Interactions of the H5 Pore Region and Hydroxylamine with N-type Inactivation in the *Shaker* K^+ Channel

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ABSTRACT Mutations at sites in the H5 region of the *Shaker* B K^+ channel were used to analyze the influence of the pore on N-type inactivation. Single-channel and two-electrode voltage clamp analyses showed that mutations at residues T441 and T442, which are thought to lie at the internal mouth of the pore, produced opposite effects on inactivation: the inactivated state is stabilized by T441S and destabilized by T442S. In addition, an ammonium derivative, hydroxylamine ($OH—(NH_3)^+$), appears to bind in the pore region of T441S and further decreases the rate of recovery from N-type inactivation. This effect relies on the presence of the amino-terminal. The effect of hydroxylamine on the T441S mutation of this K^+ channel shows several properties analogous to those of local anesthetics on the Na^+ channel. These results can be interpreted to suggest that part of the H5 region contributes to the receptor for the inactivation particle and that a hydroxylamine ion trapped near that site can stabilize their interaction.

INTRODUCTION

The duration of ion channel openings can be limited by an intrinsic inactivation mechanism that is a crucial feature of Na^+ channels and many K^+ and Ca^{2+} channels (Hille, 1992). The voltage-dependent K^+ channels encoded by the alternatively spliced *Shaker* gene (Tempel et al., 1987; Iverson et al., 1988; Schwarz et al., 1988; Timpe et al., 1988) have at least two distinct mechanisms of inactivation (Choi et al., 1991; Hoshi et al., 1991). The molecular mechanism of the typically faster component (N-type inactivation) is thought to involve the physical plugging of the cytoplasmic side of the channel by an "inactivation particle" comprising the amino terminal (Hoshi et al., 1990; Zagotta et al., 1990), and follows the conceptual model of the "ball and chain" process first suggested for the Na^+ channel (Armstrong and Bezanilla, 1977). Two regions in the first 20 amino acids determine the effectiveness of the inactivation particle (ball) (Hoshi et al., 1990). Synthetic peptides identical to the wild-type amino terminal sequence restore inactivation in N-terminal deleted mutants (Zagotta et al., 1990). These results demonstrate that the N-terminal is necessary for the typically fast inactivation process in *Shaker*.

The N-terminal, and therefore the putative receptor for the inactivation particle, lies on the cytoplasmic side of the membrane (Tempel et al., 1987; Zagotta et al., 1990). This is consistent with substantial physiological evidence linking fast inactivation with the internal mouth of the pore (Armstrong, 1971; Hoshi et al., 1990; Demo and Yellen, 1991; Solano and Lingle, 1992). One region that is thought to contribute to the inactivation particle receptor is the sequence that links the S4 and S5 transmembrane domains. Site-directed mutagenesis of the S4-S5 linker influences the rate of N-type inactivation and suggests that this region forms part of the internal vestibule, which is envisioned as a widening of the internal mouth of the pore (Isacoff et al., 1991).

Results presented here show that a part of the H5 segment strongly influences N-type inactivation. H5 is a major determinant of pharmacological and physiological properties of the pore. The H5 region extends from D431 to T449 in the *Shaker* B channel and is highly conserved among the many cloned K^+ channels (Jan and Jan, 1990). Mutations and chimeric substitutions in this region have been shown to influence the selectivity of the channel (Yool and Schwarz, 1991; Heginbotham et al., 1992), unit conductance (Hartmann et al., 1991), external binding of TEA and charybdotoxin (MacKinnon and Yellen, 1990; MacKinnon and Miller, 1989), and the binding of internally applied TEA (Yellen et al., 1991; Hartmann et al., 1991). The ability of mutations in H5 to alter sites associated with pore properties on both sides of the membrane supports the hypothesis that this region spans the narrow part of the pore. Other regions may also contribute to the pore-lining (Slesinger et al., 1993).

We have measured effects on inactivation and recovery from inactivation resulting from mutations in H5. The stabilizing effect of the mutation T441S on inactivation is increased by a simple ammonium derivative, hydroxylamine. A generally similar mechanism of action for local anesthetics on the inactivation of Na^+ channels was proposed two decades ago (Hille, 1977). We report here the ability of hydroxylamine to produce a local anesthetic-like block of the K^+ channel, and explore the interactions of hydroxylamine with the H5 mutations.

MATERIALS AND METHODS

Site-directed mutations were generated in the *Shaker* B gene, in a commercial plasmid vector (Bluescript KS) modified by the substitution of the 3′-untranslated region of *Xenopus* β-globin to enhance expression in

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oocytes, as described previously (Yool and Schwarz, 1991). The deletion of N-terminal amino acids 9–81 was accomplished with restriction sites for *Sfi*I and *Rsr*II and an 8-base oligonucleotide to bridge the splice site and maintain the open reading frame. Mutations were confirmed by DNA sequencing. To synthesize RNA in vitro, wild-type and mutant cDNA constructs were linearized by *Eco*RI in the 3′ polylinker and transcribed with T3 RNA polymerase. Oocytes from anesthetized mature female *Xenopus laevis* were obtained by the surgical removal of several lobes of ovary and prepared by techniques similar to those published previously (Zagotta et al., 1989). Follicular cell layers were removed by treatment with Sigma collagenase type IA at 1.5 mg/ml for 2–3 h in OR2 medium (NaCl 82 mM; KCl 2.5 mM; $MgCl_2$ 1.0 mM; HEPES 5 mM, pH 7.6). Prepared oocytes were injected with 10–25 ng of RNA in 50 nl of sterile water, and incubated 2 or more days at 18°C in ND96 medium (NaCl 96 mM; KCl 2.0 mM; $CaCl_2$ 1.8 mM; $MgCl_2$ 1.0 mM, HEPES 5 mM, pH 7.6) before recording. The ND96 and OR2 media contained the antibiotics penicillin (100 units/ml) and streptomycin (100 μg/ml) without Na pyruvate.

Single-channel activity was recorded at room temperature (22–23°C) in cell-attached and inside-out patches with electrodes (2–3 MΩ, VWR glass micropipettes) filled with test salines. Recording saline compositions were 100 mM of the Cl^- salt of the test ion, 4.3 mM $MgCl_2$, and 5 mM HEPES, pH 7.3. Either Na^+ or K^+ saline was used in the bath, depending on the recording configuration. Calcium was omitted from the recording salines (Zagotta et al., 1989) to eliminate the calcium-dependent Cl^- current endogenous to oocytes (Barish, 1983). Two-electrode voltage clamp (TEVC) recordings, also done at room temperature, used a virtual ground, a bath headstage, and two intracellular electrodes (0.5–1.5 MΩ) filled with 3 M KCl, and the same recording salines specified above. Hydroxylamine-containing salines were made by equimolar substitution of hydroxylamine for Na^+ or K^+. The pH of the substituted solutions was adjusted to 7.3. Bath saline was changed by perfusion with a volume ≥5 times that of the recording chamber; excess saline was removed by aspiration. For T441S, the average holding current before perfusion of 10–20 mM hydroxylamine was 33 ± 14 nA, and after perfusion it was 44 ± 13 nA (n = 9); before perfusion of 100 mM hydroxylamine it was 57 ± 21 nA, and after perfusion it was 63 ± 32 nA (n = 6). These leak currents were similar for oocytes expressing wild type or N-terminal-deleted T441S. A small increase also was observed on perfusion of control Na^+ saline (from 43 ± 26 nA before to 50 ± 26 nA after perfusion, n = 6), indicating that some change is due simply to mechanical effects. The leak is trivial in comparison to the μA scale of *Shaker* currents and was easily removed by the capacitance-leak subtraction protocol. In all recordings, the voltage-gated K^+ channels were activated with step protocols from holding potentials of −80 or −100 mV. Data were recorded with an AxoPatch or AxoClamp (Axon Instruments, Foster City, CA), filtered at 2 kHz (8-pole Bessel, Frequency Devices, Haverhill, MA), digitized at 100–200 μs (in all cases except for TEVC traces greater than 100 ms in duration, for which the sampling rate was slower), and stored on a Dell computer hard disk for subsequent analysis with pClamp software (Axon Instruments). Macroscopic inactivation rates were fit with two exponentials using least-squares regression to determine amplitude and the simplex method to determine time constants (reviewed by Motulsky and Ransnas, 1987), as provided by the pClamp Clampfit software. The fitting range was set from the position of the peak current amplitude to the end of the depolarizing step. Fast time constants for inactivation were determined from traces 100–200 ms in duration by fixing the slow time constant at 500 ms. Slow time constants were determined from traces 4–8 s in duration with the fast time constant fixed at 10 ms. This method provided reproducible fits that matched the data well, and avoided problems associated with mismatches between the scales of the time constants and the fitting ranges.

