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The Internal Quaternary Ammonium Receptor Site of *Shaker* Potassium Channels

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Summary

Quaternary ammonium (QA) compounds inhibit K^+ conductance by entering and occluding the open pore of voltage-activated K^+ channels. We characterized the effects of a series of alkyl-triethylammonium blockers on the *Shaker* K^+ channel and tested them on a series of site-directed mutants of the channel protein in order to define the structural features of the binding sites. We found that mutations in two regions of the channel protein, the pore (P) region and the last transmembrane sequence (S6), appear to alter QA binding, not through their effects on gating but perhaps through direct effects on the binding site. Several mutations in the P region affect tetraethylammonium binding but have minimal effects on longer blockers, suggesting that the hydrophobic tail contributes to binding in a nonadditive fashion. Binding of the longer blockers can be affected by varying the hydrophobicity of 1 residue within S6 by site-specific substitution, in a manner consistent with a direct hydrophobic interaction between the side chain at this site and the alkyl chains of the blocker.

Introduction

Tetraethylammonium (TEA) and other quaternary ammonium (QA) compounds are well-known blockers of voltage-activated K^+ channels. They bind within the pore after the channels are opened by depolarization and inhibit the current flow (Armstrong 1966, 1969, 1971; Armstrong and Binstock, 1965). Because of their open-pore blockade properties, QA compounds have been used as structural probes to study the permeation pathway and to estimate the pore length (Miller, 1982; Villarroel et al., 1989).

Voltage-activated K^+ channels generally have two distinct receptor sites for QA binding, located superficially at the inner and outer mouths of the channels. The receptor sites may be distinguished from each other both by their affinity for TEA and by their specificity for QA ligands. Increasing the length of one or more alkyl chains of a QA ion favors interaction with the internal site, suggesting that this site, unlike the external site, contains a hydrophobic region (Armstrong, 1971; Armstrong and Hille, 1972; French and Shoukimas, 1981; Swenson, 1981). It has not been clear whether the alkyl tails of longer QA ions interact with specific amino acids of the channel protein or penetrate nonspecifically between the subunits (French and Shoukimas, 1981; Heyer et al., 1976).

We used a series of alkyl-triethylammonium (alkyl-TEA) derivatives to probe the internal QA-binding site systematically. We studied the basic properties of these compounds to confirm that their interaction with the *Shaker* channel is essentially similar to that described for other voltage-activated K^+ channels. We then tested the effects of these compounds on a series of site-directed mutants of the *Shaker* channel in order to dissect the structural determinants of QA binding. Since internal QA blockade has a strong interaction with voltage-dependent channel gating, we distinguished direct mutational effects on QA binding from indirect effects through gating. Those mutations that were candidates for direct effects could be separated into two classes. One class primarily affected the binding of TEA and had progressively diminishing effects as the substituted alkyl chain was made longer. The other class, represented by mutations at one site in the S6 segment, affected primarily the hydrophobic binding of the long-chain derivatives.

Results

Basic Properties of Alkyl QA Blockade

Long Alkyl Tails Increase QA Affinity

To establish whether QA blockade of the *Shaker* channel was similar to other K^+ channel types, we used a series of alkyl-TEA derivatives as structural probes. A constant triethylammonium "head" was combined with a variable-length n-alkyl "tail." To avoid confusion between the effects of QA blockers and the fast inactivation process seen in wild-type *Shaker* channels, we used a mutated channel that does not inactivate during a brief depolarization pulse (here called *Sh*IR, which is equivalent to *Sh*H4: Δ6–46; Hoshi et al., 1990; Choi et al., 1991). Throughout this paper, we refer to this N-terminal deletion mutant as the wild-type channel; all other mutations are made on this genetic background.

Figure 1 shows the blockade of this inactivation-removed current by various concentrations of TEA and each derivative. For TEA and the shorter alkyl-TEA compounds, the blockade is quite rapid and shows no time dependence: the voltage-activated current is simply scaled down in size. For the longer alkyl-TEA compounds, two differences are apparent. First, much lower concentrations are required for blockade. Second, the blockade at these concentrations is slower and shows an obvious time dependence.

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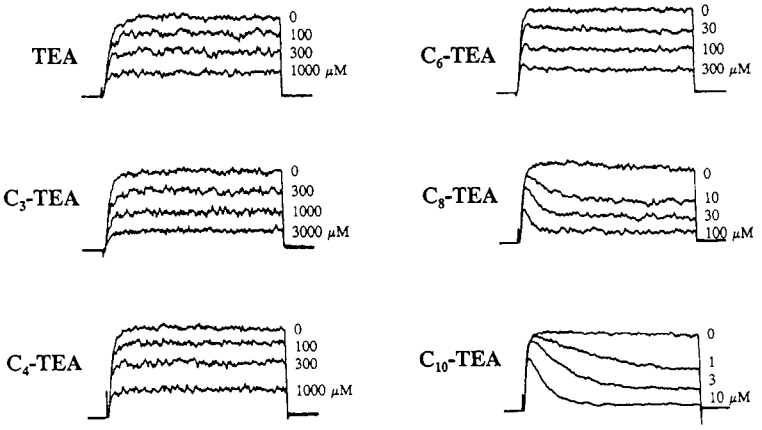

