramp response, these variables are not expected to influence the determination of the reversal potential because the zero-current point is determined by the current solely through open channels, and because the presence of inward currents during the negative voltage range of the ramp shows that a sufficient number of channels remain active to allow the reversal potential measurement.

The removal of N-type inactivation in T441S by deletion of the amino terminal does not alter the permeability effect of the pore mutation (Fig. 1 *F*); the N-terminal-deleted form of T441S also shows an increased NH_4^+ permeability that is comparable to that seen in T441S channels with the fast inactivation process intact. The reversal potential of the N-terminal-deleted T441S in NH_4^+ saline was -46.8 ± 4.4 mV (mean ± SD; $n = 12$). Thus, the increased permeability to NH_4^+ correlates directly with the mutation T441S and not with indirect effects of inactivation during the ramp.

Hybrid channels were made in which the T441S mutant was linked in tandem with wild-type to form a polypeptide that is presumed to assemble into channels that are half wild-type and half T441S mutant (Fig. 1 *G*). These hybrids have a reversal potential in NH_4^+ saline (-56.3 ± 2.7; mean ± SD, $n = 4$) that is intermediate between the parent phenotypes. The intermediate value for the hybrid may suggest that all four 441 residues in the tetrameric channel contribute to the permeability properties of the channel.

In sham-injected oocytes (Fig. 1 *H*), recordings made by the same protocol show essentially flat responses, demonstrating that currents endogenous to the oocyte do not contribute appreciably to the membrane response to voltage ramps, and that the capacitance and leak subtraction protocol adequately compensates for passive membrane properties.

Fig. 2 shows a summary of the data obtained from the ramped voltage analyses, and illustrates the effect of external K^+ on reversal potential. Each measurement is the mean reversal potential averaged from ~10 or more ramp recordings for each patch. These data are represented in a box plot as a function of the ratio of concentrations of K^+ and NH_4^+ in the external saline (K^+/NH_4^+). The greatest difference between the reversal potential values measured for wild-type and T441S channels is seen in external NH_4^+ saline without added K^+ (K^+/NH_4^+ ratio ~0). In this condition, the mean reversal potential for the mutant is ~15 mV more positive than that of wild-type. The reversal potential of wild-type is -63.1 ± 4.2 (mean ± SD; $n = 29$) and of T441S mutant is -48.1 ± 4.8 ($n = 38$). A statistical analysis indicates that the difference between wild-type and T441S mutant channels is significant ($p < 0.01$, Wilcoxon rank test) for the NH_4^+ external saline without added K^+. The calculated permeability ratios P_{NH_4}/P_K are 0.11 for wild-type and 0.21 for T441S channels.

For wild-type channels, 14 of the 29 macropatches were recorded in the inside-out configuration and yielded a mean reversal potential of -61.9 ± 4.0 mV (mean ± SD). The 15 cell-attached patches yielded a mean value of -64.3 ± 3.9 mV. This difference is consistent with a higher internal K^+ concentration in the cell-attached configuration than in the inside-out configuration (see Methods), whereas the external NH_4^+ concentration is constant. For the mutant T441S channels, all of the recordings were from cell-attached patches. The mean reversal potential of -48 mV suggests an increase in relative NH_4^+ permeability in the mutant channel compared with the wild type. However, we can consider whether an alternative explanation (variation in the oocyte intracellular K^+ concentration) is sufficient to account for the depolarized reversal potential. In this alternative scheme, we assume that the permeability ratio (P_{NH_4}/P_K) of the mutant is 0.11, and calculate that the internal concentration of K^+ would be:

$$[K_{in}] = (P_{NH_4}/P_K)([NH_{4ex}])(10^{Vrev/-60})$$

$$= (0.11)(100\ mM)(10^{48/60}) = 70\ mM.$$

Thus an average $[K_{in}]$ value of ~70 mM is needed to account for a reversal potential of -48 mV. This explana-