RESULTS

The mutation T442S antagonizes inactivation

The mutation of threonine to serine at T442 dramatically alters the inactivation of the ShB channel in a manner that is not matched by a similar mutation (T441S) at an adjacent site (Fig. 1). Macroscopic inactivation was studied by two-electrode voltage clamp in 100 mM Na^+, 0 mM K^+ bath saline at 23 ± 0.5°C. In wild type and the mutant T441S, inactivation decreases the current amplitude by 80–85% during a 100 ms step depolarization to +40 mV, whereas inactivation in the mutant T442S accounts for only 12% of the amplitude in the same conditions (Fig. 1 *A*). Macroscopic inactivation is described by two time constants, fast and slow. The time constant of the fast component at +40 mV is slightly altered for T442S as compared with wild type and T441S; the fast time constants (mean ± SD) are 6.9 ± 0.8 ms (n = 11) for T442S, 4.3 ± 0.3 ms for wild type (n = 8), and 4.2 ± 0.2 ms for T441S (n = 17). The slow component that dominates the inactivation of the T442S channel has a time constant (271 ± 44 ms, n = 4) that is similar that observed for the N-terminal-deleted ShB channel (267 ± 54 ms, n = 6), which lacks amino acids 6–46. Thus, much of the inactivation observed in T442S may be attributed to the slower C-type mechanism (Hoshi et al., 1991). This idea is supported by the observation that 30 mM external tetraethylammonium (TEA) lengthens the slow component of inactivation in T442S (Fig. 1 *C*); external TEA (10–30 mM) has been shown to compete with C-type inactivation in N-terminal-deleted *Shaker* channels (Choi et al., 1991). However, the T442S mutant retains a small fast component of inactivation, and in this respect is unlike the N-terminal-deleted wild-type channel for which the current rises to a flat plateau before C-type inactivation begins. In T442S, this residual fast component and the large steady-state current suggest that the N-terminal inactivation particle is able to bind, but the proportion of inactivated channels is reduced.

The reduction of fast inactivation in T442S is borne out by inspection of single-channel activity in cell-attached patches (Fig. 1 *B*). Unlike wild-type and T441S channels whose openings are clustered near the beginning of steps to +40 mV, the activity of T442S channels continues throughout the sweep. In wild type, the inactivated state is represented in single-channel records as a long-lived, nonconducting state that separates bursts of openings and is easily distinguished from the short closed state that occurs within bursts (Hoshi et al., 1990). The ragged traces in Fig. 1 *B* are characteristic of T442S but not wild-type or T441S channels. The basis of this channel behavior has not been studied. Both mutants, T441S and T442S, show decreased conductances as compared with wild-type channels; this observation supports the hypothesis that these sites are located near the ion conduction pathway. The voltage-dependent activation processes of wild type and T441S are similar, but a shift of approximately −15 mV in the midpoint of the conductance-voltage curve is observed for T442S (Fig. 1 *D*).

The mutation T441S slows recovery from inactivation

The consequences of the mutation T441S on inactivation in the *Shaker* channel are not obvious from a comparison of responses to simple depolarizing steps (as in Fig. 1 *A*); T441S and wild-type currents look similar in macroscopic activation