Figure 1. Increasing the Alkyl Tail Length of a QA Ion Enhances Its Blocking Potency on the Intracellular Side of the Channel

Voltage-clamped current traces from inside-out patches excised from oocytes expressing *Sh*H4 (Δ6–46) channels. The channels were activated by depolarizing pulses (30 ms) from −80 to +60 mV in the presence of a given alkyl-TEA blocker on the intracellular side. To calculate $K_{½}$, we measured the steady-state level of blockade for several different concentrations of the blocker. Currents were recorded 3–5 days after injection with *Sh*H4:(Δ6–46) mRNA. The level of control current for the patches shown are as follows: TEA, 340 pA; C_3-TEA, 460 pA; C_4-TEA, 630 pA; C_6-TEA, 1030 pA; C_8-TEA, 550 pA; and C_{10}-TEA, 1210 pA. The pulses were applied once every 15 s with an external solution containing 155 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). The sampling frequency was 10 kHz.

The half-blocking concentration ($K_{½}$) for blockade is determined from the steady-state level of blockade achieved after the time-dependent relaxation (Table 1). These values can be converted into relative apparent binding energies (ΔG) by comparison with an arbitrary standard-state concentration of 1 M: $\Delta G = -RT \times \ln(1\ M/K_{½})$, where R is the gas constant and T is temperature. We use this approach throughout to describe the effects of a blocker or channel modification on the apparent binding energy. The results for alkyl-TEA binding to the wild-type channel are shown in Figure 2.

For the longer blockers, the binding energy increased by about 1.2 RT for each additional methylene group (Figure 2). This value agrees well with the energy determined in model experiments for transferring one methylene group from a nonpolar to an aqueous medium (Tanford, 1980), as found previously for long-chain blockers of the sarcoplasmic reticulum K^+ channel (Miller, 1982). Like the delayed rectifier K^+ channel of squid axon, the *Shaker* K^+ channel seems to have a hydrophobic binding site near the inner mouth that accommodates the alkyl chain of the longer TEA derivatives.

The enhanced blocking potency observed with increasing alkyl tail length is produced by both an accelerated on rate and a slowed off rate (Table 2). The on and off rates of long-chain derivatives were calculated from the relaxation rate (equal to the sum of on and off rates) and the steady-state level of blockade (determined by the ratio of the two rates). As shown in Table 2, the addition of two methylene groups to the alkyl tail increases the specific on rate by approximately 3-fold and decreases the off rate by 4-fold.

Internal QA Blockade of Shaker *Interacts with the Gating Mechanism*

The experiments by Armstrong (1966, 1969, 1971) on squid axon delayed rectifier K^+ channels showed a substantial interaction of QA blockers with the gating mechanism. We examined whether such an interaction is present for the *Shaker* channel by analyzing the effects of long-chain QA ions on the channel activation and closing kinetics.

Upon depolarization in the presence of long-chain alkyl-TEA compounds on the intracellular side, the current rose at a normal rate to a peak and then relaxed to a lower steady-state level (see Figure 1). The relaxation process indicates that depolarization produced a large shift in the equilibrium between the blocked and unblocked channel populations. With 3 μM C_{10}-TEA, the fraction of blocked channels increased 20-fold after depolarization from −80 to +60 mV (comparing the extrapolated initial level of blockade with the steady-state level). Such a shift in equilibrium could have been produced either by a steep intrinsic voltage dependence of blockade or by coupling of blockade to voltage-dependent channel gating. The intrinsic voltage dependence of C_{10}-TEA blockade, measured in a voltage range (0 to +80 mV) in which channels are always open, is e-fold per 125 mV, which is completely inadequate to produce such a relaxation. However, if we suppose that

Table 1. The Half-Blocking Concentrations and Electrical Distances of Alkyl-TEA Blockers on the Wild-Type Channel

QA	$K_{½}$ (μM)	zδ
TEA	390 ± 11 (29)	0.16 ± 0.03
C_3-TEA	820 ± 34 (20)	0.17 ± 0.02
C_4-TEA	490 ± 10 (12)	0.18 ± 0.01
C_6-TEA	100 ± 2 (15)	0.17 ± 0.03
C_8-TEA	11 ± 0.4 (17)	0.18 ± 0.02
C_{10}-TEA	0.75 ± 0.04 (13)	0.20 ± 0.02

The steady-state levels of blockade were measured after depolarizing pulses to a voltage (+60 mV) at which all the channels were completely activated. From the fractional current remaining after blockade, the half-blocking concentrations were calculated: $K_{½} = [f_0/(1 - f_0)] \times [QA]$. The electrical distance, zδ, was determined from the voltage dependence of $K_{½}$ measured in the range of 0–80 mV using $K_{½}(V) = K_{½}(0\ mV) \times \exp(-z\delta V/RT)$. The number of determinations of f_0 is shown in parentheses.