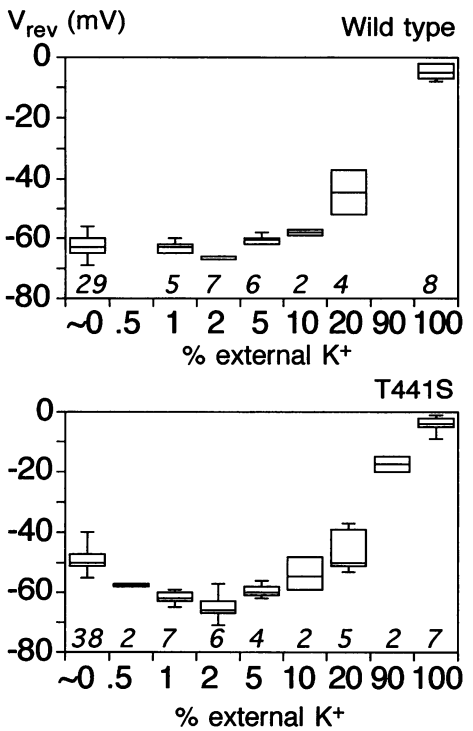

FIGURE 2 Summary of the reversal potentials measured by macropatch recordings with the voltage-ramp protocol. Data are presented as box plots for the wild type (*top panel*) and T441S (*bottom panel*), as a function of the percentage of external K^+ in the external saline (where NH_4^+ and K^+ total 100 mM, or 100%). The box plots show the mean (*center of box*), the median (*line within box*), the range occupied by 50% of the data points (*boundaries of the box*), and the full range of data (*error bars*). The number of patches in each group (n) is shown in italics above the x axis. The greatest difference between wild-type and T441S channels is evident in NH_4^+ saline with no added external K^+. With small concentrations of K^+ substituted for NH_4^+, the reversal potential in the T441S channels is depressed, creating an anomalous mole fraction effect. This effect is not evident for the wild-type channels.

tion for the positive shift in reversal potential in the mutant channel seems unlikely because it would require a large decrease in intracellular K^+ concentration specifically in those oocytes injected with the mutated channel. The most plausible explanation of the difference in reversal potential is that the apparent relative NH_4^+ permeability is increased in the T441S mutant.

We also have found that another permeant ion, Rb^+, appears to show a small shift in reversal potential that suggests an increased permeability in the T441S mutant channel, in agreement with previously published results (Yool and Schwarz, 1991). With 100 mM Rb^+ in the external saline, the reversal potential for the wild type is -19.7 ± 4.1 mV, and for the T441S mutant is -14.0 ± 1.4 mV (mean $\pm$ SD).

As is also shown in Fig. 2, a distinct anomalous mole fraction effect is observed for the T441S mutant when 1–5 mM external K^+ is present in the external NH_4^+ saline; the range and mean for the reversal potentials are shifted to values more negative than those seen with NH_4^+ or K^+ alone (~0 and 100% external K^+). The reversal potentials of wild-type channels, interestingly, do not show a mole fraction effect (Fig. 2; see also Heginbotham and MacKinnon, 1993). In the mutant, however, the mole fraction effect is sufficient to counteract the enhanced permeability of the T441S channel to NH_4^+, and causes the reversal potentials for the wild-type and mutant channels to be indistinguishable in the presence of mixed external cations.