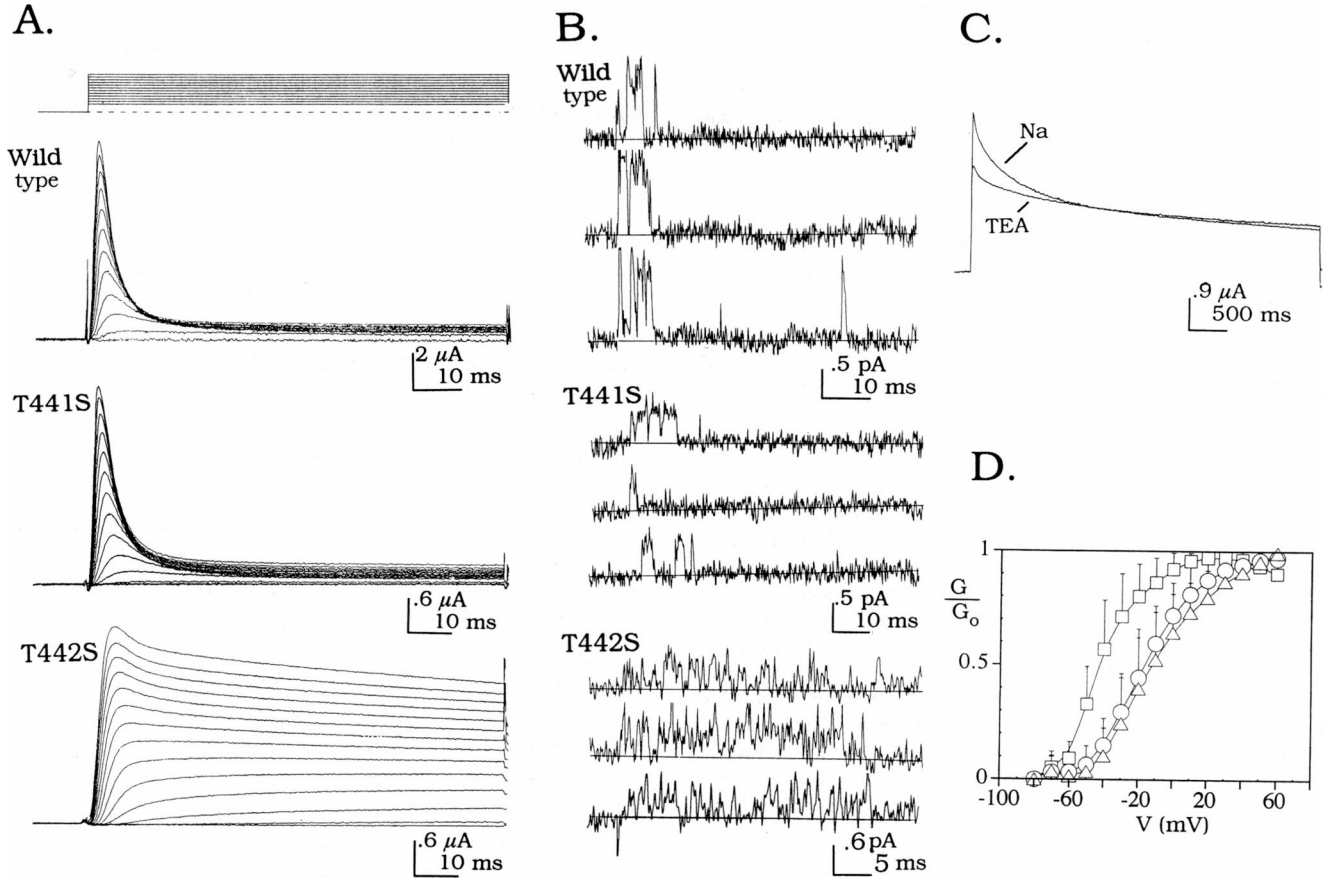


FIGURE 1 Macroscopic currents for wild-type and mutants of *Shaker* B. (*A*) Two-electrode voltage clamp recordings are shown for wild type, T441S, and T442S in 100 mM Na^+ bath saline. The traces illustrate the normal activation and inactivation of wild type and T441S, and the much slower inactivation of T442S. Voltage steps (+60 mV; increment −10 mV) were applied at 11 s interpulse intervals. Leak and capacitance were subtracted using P/4 or P/5 techniques. Holding potentials were −80 mV except for T442S, which was recorded from a holding potential of −100 mV to speed deactivation. (*B*) Single-channel records at voltage steps to +40 mV are shown for cell-attached patches containing wild-type, T441S, and T442S channels. The records illustrate initial bursts of activity followed by inactivation for wild-type and T441S channels, and prolonged activity associated with the lack of inactivation of T442S. The ragged traces are characteristic of T442S channels; the basis of this behavior is unknown. Voltage steps to +40 mV were applied from a holding potential of −100 mV with 100 mM Na^+ saline in the pipette. Leak and capacitance were subtracted using blank records. (*C*) Slow inactivation of T442S is antagonized by external tetraethylammonium (TEA) at 30 mM added as a chloride salt to standard Na^+ bath saline. The crossover in the control and TEA traces indicates that external TEA competes with the slow inactivation and suggests that this process is similar to the C-type inactivation of wild-type *Shaker* channels (see text). The voltage steps are −20 mV, for traces are superimposed from the same oocyte to show the current after perfusion of 30 mM TEA (TEA) and after recovery by perfusion with control Na^+ bath saline (Na). (*D*) Conductance-voltage plots for wild-type (○), T441S (△), and T442S (□) in Na^+ saline. Conductance (G) was calculated from peak current assuming a reversal potential of −80 mV, and was standardized to the maximal conductance (G_o). Data are mean ± SD; for n = 10 (wild type), 16 (T441S), and 12 (T442S).

and inactivation properties. Small variations in macroscopic inactivation rates and steady-state levels for wild type and T441S were apparent among different oocytes but did not show any consistent differences. However, in the double-pulse protocol used to measure the recovery of channels from inactivation, an effect of T441S on inactivation appears that contrasts diametrically with that of T442S. Although the wild-type ShB channel recovers rapidly at −80 mV from N-type inactivation (Fig. 2) with a time constant of 14.3 ± 0.5 ms, recovery in the same conditions (external Na^+ saline) is slower for T441S, with a time constant of 24.0 ± 0.8 ms (see Fig. 6 for graphs of mean ± SD). In addition, a slower component of recovery was apparent for T441S (Figs. 2 and 6). The use of a short conditioning step (40 ms) produces little C-type inactivation and allows the analysis of recovery from primarily N-type inactivation. In contrast to T442S, which appears to destabilize the inactivated state, the mutation T441S appears to enhance inactivation in that it decreases the rate of return of channels from the inactivated state. A slowing of recovery from inactivation might have given rise to a slowing of the macroscopic inactivation in T441S, but this was not observed.

Hydroxylamine: interactions with the *Shaker* channel

The analysis of inactivation in the mutant T441S was facilitated by an observation that hydroxylamine strongly influences the behavior of this channel. Hydroxylamine affects conductance and creates a dramatic effect on inactivation in

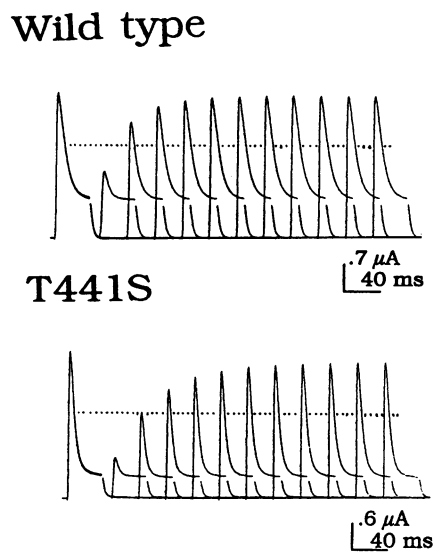


FIGURE 2 Recovery from N-type inactivation is slower for T441S than for wild type. Recovery from fast inactivation was measured with a double-pulse protocol for wild type and T441S in Na^+ bath saline. The short step durations provided substantial fast (N-type) inactivation while minimizing the development of slow (C-type) inactivation. The conditioning and test pulses each were +40 mV for a duration of 40 ms; the recovery potential was −80 mV for varied durations; the interpulse interval was 11 s.

T441S channels. An ion used previously to probe the dimensions of the Na^+ channel pore (Hille, 1975), hydroxylamine, also is permeant, although poorly, through the wild-type and mutant ShB K^+ channels (as determined from inward tail currents at voltages below −100 mV in macropatch recordings with 100 mM external hydroxylamine; not shown). In single-channel studies of uninjected oocytes, we have found that the charged form of hydroxylamine carries an inward current, although during two-electrode voltage clamp, the contribution of an inward hydroxylamine current was not obvious. Given its ability to deprotonate, hydroxylamine also is predicted to cross the lipid bilayer directly in the neutral form. If this compound (pK_a 8) entered the cell only in the neutral form, an alkalization of the cytoplasmic pH would be predicted. This effect should be mitigated by the ability of the ion to cross the membrane in the charged form. We have observed that macroscopic currents in patches that have been excised into pH-buffered hydroxylamine saline show the same effects as described for the whole cell currents, indicating that possible changes in cytoplasmic pH do not account for the effects of hydroxylamine. Hydroxylamine did not alter the leak significantly (see Materials and Methods).

In the following sections, we will show that the effects of hydroxylamine on two properties of the *Shaker* channel, conduction and inactivation, depend on the nature of site 441. With regard to conduction, hydroxylamine causes transient pore block in T441S, as determined from a decrease in mean open time (Fig. 3). With regard to inactivation, hydroxylamine further stabilizes fast inactivation in T441S (Figs. 4–7). In certain stimulation paradigms (prolonged depolar-