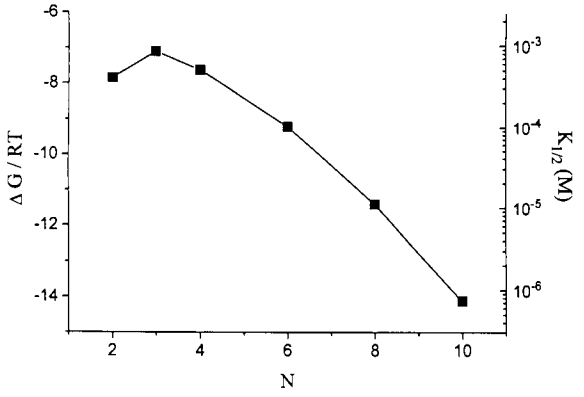

Figure 2. Binding Energy of a Long-Chain Blocker Increases Exponentially As Its One Alkyl Side-Chain Is Lengthened

The binding energy of each blocker in the series was calculated from the half-blocking concentration, $K_{1/2}$, by using an arbitrary standard-state concentration of 1 M: $\Delta G = -RT \times \ln(1\ M/K_{1/2})$. The binding energies were plotted in RT units ($RT \approx 600$ cal/mol). The slope of the line through the binding energies of long-chain blockers represents a gain of 1.2 RT in binding energy with each additional methylene group in the alkyl tail. N represents the number of methylene groups in the tail. All the errors are smaller than the size of the symbols.

the blockade is state dependent and requires the channel to open, we expect to see the binding relaxation in spite of the small intrinsic voltage dependence.

We then tested whether QA ions interact with gating during channel closure. After a depolarizing pulse that produced a steady-state outward current, repolarization to negative potentials resulted in an inward "tail current" owing to the instantaneous change in the driving force. The tail current then declined single-exponentially in time because of channel closing (Figure 3). In the presence of a high concentration of internal C_{10}–TEA, the inward tail current first increased and then declined at a much slower rate. These effects are consistent with observations for the delayed rectifier K^+ channel (Armstrong, 1969, 1971).

To explain the effects of C_{10}–TEA on the tail current kinetics, we tested a state-dependent blockade model. In this model, channels must be open before QA ions can bind, and the QA ions must unbind before the channels can close again (Armstrong, 1966, 1971):

$$\begin{array}{c} C \underset{\beta}{\rightleftharpoons} O \\ \kappa \Big\Downarrow\Uparrow \lambda \\ B \end{array}$$

This model predicts that the tail currents will be fitted by a double-exponential function which depends on four parameters: β, the intrinsic closing rate; κ and λ, the block and unblock rates; and f_o, the initial fractional current upon repolarization. β and f_o can also be determined independently: β from the macroscopic tail current decay upon repolarization in the absence of blocker and f_o from the fractional current immediately before repolarization in the presence of blocker.

The tail currents were indeed well fitted by a double-exponential function (Figure 3, smooth curve). The extracted values for β and f_o agreed well with those measured experimentally (Figure 3), and reasonable values were obtained for block and unblock rates (though these could not be measured directly at the negative voltages used). Thus, the model illustrates that channel closing can be slowed when a QA ion bound in the pore acts as a "foot in the door" and interferes with closing of activation gates (Armstrong, 1971).

Mutations That Alter Gating Affect QA Blockade Predictably

To establish further the interaction between channel gating and QA blockade, we examined how changing the open probability of the channel would affect the degree of blockade. We manipulated the open probability by introducing either of two mutations in the "voltage sensor" S4 region that were previously shown to affect the voltage dependence of activation (Liman et al., 1991; Logothetis et al., 1992; Papazian et al., 1991). Our analysis of these mutants, R377K and H378Q, shows that their voltage dependence of activation is shifted by +60 mV compared with that of the wild type. We characterized the voltage dependence of TEA and C_{10}–TEA blockade on these mutants.

At positive voltages that maximally activate the channels, a given concentration of either TEA or C_{10}–TEA blocked the wild-type and H378Q channels to the same extent. By contrast, at voltages at which the wild type was fully activated but the mutant was not, the mutant was less blocked than the wild type (Figure 4). Similar observations were made with the mutation R377K (data not shown).

The decreased level of blockade for the mutants at

Table 2. Enhanced Blocking Potency of the Long Alkyl-TEA Compounds Results from Faster On Rate and Slower Off Rate

	On Rate ($\mu M^{-1}\ ms^{-1}$)		Off Rate (ms^{-1})	
QA	$[K_{out}]$ = 5 mM	$[K_{out}]$ = 160 mM	$[K_{out}]$ = 5 mM	$[K_{out}]$ = 160 mM
C_8–TEA	0.012 ± 0.001 (8)	0.010 ± 0.001 (7)	0.13 ± 0.01 (8)	0.26 ± 0.03 (7)
C_{10}–TEA	0.030 ± 0.002 (13)	0.026 ± 0.002 (10)	0.023 ± 0.001 (13)	0.048 ± 0.002 (10)

The on (κ) and off (λ) rates of C_8–TEA and C_{10}–TEA were calculated from the relaxation time constants ($\tau = (\kappa + \lambda)^{-1}$), and the steady-state level of blockade (f_B) was determined by the ratio of the two rates: $\kappa = f_B/(\tau \times [QA])$ and $\lambda = (1/\tau) - \kappa \times [QA]$, where [QA] is the blocker concentration used. Measurements were at +60 mV at the indicated external K^+ concentrations. High external $[K^+]$ solution was 160 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4). The number of determinations for each value is given in parentheses.