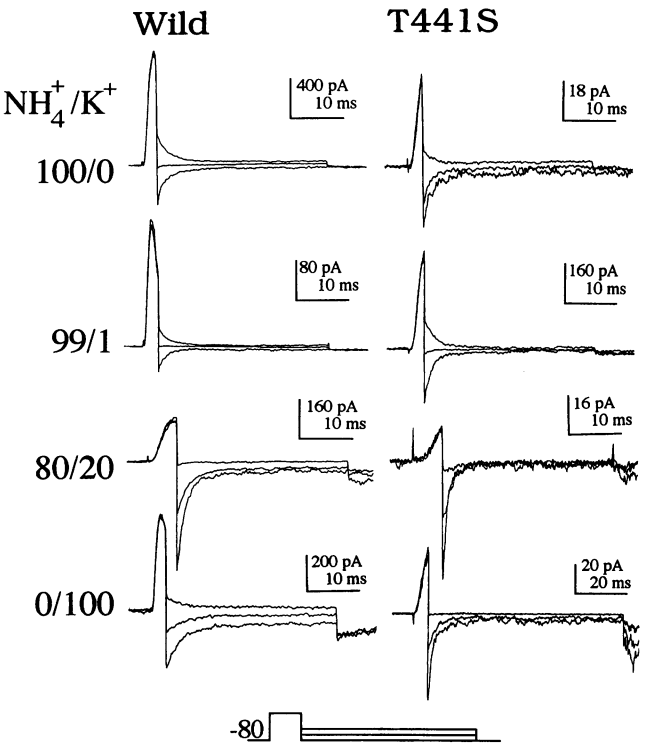

FIGURE 3 Tail currents recorded for wild-type and mutant T441S channels in macropatches with different ratios of NH_4^+ and K^+ in the external salines. Traces from eight different macropatches are shown for wild-type (*left*) and T441S (*right*) channels. The ratio of NH_4^+ to K^+ in the external saline is listed on the left (in mM). Selected steps illustrate tail currents near the reversal potential. In traces with NH_4^+ as the principal external cation (100/0, 99/1, 80/20), the tail steps were to -40, -60, and -80 mV. In high K^+ saline (0/100), the tail steps were to 0, -20, and -40 mV. The holding potential was -80 mV and the activation steps were $+40$ mV (for 100/0, 99/1 and 0/100) or -20 mV (for 80/20). A difference in reversal potential between wild-type and T441S channels is evident in the NH_4^+ external saline (100/0); at -60 mV the mutant shows inward current, whereas the wild type shows little net current. With 1 mM K^+ present (99/1), the reversal potentials of both wild-type and mutant channels are comparable. (Note the different time scale for T441S, 0/100.)

Tail current analyses

Tail currents after a brief activation step were measured in macropatch recordings over a range of voltages. Fig. 3 uses selected voltage steps to illustrate differences in the tail currents that were recorded from wild-type and T441S mutant channels. These tail currents demonstrate the altered reversal potential of T441S in external NH_4^+ saline without added K^+. Thus, whereas tail currents for the wild-type channel are at their reversal potential at -60 mV, T441S shows a clear inward tail at that potential. Addition of 1 mM K^+ to the external saline suppresses this difference because of the mole fraction effect, in a manner consistent with the results of the ramp experiments described above. The peak tail currents were standardized to the maximal outward current recorded during the activation step to $+40$ mV to account for differences in the number of channels present in different macropatches. Plotted against voltage, these tail currents show a positive shift in the reversal potential for T441S mutant channels in NH_4^+ saline, as compared with wild-type channels (Fig. 4 *A*). In tests with NH_4^+ saline for wild-type channels, 3 of 5 patches were in the cell-attached configuration and yielded interpolated reversal potential values of -58, -60, and -60 mV; the two patches in the inside-out configuration yielded values of -62 and -64 mV. In tests of NH_4^+ saline with the mutant T441S, all of the patches were in the cell-attached configuration. With 1 mM external K^+, no difference is apparent in the reversal potentials (Fig. 4 *B*).

The reversal potential values that were interpolated from third-order polynomial fits of the data are shown in Fig. 4 *C*. The mole fraction effect is observed with 1–5 mM external K^+, which depresses the reversal potential in the T441S mutant to a level similar to that of the wild type. A similar trend is suggested for the relative conductance of the inward tail currents (Fig. 4 *D*). The conductance values were measured from the slopes of linear fits of the peak tail current data (G), in a voltage range spanning 30 mV immediately below the reversal potential in each saline condition. The ranges of values overlapped, but suggested that the mutant T441S had on average a slightly higher conductance than wild-type in NH_4^+ saline; with 2 mM K^+ externally, the average conductance for the mutant was decreased to a level comparable to wild-type channel conductance.