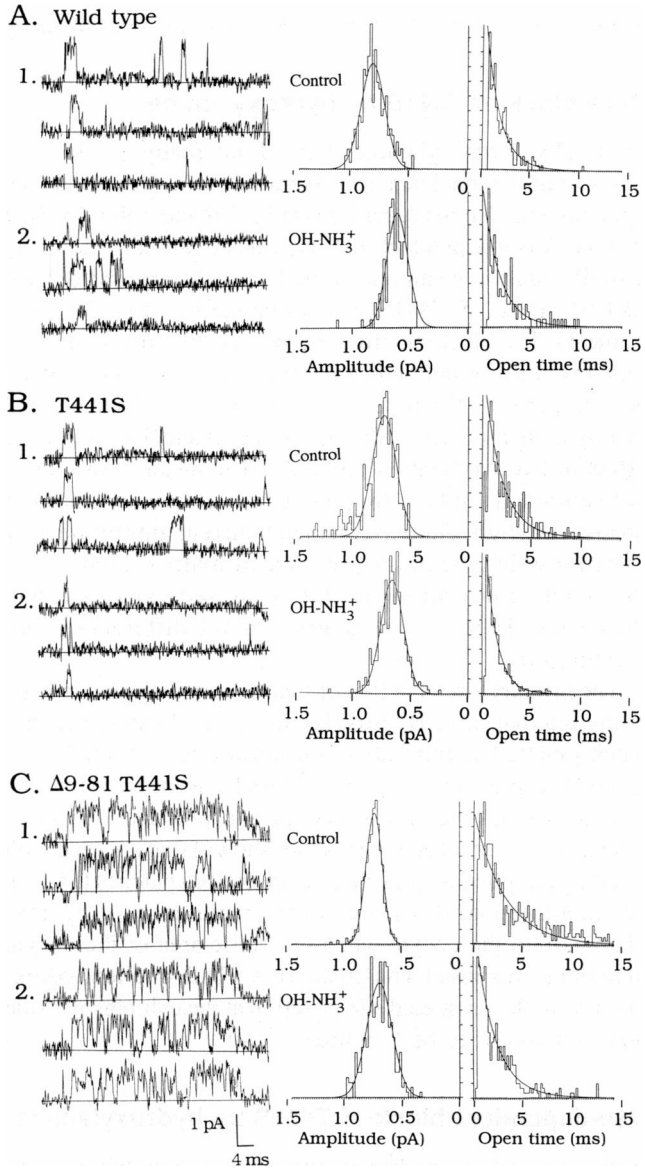


FIGURE 3 Effects of internally applied hydroxylamine on single-channel amplitude and open-time for wild-type and mutant channels. Channel activity elicited by voltage steps to 0 mV (*left* panels) is shown for (*A*) wild-type, (*B*) T441S, and (*C*) (Δ9–81)-T441S channels, which lack fast inactivation. Traces were recorded with Na^+ saline in the pipette and (1) control K^+ bath saline, or (2) 10 mM hydroxylamine in K^+ bath saline. Hydroxylamine causes a decrease in open time for T441S and (Δ9–81)-T441S channels. Histograms and frequency distributions for single-channel data are shown in the right panels, constructed from patches with the number of events indicated below

Preparation	Saline amplitide	Open time (# Events)	(# Events)
Wild type	Control	213	213
	$(OHNH_3)^+$	153	125
T441S	Control	269	251
	$(OHNH_3)^+$	367	355
(Δ9–81)-T441S	Control	595	553
	$(OHNH_3)^+$	328	337

Mean amplitude and open time values, combined for multiple patches, are presented in Table 1.

izations), the effect on inactivation has the attendant consequence of a virtually irreversible use-dependent block.

Pore block of T441S by hydroxylamine

In T441S, hydroxylamine applied internally produces a block of the channel that is measurable as a decrease in mean open time in channel recordings (Fig. 3 *B* and Table 1). That this block is independent of N-type inactivation is shown by a similar shift in mean open time for the N-terminal-deleted (Δ9–81) form of T441S in hydroxylamine (Fig. 3 *C* and Table 1). The blocking effect occurs whether the amino terminal is intact or not, indicating that the open channel block is a property of the pore region. In both conductance and inactivation properties, the wild-type channel shows little effect of hydroxylamine (Fig. 3 *A*). The mean open time is not shortened, and the frequency of blank traces does not change. A slight decrease in mean current amplitude may exist for wild type in the presence of hydroxylamine, but the standard deviations for the measured values overlap the means (Table 1) and suggest that the difference is not meaningful.

The primary site of action of hydroxylamine appears to be at the internal face. Hydroxylamine applied externally in a patch pipette has little effect on inward current amplitude. Yet bath-applied hydroxylamine does have access to the presumed cytoplasmic site of action, and produces the same effects in cell-attached patches (not shown) that are seen with internally applied hydroxylamine in excised patches (Fig. 3). We presume that this is due to the rapid entry of hydroxylamine into the oocyte when the entire area of the oocyte membrane is exposed. Thus, the access of bath-applied hydroxylamine to the presumed site of action at the cytoplasmic side does not appear to be a limiting factor.

Use-dependent block of T441S by hydroxylamine

In the presence of hydroxylamine, successive depolarizing steps produce a progressive decrease in the peak amplitude of T441S currents, but not in wild-type currents (Figs. 4 *A1* and 5 *A*). The development by hydroxylamine of this irreversible type of block in T441S is use-dependent; it requires activation of the channels by depolarization and is not simply a function of time of exposure. For example, in Fig. 4 *A1*, traces are superimposed for steps before and after the introduction of hydroxylamine. During the period of ~1.5 min of exposure to hydroxylamine before the second trace, depolarizing pulses to +40 mV were applied every 11 s and elicited use-dependent block in T441S. In contrast, no decline in T441S current amplitude occurs in hydroxylamine in the absence of depolarizing pulses. The first current evoked in hydroxylamine after a 5 min period of inactivity at −80 mV is unchanged in amplitude from the last control response (Fig. 4 *A2*), and the decline in amplitude resumes with the initiation of repeated depolarizations (Fig. 5 *B*). Thus, this irreversible type of block develops during the open or inactivated state, but not during the closed state of the T441S channel.

The rate of decline in peak current for T441S in hydroxylamine depends on the duration of the depolarizing steps at +40 mV (Fig. 5 *A*). The analyses of block described above use step durations of 100 ms. For shorter depolarizations (20–50 ms), irreversible block develops much more slowly. Thus, shorter depolarizing steps provide a useful means for assessing other effects of hydroxylamine (as in tests of recovery from inactivation and prepulse inactivation; see below) with minimal involvement of irreversible block. For very prolonged depolarizations at +40 mV (>1 s), a substantial decline is achieved after a single pulse. We have referred to this use-dependent blocked state as irreversible because (i) currents cannot be recovered with very long interpulse intervals (10 min or more) or with strongly hyperpolarized holding potentials (−100 to −140 mV), and (ii) channels remained blocked for at least several h after the removal of hydroxylamine from the bath. At less depolarized potentials, irreversible block develops much more slowly (see results of prepulse inactivation tests below).

Wild-type channels display no use-dependent block by hydroxylamine. In fact, wild-type currents remain stable or increase slightly with the introduction of hydroxylamine into the Na^+ bath saline (Fig. 4 *A1* and *A2*). The difference between wild type and T441S is not an indirect result of a difference in the ability of hydroxylamine to access the internal surface of the channel. For macroscopic currents from inside-out patches (Fig. 4 *B*), hydroxylamine perfused into the bath during the recording does not block wild type, but does cause use-dependent block of T441S. Even with extremely long depolarizations block does not occur in wild-type channels; multiple traces superimposed for 8000 ms steps before and after perfusion with 100 mM hydroxylamine show no difference in magnitude or kinetics (not shown). Thus, the apparent insensitivity of wild-type channels is not simply a difference in access of hydroxylamine to an internal site or the amount of inactivation achieved in the test pulses. These results confirm that the use-dependent block observed in T441S is a consequence of state-dependent changes in the

TABLE 1 Single channel effects of internally applied hydroxylamine

Parameter*	$[OHNH_3^+]_{in}$ (mM)	Wild	T441S	Δ9–81 T441S
Amplitude (pA)	0	0.91 ± 0.2	0.68 ± 0.1	0.68 ± 0.1
	10	0.91 ± 0.1	0.61 ± 0.1	0.69 ± 0.1
Mean open time (ms)	0	1.3 ± 0.2	1.6 ± 0.2	1.7 ± 0.1
	10	1.5 ± 0.1	0.85 ± 0.04	0.87 ± 0.05
Burst duration‡ (ms)	0	2.5 ± 0.6	2.1 ± 0.4	N.A.
	10	2.2 ± 0.3	2.2 ± 0.3	N.A.
Latency (ms)	0	1.7 ± 0.3	1.8 ± 0.2	3.3 ± 0.1
	10	1.9 ± 0.2	1.9 ± 0.2	3.0 ± 0.2

* Single-channel data from inside-out patches with Na^+ saline in the pipette, and K^+ saline (with and without hydroxylamine) in the bath, analyzed for voltage steps to 0 mV; see legend.

‡ N.A., not analyzed. For Δ9–81-T441S, bursts often extended to the end of the voltage pulse; frequency distributions were not constructed from these data.