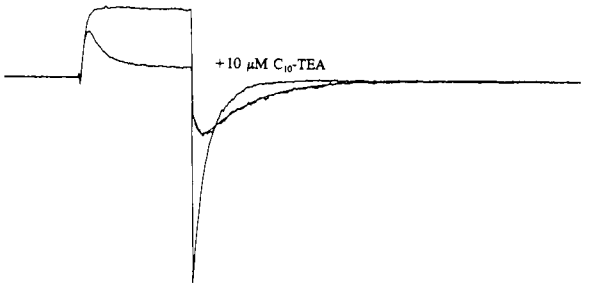

Figure 3. Channel Closing Rate Is Slowed by C_{10}-TEA Blockade

Tail current kinetics were measured with symmetric 160 mM K^+. Depolarizing pulses (20 ms) to +60 mV were given to activate the channels, followed by repolarization to −100 mV. We measured the channel closing rate, β, by fitting the control inward current decay as a single-exponential function. With 10 μM C_{10}-TEA on the intracellular side, the altered tail current kinetics were best fitted by a double-exponential function as predicted by the model presented in the text. Four parameters were calculated on the basis of the time constants and their coefficients of the double-exponential function fitted to the tail current: β, the intrinsic closing rate; κ and λ, the block and unblock rates; and f_o, the initial fractional current upon repolarization. The experimental and model-extracted values for β were 0.34 and 0.37 per ms, respectively, and for f_o were 0.16 and 0.18, respectively. The extracted values for block and unblock rates were on the same order of magnitude as those measured at positive potentials. The thin, smooth line (which represents the tail current fitted with extracted values from the model) matches well the experimental current trace. Bar, 1000 pA; 10 ms.

the lower voltages can be explained by assuming that the blockade can only occur when channel gates are open, as in the model described above. Since the blockade is coupled to the channel gating, the fractional current ($f_o = I_{blocker}/I_{ctrl}$) produced by a given concentration of a blocker depends in general on both gating and blockade equilibrium constants (K_g is the opening rate divided by the closing rate; $K_{½} = \lambda/\kappa$; $f_o = (1 + K_g^{-1})/(1 + K_g^{-1} + [QA]/K_{½})$, where [QA] represents the blocker concentration of QA). At a voltage at which channels are fully activated ($K_g >> 1$), the fractional current with blocker reflects a true equilibrium between only the open and blocked states (given by $K_{½}$). At lower voltages, at which the channel activation is incomplete ($K_g \approx 1$), the blocker will appear to be less effective. As shown in Figure 4B, experimentally measured fractional currents were in close agreement with the calculated values for f_o, estimated on the basis of mutant K_g and the wild-type $K_{½}$.

Localization of Amino Acid Residues That Contribute to the QA-Binding Site

Putative Structure of the Channel and the Location of the Binding Site

Recent studies have shown that substitutions of a few specific amino acid residues in the *Shaker* K^+ channel can alter its sensitivity to TEA and its ion selectivity. The *Shaker* channel protein is approximately 600 amino acids in length and contains six proposed transmembrane sequences, designated S1 to S6. A short, 20 amino acid stretch between S5 and S6, called the pore (P) region, appears to form the K^+-selective pore (Hartmann et al., 1991; Yellen et al., 1991; Yool and Schwarz, 1991). Mutation of conserved threonine in the middle of the P region alters the sensitivity to internal, but not external, TEA (Yellen et al., 1991) and may modify the ion selectivity (Yool and Schwarz, 1991). In contrast, 2 residues, 1 at each end of the P region, appear to determine the external TEA sensitivity without affecting the internal TEA sensitivity (MacKinnon and Yellen, 1990). These findings support the notion that the two receptor sites for TEA are distinct and that the P region may fold into the membrane and line the pore of the channel.

To define the QA-binding site more clearly, we made mutations in two different regions of the *Shaker* channel protein: first, the P region, and second, S6 and the adjacent C-terminal region. We selected the P region since mutational dissection had previously revealed its role in modulating TEA sensitivity and ion selectivity (Hartmann et al., 1991; Yellen et al., 1991; Yool and Schwarz, 1991). The S6 region was chosen because a heuristic model of the *Shaker* channel protein predicts that it may be physically close to the P region, possibly forming the vestibule of the channel's inner mouth (Guy and Conti, 1990).

In addition to internal QA affinities, we routinely measured several other parameters for each mutant. The voltage dependence of activation was measured from the macroscopic tail current. The maximum open channel probability and single-channel conductance were measured in single-channel experiments. We examined external TEA sensitivities to test whether any observed changes in QA affinities were specific to the internal QA site (see Table 5). Several mutations induced changes as large as 2-fold for the binding of external TEA, as well as some changes in single-channel conductance and gating. These changes are smaller than those that we frequently observed for internal TEA binding, but they do compel the usual caution in interpreting mutational effects as highly specific and local changes in channel structure.