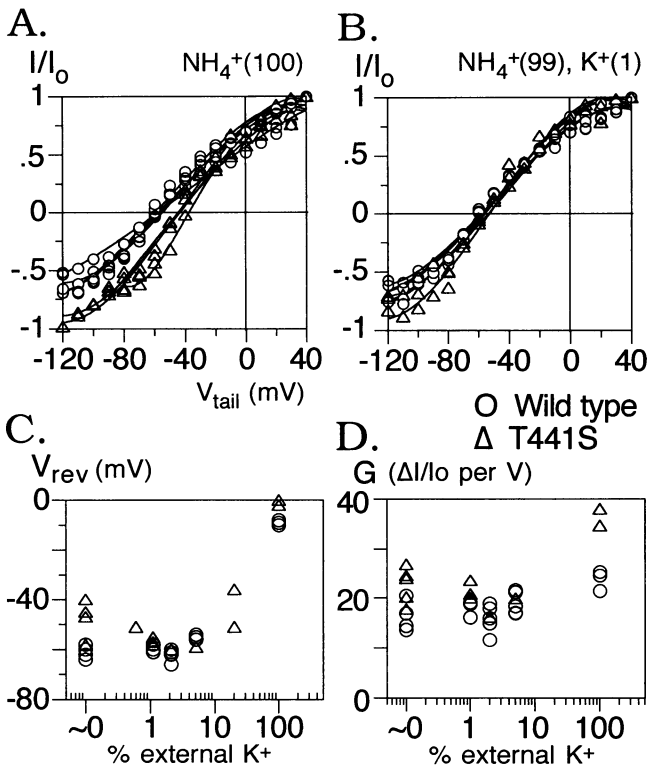

FIGURE 4 Tail currents recorded from wild-type and T441S mutant channels in macropatches. Current-voltage relationships show the peak tail currents recorded with 100 mM NH_4^+ external saline (*A*), or 99 mM NH_4^+ with 1 mM K^+ saline (*B*) for wild-type (○) and T441S (△) channels. Data were fitted by third order polynomials. Each curve represents a separate patch. (*C*) Reversal potentials were determined from the polynomial fits of the data, and are plotted as a function of the percentage of K^+ in the external saline, where NH_4^+ and K^+ total 100%. The *x* axis is shown on a log scale for clarity. (*D*) Relative conductances were determined from plots of peak tail current versus voltage in the different external K^+ and NH_4^+ salines. Values for conductance G were determined from the slope of linear fits of the I-V plots over a 30-mV range immediately below the reversal potential, and represent ΔI/Io per V.

DISCUSSION

In these experiments, the mutation T441S in the H5 region of the *Shaker* channel was used to investigate the significance of residue 441 for the permeation properties of the pore. Reversal potentials were determined by a voltage ramp method to minimize the uncertainties associated with curve fitting and interpolation, and to provide fast voltage control in a delimited membrane area. A high signal-to-noise ratio in macropatch recordings was obtained because the expressed *Shaker* channels vastly outnumbered the endogenous oocyte channels. The analysis of these ramp recordings allowed us to determine the effect of the T441S channel on apparent relative NH_4^+ permeability more precisely than in our previous estimate (Yool and Schwarz, 1991). The relative permeability ($P_{NH_4^+}/P_{K^+}$) for the wild-type channel is 0.11, and for the T441S channel 0.21.

In the background of an enhanced permeability to ammonium in the mutant T441S channel, we found that a mole fraction effect became evident in the permeability ratio of NH_4^+ and K^+. A similar effect was suggested from measurements of macroscopic slope conductance. The mole fraction effect was sufficient to counteract the enhanced permeability of the T441S channel to NH_4^+, and caused the reversal potentials for the wild-type and mutant channels to be indistinguishable in the presence of even a low concentration of external K^+. In the reversal potentials of wild-type channels we did not see this mole fraction effect (Fig. 2; see also Heginbotham and MacKinnon, 1993; Perez-Cornejo and Begenisich, 1994). In higher ionic strength solutions of K^+ and NH_4^+, however, a mole fraction effect on single-channel conductance has been described for wild-type channels (Heginbotham and MacKinnon, 1993). The dependence of a mole fraction effect on a specific amino acid side chain has precedent in studies of the CFTR (cystic fibrosis transmembrane conductance regulator) chloride channel; the mutation of arginine to aspartate alters halide selectivity and abolishes an anomalous mole fraction effect (Tabcharani et al., 1993).