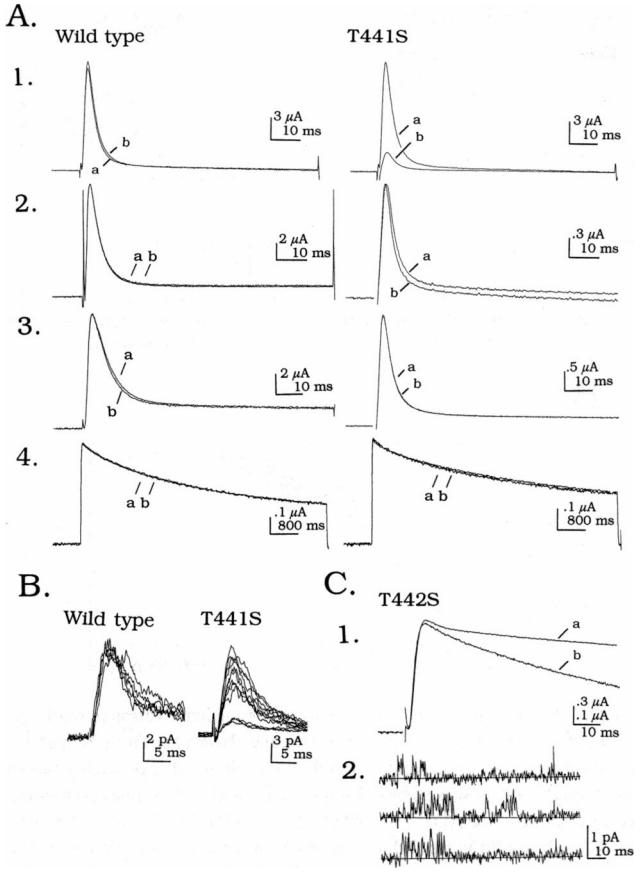


FIGURE 4 Effects of hydroxylamine on macroscopic properties of wild-type, T441S, and T442S currents. (*A*) Traces from steps to +40 mV are shown for wild-type and T441S currents. For each experiment (1–4), control Na^+ saline (a) was followed by perfusion with saline containing hydroxylamine substituted for Na^+ (b). (1) Hydroxylamine at 10 mM caused use-dependent block of T441S currents. Depolarizing steps were made at 11 s intervals before (a) and during perfusion with hydroxylamine. In contrast to wild-type currents that remained stable indefinitely, T441S currents declined progressively with each step. By the ninth pulse (b), taken 100 s after the addition of hydroxylamine, the amplitude of the current was decreased to 15% of control. (2) The block of T441S by 10 mM hydroxylamine did not develop in the absence of depolarizing steps. The test traces (b) were taken 5 min after perfusion with hydroxylamine; during this exposure period, the membrane was held continuously at −80 mV. Unlike T441S subjected to repetitive depolarizations as in (1), no decline in the peak current amplitude was observed. (3) External K^+ prevented the use-dependent block by10 mM hydroxylamine. The experiment was done as in (1) but with 10 mM K^+ substituted for Na^+. (4) The effect of hydroxylamine on T441S required the presence of the N-terminal inactivation particle. Long depolarizing steps (8 s) showed that 100 mM hydroxylamine exerted no blocking effect on (Δ9–81)-wild-type or on (Δ9–81)-T441S currents. Consecutive traces at 1 min intervals showed no differences in amplitude and kinetics, except for a slight increase in amplitude for (Δ9–81)-T441S after perfusion. (*B*) Macropatch currents were recorded in inside-out patches with internally applied 10 mM hydroxylamine (in K^+ bath saline, pH 7.3) for wild-type and T441S channels. Each record shows two sets of superimposed traces: one set recorded immediately after perfusion, and the second set after 3 min of repetitive depolarizing pulses. Progressive block occurred for T441S but not wild-type currents, and indicated that the difference in susceptibility was not an indirect effect of differences in access of the cation to the putative internal binding site. Voltage steps were from −80 to +40 mV for wild type, and to 0 mV for T441S. Depolarizing steps were 100 ms in duration; only the initial 20 ms are shown. (*C*) Inactivation in the mutant T442S was increased in the presence of hydroxylamine. (1) Traces illustrate currents evoked by steps to +40 mV from a holding potential of −100 mV in (a) 100 mM Na^+; and (b) 100 mM hydroxylamine. (2) Single-channel records of T442S in an inside-out patch exposed to 10 mM hydroxylamine in K^+ bath saline showed the occurrence of bursts of activity followed by inactivation, in contrast to control records (see Fig. 1 *B*) that showed prolonged bursts of activity with little inactivation.

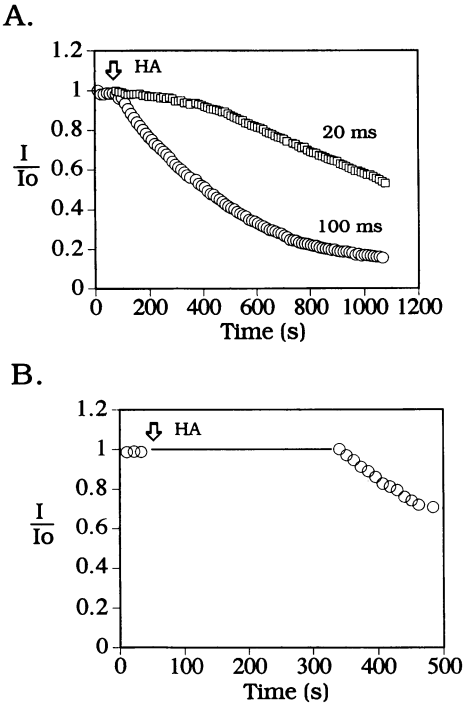


FIGURE 5 Use-dependent effect of hydroxylamine on T441S. (*A*) Currents were evoked at +40 mV for steps of 20 ms (□) or 100 ms (○) in duration. Steps were applied once per 11 s from a holding potential of −80 mV. Data points indicate peak current amplitudes standardized to the initial control response (I_o); different oocytes were used for each series. Hydroxylamine at 10 mM in Na^+ bath saline was perfused in at the time indicated by the arrow, and remained present through the remainder of the experiment. (*B*) Currents were evoked with +40 mV steps, 100 ms in duration, as described in *A* except that the oocyte was held at −80 mV (——) during and after the perfusion of 10 mM hydroxylamine (*arrow*). Test steps were resumed after 5 min. Use-dependent block by hydroxylamine does not occur in T441S in the absence of depolarizing voltage steps.

channel and not an artifact of depolarization-dependent access of hydroxylamine to the internal side.

The use-dependent blocking effect of hydroxylamine is at present unique to the combination of this ion with the mutant channel T441S. Ammonium and methylamine do not produce this type of block in wild type or T441S; hydroxylamine tested with another mutant, F433S, also is ineffective (not shown). The sensitivity of T441S to this type of block is eliminated by the presence of permeant external ions such as K^+ (Fig. 4 *A3*). With external K^+ (at 10 mM replacing bath Na^+), channel behavior in the presence of hydroxylamine is indistinguishable from that in the control condition (also with 10 mM K^+). Thus, despite the fact that the effects of hydroxylamine cannot be reversed by washout, the "normal" behavior of the T441S channel in hydroxylamine with K^+

strongly suggests that the dramatic effects of hydroxylamine alone cannot be attributed to nonspecific damage of the protein. This conclusion also is supported by the lack of block in wild-type channels.

The rates of fast macroscopic inactivation for wild type and T441S at +40 mV show little or no change in hydroxylamine (Fig. 4 *A2*). In Na^+ bath saline, the fast components of macroscopic inactivation (mean ± SD) are comparable for wild type (4.3 ± 0.3 ms) and T441S (4.2 ± 0.2 ms), as summarized above for Fig. 1. With hydroxylamine, the inactivation rates for the same cells are: T441S (5 mM, 4.0 ± 0.2 ms; 10 mM, 3.6 ± 0.2 ms; n = 17); and wild type (100 mM, 3.9 ± 0.2 ms; n = 7).