Longer Alkyl Tails Appear to Meliorate the Effect of Some Binding Site Mutants

Two mutations in the middle of the P region, M440I and T441S, alter the internal TEA sensitivity dramatically. The internal TEA sensitivity is decreased by 13-fold for the mutation T441S, as previously reported (Yellen et al., 1991), and by 54-fold for the mutation M440I (Table 3). We examined the effects of these mutations on the binding of other alkyl-TEA blockers. We were surprised to find that the mutational effects gradually disappear as the alkyl tail is lengthened; C_{10}-TEA binds about equally well to both wild-type and mutant channels (Figure 5). The presence of the alkyl tail appears to "buffer" the effect of the mutation on QA binding.

This result is inconsistent with a scheme supposing both that the head groups of the various blockers bind to the same site and that the binding energy for the

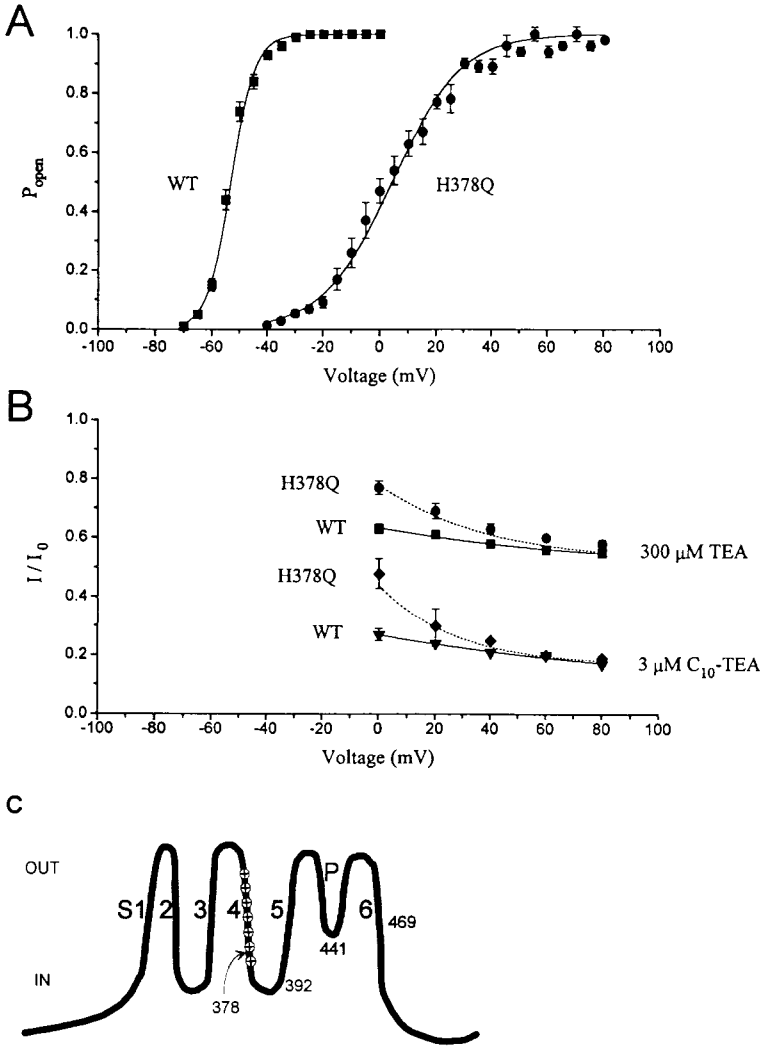

Figure 4. Mutations in S4 That Shift the Voltage Dependence of Activation Affect the Apparent Affinity for QA Blockers

(A) The voltage dependences of activation for the wild type and the mutant H378Q were measured from the macroscopic tail current experiments with symmetric 160 mM K^+. The currents were normalized to their respective maximum currents. The curves were fitted by a Boltzmann function: $I/I_o = 1 - [1/[1 + \exp(V_{mid}/\Delta V)]]$. The activation V_{mid} values for the wild type and mutant were -54 ± 1 mV and 6 ± 2 mV with slopes of 4.1 ± 0.3 mV and 13 ± 0.4 mV per e-fold change, respectively. Each point represents the mean plus or minus the standard error of the mean of at least three determinations.

(B) The fractional currents remaining after blockade by either 300 μM TEA or 3 μM C_{10}-TEA were compared between the wild type and the mutant. The dotted lines represent our predictions for the apparent fractional currents of the mutant channels, taking into account the observed altered gating of the mutant but assuming wild-type affinity for the blocker (see text for details).

(C) The locations of various mutation sites are indicated on a schematic of the hypothetical transmembrane folding pattern for a single *Shaker* channel subunit.

hydrophobic tail is additive with the head binding energy. At least one of these assumptions must be wrong.