In work by Heginbotham and colleagues (1994), the mutant T441S channel analyzed by voltage clamp did not show a difference in permeability to NH_4^+ as compared with wild-type. In general, it is conceivable that large or sustained outward currents could contribute to local increases in external K^+ concentration, and may suppress small differences in permeability because of mole fraction effects in sensitive channels. No mole fraction effect for the permeability ratio ($P_{NH_4^+}/P_{K^+}$) is evident for the wild-type channel, in agreement with other published results (Heginbotham and MacKinnon, 1993; Perez-Cornejo and Begenisich, 1994). The mole fraction effect on the permeability ratio in T441S channels is observed when small amounts of K^+ are mixed with NH_4^+ in the external saline, and is consistent with the idea that the *Shaker* K^+ channel is a multi-ion pore (Newland et al., 1992; Heginbotham and MacKinnon, 1993; Perez-Cornejo and Begenisich, 1994) and that the residue T441 interacts with permeating ions.

Many features of permeation can be modeled successfully with multiple barriers and binding sites, and a mole fraction effect can be explained as an interaction between ions that depends on the relative occupancy of the binding sites (Hille and Schwarz, 1978). Generalizations from a three-barrier two-well model of a voltage-dependent K^+ channel in squid axon indicate that both conductance magnitude and permeability ratios are sensitive to the barrier heights and the depths of the wells (Wagoner and Oxford, 1987). Depending on the energy profiles for transitions through the pore for each ionic species, there may be no anomalous effect, or an effect on conductance, reversal potential, or both. Anomalous effects on reversal potential, like those shown here, are known for inward rectifier, delayed rectifier, and Ca^{2+}-dependent K^+ channels, and for squid axon Na^+ channels (Hagiwara et al., 1977; Eisenman et al., 1986; Begenisich and Cahalan, 1980; Shapiro and DeCoursey, 1991).

The mole fraction effect described here has implications for the structure of the *Shaker* channel. Our data add to a growing list of results that supports the idea that this channel is a multi-ion pore (Newland et al., 1992; Heginbotham and MacKinnon, 1993; Perez-Cornejo and Begenisich, 1994). The mutation of threonine 441 to serine could act by altering either a barrier or a binding site in the permeation pathway. An effect on a binding site would be consistent with the results of other studies using ammonium derivatives; for example, the same mutation alters the sensitivity of the channel to block by internal tetraethylammonium (Yellen et al., 1991), and induces sensitivity to block by hydroxylamine and clofilium (Yool, 1994; Yool and Schwarz, 1995). By analogy with other studies (Hille and Schwarz, 1978; Hess and Tsien, 1984), our results suggest that the NH_4^+ ion may bind more strongly in the T441S channel than in the wild-type channel. Similarly, hydroxylamine appears to have higher binding affinity in the mutant channel than in the wild-type channel, as determined from a decrease in mean open time of T441S channels in the presence of the blocker (Yool and Schwarz, 1995).

In summary, our results provide additional support for the hypothesis that the *Shaker* K^+ channel is a multi-ion pore in which permeant ions can compete or interfere with one another during transit through the conduction pathway. The mutation T441S in the H5 segment increases permeability to NH_4^+ and causes a mole fraction effect in the permeability ratio. We can speculate that this residue contributes to a binding site (particularly for NH_4^+ and its derivatives), which is located near the internal mouth of the pore, and that it is one of a series of binding sites that permeant ions must traverse as they pass through the channel.

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