Use-dependent block of T441S requires the amino terminal

The removal of N-type inactivation by deletion of amino acids 9–81 in the amino terminal abolishes use-dependent block (Fig. 4 *A4*). At concentrations up to 100 mM hydroxylamine, no difference is observed for (Δ9–81)-wild type or (Δ9–81)-T441S. Unlike the pore block described above, the use-dependent block of T441S by hydroxylamine requires the presence of the inactivation particle. In agreement with macroscopic data, single-channel records of T441S with hydroxylamine show an increase in the frequency of blank traces in which channels fail to open in response to depolarization, whereas the wild-type channels and the (Δ9–81)--T441S channels do not show any obvious changes in frequency of blanks (not shown). The idea that hydroxylamine stabilizes the binding of the inactivation particle may be supported by the absence of an effect of hydroxylamine in the presence of external K^+ (as described above); external K^+ has been shown to antagonize fast inactivation (Demo and Yellen, 1991). In addition to demonstrating that use-dependent block requires the amino terminal, these data also demonstrate that hydroxylamine does not have general or indirect deleterious effects on the T441S channel.

Fast inactivation is stabilized by hydroxylamine

The data presented above for T441S demonstrate that use-dependent block occurs during prolonged depolarizations. Using shorter duration pulses (in which the development of use-dependent block is minimized) we have determined that hydroxylamine directly alters N-type inactivation in T441S. This effect is rapidly produced and reversible, and thus is clearly separable from the subsequent development of use-dependent block. Three approaches were used to determine the effects of hydroxylamine on N-type inactivation, including the analyses of steady-state current amplitudes (Fig. 4), recovery from inactivation (Fig. 6), and prepulse inactivation (Fig. 7).

Steady-state current

Current amplitudes measured during the late phase of 100 ms depolarizing steps were used to estimate steady-state current

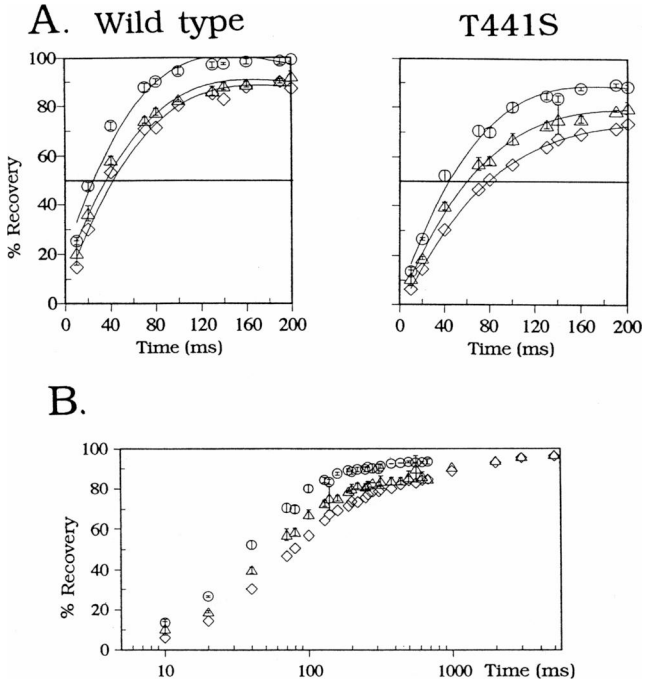


FIGURE 6 Slowing of the recovery from N-type inactivation in wild type and T441S in the presence of hydroxylamine. Recovery from N-type inactivation was measured with a double-pulse protocol. For each channel type, a single oocyte was perfused sequentially with bath salines containing hydroxylamine at 0 mM (○); 20 mM (△); and 100 mM (◇). Recovery was at −80 mV; conditioning and test pulses each were at +40 mV for 40 ms. These short steps provided fast inactivation (see Fig. 2) while minimizing the development of slow inactivation and use-dependent block. The data, calculated for the peak test amplitude (second pulse) as a percentage of the peak conditioning amplitude (first pulse), are presented as mean ± SD. (*A*) Recovery was slower for T441S than for wild type, both in Na^+ saline and in the presence of hydroxylamine. (*B*) An extended time scale showed that recovery for T441S, although slow, is virtually complete by 10 s.

amplitude. The term "steady-state" used here is defined as a time when fast inactivation is close to apparent equilibrium, but before slower C-type inactivation and irreversible block have become significant factors. Calculated as a percentage of the peak outward current, the steady-state current ranges from 15 to 20% in wild type and in T441S at +40 mV in Na^+ saline. For wild type, the presence of hydroxylamine does not cause an obvious change in the steady-state amplitude (Fig. 4 *A2*). In contrast, the presence of hydroxylamine decreases the steady-state current for T441S (Fig. 4 *A2*). The percent steady state (mean ± SD) for T441S was 17 ± 3 in Na^+ saline, and 8 ± 1 in 10 mM hydroxylamine. The percent steady state for wild type was 14 ± 2 in Na^+ saline, and 11 ± 4 in 100 mM hydroxylamine. The steady state is viewed as an equilibrium between the opposing processes of inactivation and recovery from inactivation; the amplitude reflects in part the rate of channel re-opening from the inactivated state (Zagotta et al., 1989). For T441S, the effect of hydroxylamine on steady-state current amplitude suggests that it decreases the likelihood of recovery from inactivation during a depolarizing pulse. As with the use-dependent effects described above, the changes in macroscopic inactivation and in the steady-state current are prevented by the presence of external K^+. Because external permeant ions

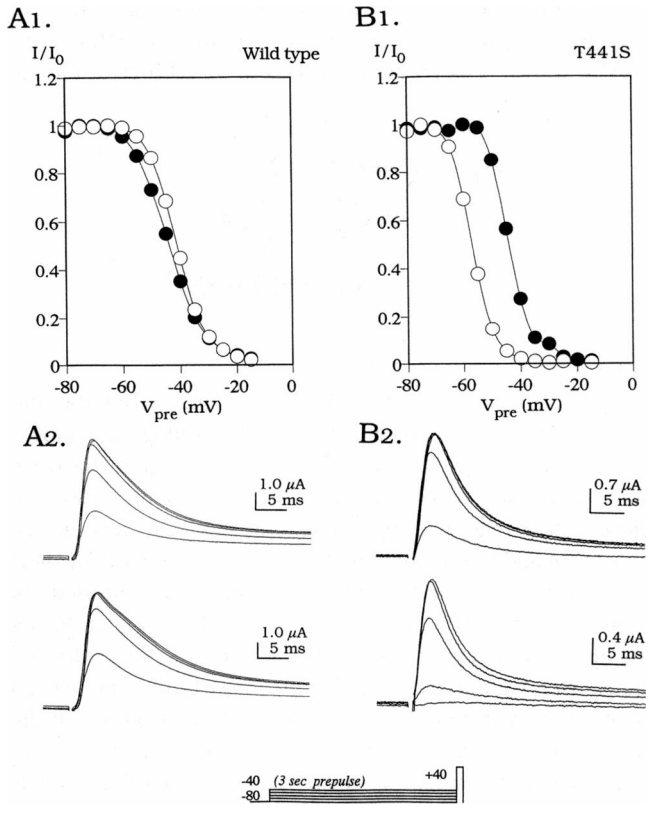


FIGURE 7 Prepulse inactivation of T441S but not wild type is shifted by hydroxylamine. Prepulse inactivation plots and traces are shown for (*A*) wild type and (*B*) T441S. The magnitude of current remaining noninactivated after a 5 s prepulse step to various conditioning potentials was measured with a short test pulse (+40 mV, 35 ms duration). (1) Hydroxylamine caused a negative shift in the curve midpoint for T441S but not wild type. Single oocytes were tested with control Na^+ saline (●), and then perfused with 10 mM hydroxylamine (○). Standardized net peak currents are plotted as a function of prepulse potential for (*A1*) wild type, and (*B1*) T441S. Net peak amplitudes (*I*) were measured as (peak) − (steady state) and were standardized to the net peak amplitude (I_o) evoked from the prepulse potential of −80 mV. (2) Traces corresponding to the graphed data above show the test pulse recorded at the end of the prepulse for (*A2*) wild type and (*B2*) T441S. Traces are shown for prepulse voltages of −80 to −40 mV (increment +10 mV). The upper sets of traces were recorded in control Na^+ saline; the lower sets were recorded in 10 mM hydroxylamine.

destabilize inactivation, this observation is consistent with our interpretation that hydroxylamine in the pore causes a stabilization of inactivation.