Two results indicate that the head groups of the various blockers bind in similar if not identical positions. First, the blockers show approximately equal voltage dependence (see Table 1), indicating that they penetrate the membrane by about the same amount. Second, we find that TEA and C_{10}-TEA compete for binding: TEA slows the relaxation produced by C_{10}-TEA by an amount equal to the degree of TEA blockade (data not shown). This result is similar to the previous observation of internal TEA competition with the *Sh*H4 fast inactivation process (Choi et al., 1991) and implies that TEA and C_{10}-TEA bind to the same or overlapping sites.

The buffering effect might be explained by supposing that the two binding determinants, head and tail, are at odds with each other: they cannot both bind to their optimal sites at the same time. In this case, a mutation that reduced the binding affinity of the head might permit the tail (if present) to bind more tightly and thus provide some compensatory binding energy to offset the effect of the mutation.

A Residue in S6 May Contribute to the Hydrophobic Binding Pocket for Alkyl-TEA Blockers

Although most of the mutations in S6 and its vicinity showed negligible effects on TEA and C_{10}-TEA affinities (V476I, S479N, and N482S), substitutions at position 469 within S6 produced prominent effects on affinities of long-chain QA ions but only small changes in TEA affinity (Table 4). Mutations at this position appear to modify specifically the binding of more hydrophobic blockers, opposite to the effects of mutations in the P region.

Table 3. Comparison between TEA and C_{10}-TEA Affinities for the P Region Mutant Channels

Channel Type	TEA (μM)	C_{10}-TEA (μM)
Wild type	390 ± 10 (28)	0.72 ± 0.15 (13)
V437T	420 ± 30 (9)	0.61 ± 0.07 (10)
T439S	330 ± 40 (10)	0.77 ± 0.08 (12)
M440I	21,000 ± 1,000 (12)	2.1 ± 0.1 (24)
T441S	5,100 ± 100 (12)	0.48 ± 0.01 (14)

Half-blocking concentrations at +60 mV were calculated as described in Table 1. The number of determinations for each value is given in parentheses.

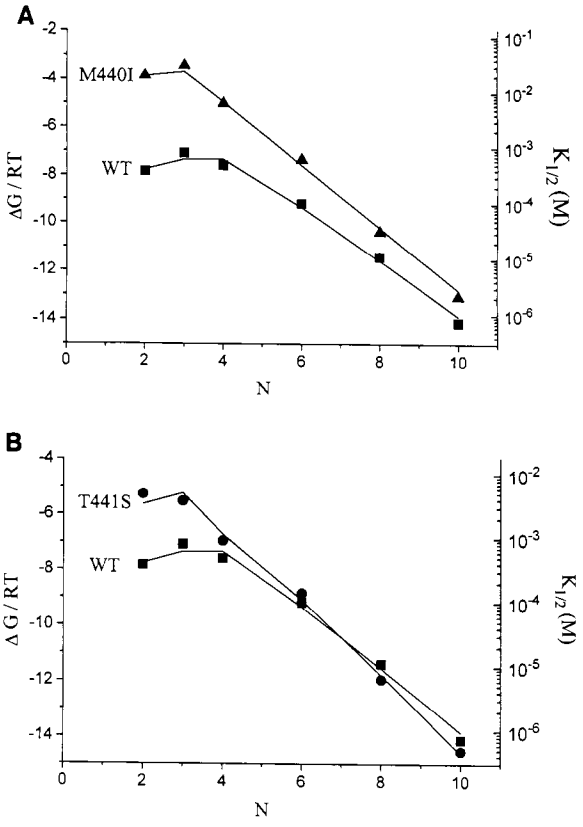

Figure 5. Mutations in the P Region That Dramatically Decrease the Internal TEA Affinity Have Small Effects on the Affinities of the Long-Chain Derivatives

(A) The energies for alkyl-TEA binding to the mutant M440I are plotted and compared with those for alkyl-TEA binding to the wild type. (B) ΔG values for the mutant T441S and for the wild type are compared. All the errors are smaller than the size of the symbols. N represents alkyl tail length.

Substitution of valine for the wild-type residue threonine increases the affinity for C_8-TEA by 27-fold but alters TEA affinity by only 2-fold (Figure 6A). This substitution involves almost no change in side chain volume but substitutes a hydrophobic CH_3 group for a polar OH group. Three other amino acid substitutions at this site (serine, alanine, and isoleucine) show consistent changes in hydrophobic binding. The changes in the binding energies of alkyl-TEA compounds ($\Delta\Delta G = \Delta G_{mutant} - \Delta G_{ShIR}$) due to substitutions at position 469 are plotted in Figure 6B. Decreasing the hydrophobicity of the site (as for T469S) lowered binding energies of all QA ions, whereas increasing the hydrophobicity of the site (as for T469A, T469V, and T469I) preferentially raised binding energies of longer QA ions.

Discussion

QA Ions Bind in the Pore

Our observations with the alkyl-TEA series are consistent with the notion first proposed by Armstrong (1966, 1969; Armstrong and Binstock, 1965) that QA ions inhibit K^+ conductance by entering and occluding the permeation pathway after the activation gates have opened. We find, as he did, first, that the QA blockade is slightly voltage dependent, in a manner consistent with a receptor site located about 15%–20% into the membrane field, and, second, that increasing external K^+ speeds the off rate of internal QA ion without affecting its on rate (see Table 2), as though increased K^+ influx can sweep the QA ion out of the pore.

The ability of a permeant ion to relieve blockade produced by open-pore blockers has been observed for a variety of K^+ channel types. Both hyperpolarization and increased external K^+ concentration speed recovery from the internal QA blockade on the squid axon K^+ channels (Armstrong, 1971). Increasing external K^+ relieves internal Na^+ block of the large conductance Ca^{2+}-activated K^+ channel (Yellen, 1984), and increasing internal K^+ can speed the dissociation of externally applied charybdotoxin (MacKinnon and Miller, 1988). Although we do not know the exact mechanism by which K^+ speeds the exit rate, we suspect that electrostatic repulsion between K^+ and the positively charged blockers leads to the increased exit rates of the blockers.

The Interaction of QA Ions with Channel Gating Resembles That of the Fast *Sh*H4 Inactivation Process

Blockade by C_{10}-TEA mimics the fast inactivation of the *Sh*H4 channel, suggesting that a QA ion may interact with the channel activation gates in the same way as the inactivation particle. Both processes produce a relax-

Table 4. Substitutions by More Hydrophobic Residues for the Threonine at 469 within S6 Preferentially Improve the Binding of the Longer Blockers

Channel Type	TEA (μM)	C_8-TEA (μM)	C_{10}-TEA (μM)
Wild type	390 ± 10 (28)	11 ± 0.4 (17)	0.72 ± 0.15 (20)
T469A	570 ± 20 (12)	2.9 ± 0.5 (19)	0.22 ± 0.06 (26)
T469I	140 ± 9 (12)	0.06 ± 0.02 (19)	0.033 ± 0.009 (14)
T469S	2500 ± 200 (12)	19 ± 1 (15)	1.8 ± 0.1 (31)
T469V	200 ± 9 (11)	0.30 ± 0.03 (10)	0.13 ± 0.05 (22)
V476I	930 ± 50 (9)	ND	0.85 ± 0.09 (8)
S479N	320 ± 8 (15)	ND	0.95 ± 0.05 (8)
N482S	860 ± 20 (13)	14 ± 0.6 (8)	0.92 ± 0.04 (15)

Site-specific mutations were introduced one at a time within and following the S6 region. The number of determinations for each value is given in parentheses. ND, not determined.

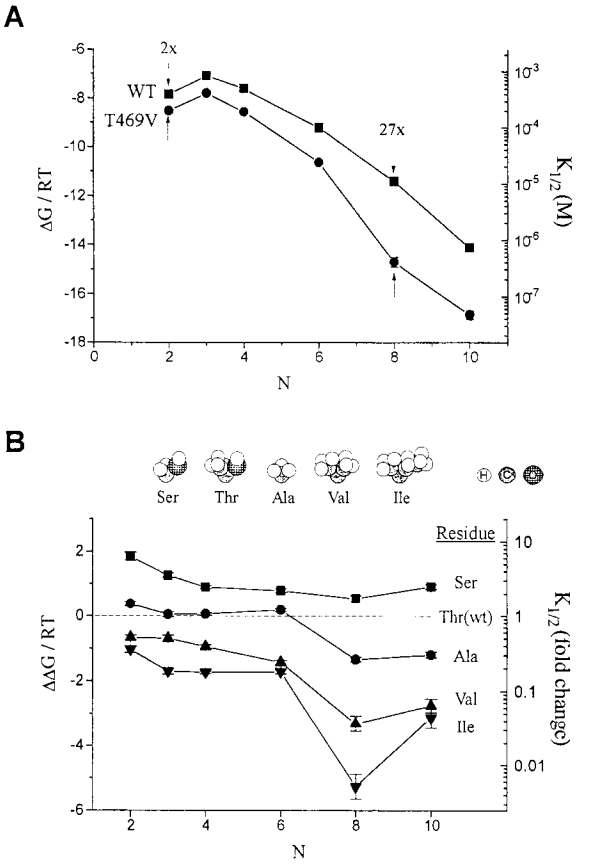

Figure 6. Varying the Hydrophobicity of Residue 469 within S6 Alters the Binding Energies of the Long-Chain Blockers Preferentially

(A) The binding energies of alkyl-TEA compounds for mutant T469V and wild-type channels are plotted. (B) Four different amino acids (serine [Ser], alanine [Ala], valine [Val], and isoleucine [Ile]) were substituted at position 469. The difference in the binding energies of alkyl-TEA compounds between wild-type and mutant channels are plotted ($\Delta\Delta G = \Delta G_{mutant} - \Delta G_{wt}$). N is alkyl tail length.

ation to reduced current after depolarization and activation, which can be explained either by a steep intrinsic voltage dependence of blockade or by coupling of blockade to voltage-dependent channel gating. There are three pieces of evidence to support the latter notion: first, both processes have little intrinsic voltage dependence (Zagotta and Aldrich, 1990); second, the voltage dependence of the first channel opening is sufficient to produce the voltage dependence of inactivation; and third, the tail current kinetics of both processes suggest that the channel activation gates usually do not close until either QA ions or inactivation particles are cleared from the pore (Demo and Yellen, 1991).