Hydroxylamine also decreases the percent late current for T442S (Fig. 4 *C*) from 75 to 40% when measured at steady state as defined above. However, unlike T441S, the macroscopic rate of inactivation is altered as well. Single-channel recordings in inside-out patches of T442S exposed to hydroxylamine show an increase in the occurrence of long-lasting, nonconducting states resembling inactivation (Fig. 4 *C2*). The small fast component of macroscopic inactivation that is expressed by this poorly inactivating mutant in control conditions seems to be enhanced in hydroxylamine (Fig. 4 *C1*), although it is still much slower than in wild type or T441S. Macroscopic inactivation in hydroxylamine is fit by a single exponential with a time constant about half that of the control slow component. The effect of hydroxylamine is difficult to interpret with certainty in T442S because this apparently single component may reflect a mixture of C-type and N-type inactivation processes. However, because the N-terminal inactivation particle is intact in this mutant, the most likely explanation is that hydroxylamine also stabilizes N-type inactivation in T442S, although much less effectively than in T441S. For T442S, hydroxylamine may enhance the rate of entry into the inactivated state as well as decrease the rate of recovery; these possibilities remain to be analyzed at the single-channel level.

Recovery from inactivation

The mutation of T441S shows a slower recovery from inactivation than wild type (see Fig. 2). Hydroxylamine, in a manner consistent with its effect on steady-state currents (described above), further slows the rate of recovery from fast inactivation at −80 mV (Fig. 6). In the double-pulse protocol, the mean current amplitudes are standardized for each test peak (second pulse) as a percentage of each respective conditioning peak (first pulse). As summarized above, the time constant of recovery for the wild-type current in Na^+ saline is 14.3 ± 0.5 ms, as determined from fitting of the averaged data. The slower recovery of T441S shows a time constant of 24.0 ± 0.8 ms. Hydroxylamine lengthens these recovery times. For wild type, the time constant is 18.6 ± 0.6 ms in 20 mM hydroxylamine and 21.6 ± 1 ms in 100 mM hydroxylamine. For T441S, the time constant is 32.8 ± 1.6 ms in 20 mM hydroxylamine and 45.8 ± 2 ms in 100 mM hydroxylamine. The fast component of recovery accounted for ≥97% of the fitted curve for wild type with and without hydroxylamine present. For T441S, the amplitude of the fast component of recovery was decreased slightly in hydroxylamine; in control conditions the fast component accounted for 98 ± 0.6% of the fitted recovery curve, but with 20 mM hydroxylamine the fast component accounted for 93 ± 0.9%, and with 100 mM hydroxylamine 92 ± 1% of the observed recovery. Although recovery at −80 mV was slower for T441S in the presence of hydroxylamine, it was ~99% complete by 10 s after the short depolarization step (Fig. 6 *B*). Interpulse intervals of ≥11 s were used for all of the protocols presented in this paper. The presence of external K^+ in the bath saline, which speeds up the rate of recovery from inactivation, prevents the effect of hydroxylamine and enables recovery equivalent to control T441S and wild type exposed to the same concentration of external K^+ (not shown). Thus, the inactivation and recovery mechanisms remain intact with hydroxylamine treatment. Other permeant cations (Rb^+ and $(NH_4)^+$) added externally also serve to restore normal recovery (not shown).

Prepulse inactivation

Further evidence for the stabilization of the inactivated state of T441S by hydroxylamine was observed in prepulse inactivation experiments. The voltage dependence of steady-state inactivation is shifted to more negative potentials for

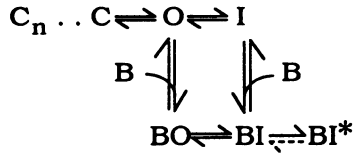
T441S in hydroxylamine (Fig. 7). Prolonged conditioning steps (5 s) at various voltages establish a steady state level of inactivation; the amount of inactivation is then measured from peak current in subsequent short test depolarizations (35 ms). Wild-type current shows no change in the midpoint potential with hydroxylamine; T441S midpoint voltages are shifted by -12 ± 3 mV (mean $\pm$ SD; $n = 4$). As expected for a stabilization of inactivation, this shift for T441S does not occur when permeant ions are present externally (2 mM K^+; or 10 mM $(NH_4)^+$ or Rb^+). The order of prepulse voltages applied in this analysis was from negative to positive. The short test steps themselves generated virtually no use-dependent block. The possible contribution of the prepulse steps to irreversible block of T441S was determined in a separate experiment by repeating the protocol multiple times from the same prepulse potential. For the same protocol used in Fig. 7, the mean percentage of irreversible block per trial at each prepulse voltage was 0% at -80 mV, 1% at -70 mV, 3% at -65 mV, and 5% at -60 and -55 mV. In relating these data to Fig. 7 *B1* for the prepulse step to -55 mV, for example, the maximum decrease in test current amplitude that can be attributed to irreversible block is less than 14% of the maximal current (I_o). The actual decrease in current for T441S in hydroxylamine is 60%. These results show that steady-state inactivation of T441S is affected by hydroxylamine independently of its ability to cause irreversible block.

DISCUSSION

We have observed effects of mutations in the pore on inactivation of the *Shaker* B channel. These mutations, threonine to serine, are conservative; the substitution removes a methyl group while preserving the hydroxyl moiety and polar character of the side chain. This subtle structural difference has dramatic effects on the properties of the *Shaker* B K^+ channel when introduced at sites 441 and 442. The mutant T441S shows alterations in ionic selectivity (Yool and Schwarz, 1991), internal TEA binding (Yellen et al., 1991) and, as shown here, binding of another ammonium derivative and recovery from N-type inactivation. The time to 50% recovery from inactivation is doubled by the mutation. The same substitution at the adjacent site, T442S, produces a dramatic destabilization of the inactivated state; only a small component of fast inactivation remains.

Additional interactions of the T441 residue with N-type inactivation were revealed by application of hydroxylamine. Our single-channel data show that this ion binds in the permeation pathway when threonine at 441 is mutated to serine. This binding has important consequences for N-type inactivation in T441S that are evident from four effects: (i) use-dependent block that requires the presence of the N-terminal sequence (Fig. 4 *A*); (ii) a decrease in the steady-state current (Fig. 4 *A*); (iii) a slowing of recovery from N-type inactivation (Fig. 6); and (iv) a shift in steady-state inactivation to more negative potentials (Fig. 7). All of these changes could arise from a slowing of the off-rate of the inactivation particle in hydroxylamine.

A simplified state diagram illustrates our view of how hydroxylamine could create the multiple effects observed for the T441S channel. Based on a kinetic scheme



Scheme 1

proposed for the A-type *Shaker* channel (Zagotta and Aldrich, 1990), C_n represents at least four voltage-dependent closed states, and C, O, and I are the last closed state, the open state, and the N-inactivated state, respectively. Because closed state inactivation is not appreciable at depolarized potentials and C-type inactivation is not appreciable in short test pulses, these additional kinetic states are omitted. In T441S channels, the binding of hydroxylamine (B) during the open and inactivated states leads to at least three blocked states, BO, BI, and BI*. The ion also may bind in the closed states; however, because no changes were observed in the rising phase of the macroscopic currents or in the single-channel first latencies, no influence of hydroxylamine has been included for the steps leading to channel opening. BO (the blocked open state) is a transient state that was manifest in T441S as a decrease in the mean open time. In the mutant T441S, the transient blocked state is stabilized sufficiently to yield brief closings that shorten the mean open time. BI (the inactivated state with hydroxylamine bound) is recognized by the slowing of recovery from inactivation induced by hydroxylamine. In T441S, this also results in a decrease in the steady-state current level and a shift in steady-state inactivation to more negative potentials. BI* (the use-dependent blocked state) is an absorbing blocked state from which recovery was not detected. It is observed only in T441S channels in response to long depolarizations. Wild-type channels do enter BI, showing a slower recovery from inactivation in hydroxylamine, but are not subsequently trapped in BI* even with very prolonged depolarizations. Although a direct transition between BO and BI is shown, we do not know if this process occurs, because the single-channel burst duration indicating the transition into the inactivated state is not substantially affected by hydroxylamine.

We have not directly shown an interaction of T441 or T442 with the inactivation particle. Although these residues could form part of the binding site for the particle, their effects may instead be indirect. Allosteric changes may alter protein structure. In addition, ions in the pore influence inactivation, so differences in channel occupancy may contribute to the observed effects of the mutations. For T442S, the lack of inactivation may be attributed to an unusual open state that interferes with the binding of the inactivation particle. Nevertheless, both the direct effects of the mutations themselves as well as the pharmacological influence of hydroxylamine

could be explained if the residues T441 and T442 contribute to part of the receptor for the inactivation particle. The observed ability of the *Shaker* N-terminal peptide to induce inactivation in typically noninactivating K^+ channels (Foster et al., 1992; Toro et al., 1992) indicates that a binding pocket exists not only in *Shaker*, but also in other K^+ channels. For many K^+ channels, the amino acids at positions equivalent to T441 and T442 are conserved (Jan and Jan, 1990) and could be logical candidates for a part of the inactivation particle receptor. The hypothetical scheme presented below incorporates our results and published data into a model based on this suggestion.

Model

The sequence linking transmembrane segments S4 and S5 already has been shown to influence inactivation (Isacoff et al., 1991). With the demonstration here that the residues T441 and T442 located in the H5 segment also influence inactivation, we can hypothesize that a more extensive receptor pocket encompasses portions of at least two segments from each of the four subunits. Given the likely size of the inactivation particle, multiple sites of interaction with residues in the channel protein would not be surprising.

Our working model (Fig. 8) shows T441 located at the internal mouth of the pore, with T442 immediately adjacent. Indeed, others have proposed that T441 forms the innermost ion binding site in the pore (Eisenman et al., 1993). Our single-channel data suggest that the mutation T441S induces a greater affinity for hydroxylamine; a simple explanation is that T441 serves directly as the hydroxylamine binding site and that mutation to serine increases its affinity for the ion. This pore block is independent of N-type inactivation, as seen in the effect on (Δ9–81)-T441S.

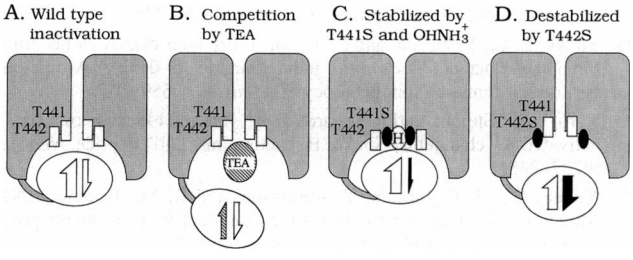


FIGURE 8 Simplified model of the *Shaker* K^+ channel pore and the interaction with the N-terminal inactivation particle (see text for explanation and references). The diagram shows: (*A*) the native interaction of the N-type inactivation particle with its wild-type receptor; (*B*) the competitive inhibition of inactivation by internal TEA, a blocker whose binding is influenced by mutation of the T441 residue; (*C*) the stabilized binding of the inactivation particle in the mutant T441S, which is further enhanced when hydroxylamine (H) is present; and (*D*) the destabilized binding of the inactivation particle in the mutant T442S. The effects of the mutations are interpreted primarily as changes in off-rates, although changes in on-rates may occur as well. The position of site 441 at the cytoplasmic mouth of the pore is based on several lines of evidence (including its ability to influence N-type inactivation). Other regions also participate in the binding of the inactivation particle. For clarity, only two subunits and one inactivation particle are drawn.

The site T441 at the internal pore mouth may participate in normal inactivation by interacting with the N-terminal particle (Fig. 8 *A*). The mutation T441S itself influences internal TEA binding (Yellen et al., 1991). Occupancy of the inner mouth of the pore by TEA interferes with binding of the N-terminal particle (Choi et al., 1991), and suggests that they compete for the same site (Fig. 8 *B*). This site is in close proximity to the pore; both inactivation and TEA blockade are antagonized by permeant ion entry (Armstrong, 1971; Demo and Yellen, 1991).

The dramatic effect of hydroxylamine on N-type inactivation in T441S suggests that a uniquely stable configuration can be achieved by this particular combination of components (Fig. 8 *C*). The effect of hydroxylamine on recovery from inactivation is most easily explained if it is bound at the inner mouth of the pore and interacts there with the N-terminal particle. This stable configuration is reversed by permeant ion entry, which could "push" the hydroxylamine molecule out of its binding site, and free the channel from hydroxylamine-stabilized inactivation. Explaining the influence of hydroxylamine on recovery from inactivation without invoking the trapping of the ion in the inactivated state would require a more elaborate scheme.

The mutation T442S causes a destabilization of inactivation, resulting in a small fast inactivating component and a large steady-state current dominated by a slow form of inactivation (Fig. 8 *D*). Several possible explanations exist. The flickery openings of T442S channels might decrease the probability of inactivation from the open state, although this explanation seems unlikely because the total percent open time for T442S is much greater than for wild type. An allosteric effect of T442S may interfere with binding of the inactivation particle. Alternatively, as shown in the model, the mutation could promote rapid dissociation of the particle by directly destabilizing the inactivated state. We favor this explanation as consistent with the observation that a small fast inactivating component persists in the T442S macroscopic currents. The reduced inactivation of T442S is somewhat restored, albeit inefficiently, by hydroxylamine.

Use-dependent block: similarity to the effects of local anesthetics

The use-dependent component of block by hydroxylamine does not occur without an intact N-type inactivation particle. The block appears to be a unique feature of the combination of hydroxylamine, the N-type inactivation particle, and the mutation T441S. Several properties of this block are reminiscent of the use-dependent blocking effects of local anesthetics, which are proposed to act by stabilizing inactivation in the Na^+ channel (Hille, 1977, 1992; Chernoff and Strichartz, 1990). Local anesthetics such as lidocaine, benzocaine, procaine, and internally applied QX-572 cause a negative shift in the prepulse inactivation curve and slow the rate of recovery from inactivation (Courtney, 1975; Hille, 1977). The use-dependent block depends on the frequency of activation (Chernoff and Strichartz, 1990; Courtney, 1975).

It has been suggested that a single local anesthetic binding site in the pore of the Na^+ channel may cause a block of conduction and the stabilization of inactivation (Hille, 1992). This hypothesis is parallel in virtually all aspects to our concept for the mechanism of action of hydroxylamine on T441S, as described in the model above.

Local anesthetics and hydroxylamine are similar in their structural dependence on an amine group and their ability to gain access to the channel both in charged and uncharged forms, but they differ greatly in target specificity. Local anesthetics for Na^+ channels affect K^+ channels only at high doses (Hille, 1966). Hydroxylamine is effective on the *Shaker* T441S channel, but has not been reported to have any similar action on Na^+ channels. Although only those K^+ channels with serine at the position equivalent to 441 may be expected to show sensitivity to hydroxylamine, it is possible that compounds structurally related to hydroxylamine might provide selective blockers of inactivating K^+ channels with research and clinical applications.

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