The behavior of the mutations in the voltage sensor S4 domain provides further evidence for coupling between blockade and gating. The mutations R377K and H378Q, which affect the voltage dependence of activation, alter TEA and C_{10}-TEA blockade in a manner consistent with blockade being directly coupled to the voltage-dependent channel opening. Furthermore, the mutation R377K and several others in S4 that shift the voltage dependence of activation also shift that of inactivation by about the same amount (Papazian et al., 1991). These results all indicate that QA blockade and inactivation are strongly coupled to channel activation and that TEA, C_{10}-TEA, and the inactivation particle interact with channel gating in a similar manner.

Tension between Multiple Binding Determinants

The effect on QA binding of two mutations in the P region, M440I and T441S, is reduced or eliminated when the QA ion has a long alkyl tail. This might be explained if the head and alkyl tail sites are separated by some distance, creating a tension between the two binding determinants. We wondered whether we could release the tension between the two binding determinants by adding a nonhydrophobic spacer such as an ether group between the head and tail moieties. This

Table 5. Gating Properties and External TEA Sensitivity of the Mutants

Channel Type	γ (pS)	P_{open}	V_{mid} (mV)	mV/e-fold	TEA_{out} (mM)
Wild type	11 ± 0.4	0.85 ± 0.1	−54 ± 1	4.1 ± 0.3	25 ± 3
R377K	13 ± 0.6	0.68 ± 0.02	54 ± 4	24 ± 1	15 ± 1
H378Q	12 ± 0.5	0.70 ± 0.02	6 ± 2	13 ± 0.4	26 ± 2
M440I	13 ± 0.4	0.72 ± 0.02	−45 ± 1	6.5 ± 0.7	12 ± 3
T441S	11 ± 1.0	0.87 ± 0.01	−44 ± 1	4.8 ± 0.2	32 ± 2
T469A	25 ± 0.5	0.93 ± 0.02	−55 ± 5	3.2 ± 0.6	22 ± 1
T469I	23 ± 0.9	0.92 ± 0.01	−44 ± 1	4.5 ± 0.2	32 ± 3
T469S	16 ± 0.3	0.87 ± 0.02	−37 ± 7	9.7 ± 3.2	22 ± 2
T469V	18 ± 0.4	0.88 ± 0.01	−42 ± 0.4	5.2 ± 0.5	22 ± 1

The single-channel conductance (γ), the maximum open probability of the channel measured at +60 mV (P_{open}), the midpoint of voltage dependence of activation (V_{mid}), and its slope over the linear range. The single-channel conductances were measured from for single-channel currents in the range 40 mV to +80 mV. The values for P_{open} were calculated for the period from 6 to 30 ms after the depolarizing step; each digitized point (0.1 ms per point) was counted as either open or closed according to a 50% current threshold. Voltage dependence of activation was measured from the size of macroscopic tail currents following 30 ms activation pulses to various voltages in the presence of 160 mM external K^+. The voltage dependence of the normalized current was fitted by a Boltzmann curve to estimate V_{mid} and mV/e-fold change. Each value is given as mean ± the standard error of the mean and is based on determinations from at least three patches.

might enable both determinants to bind at their optimal sites without any strain, because tail binding would no longer be improved by shifting the head. Consequently, those mutations in the P region that markedly decrease internal TEA sensitivity should also reduce binding of the new blocker without the buffering effect usually seen for long-chain derivatives.

We tested our hypothesis by using 3-oxa C_8-TEA ($[C_2H_4]_3N^+CH_2CH_2OC_5H_{11}$), which contains an ether group instead of a methylene group at the third position away from the charged nitrogen group. Contrary to our hope, we found that 3-oxa C_8-TEA behaves like the other long-chain derivatives: the effects of the mutations M440I and T441S on 3-oxa C_8-TEA binding are much smaller than those on TEA binding ($K_{½}$ for 3-oxa C_8-TEA is 49 ± 1 μM, 270 ± 10 μM, and 51 ± 3 μM for the wild type, M440I, and T441S, respectively). Perhaps adding an ether linker does not provide enough physical separation between the charged head and the alkyl tail of a long QA ion for both determinants to bind optimally at the same time.

For another voltage-activated K^+ channel related to the *Shaker* channel (Kv2.1), mutations at 2 other residues in the P region (corresponding to *Shaker* V438 and V443) have been shown to affect internal TEA affinity (Kirsch et al., 1992).

Nature of the Hydrophobic Binding Pocket

We have identified a specific amino acid residue at position 469 within S6 that appears to contribute to the hydrophobic binding pocket for the tails of the alkyl-TEA derivatives. Varying the hydrophobicity of this position by amino acid substitutions modulates the binding energies of the longer QA ions in a manner consistent with a direct hydrophobic interaction between the side chain of this residue and the alkyl tail. Each additional methylene group in the side chain of the residue at 469 (substitutions by valine and isoleucine) increases hydrophobic interaction surface and produces a gain of approximately 2 RT in binding energy for C_8-TEA. This effect is remarkably large: it is even larger than the 1.2 RT incremental interaction energy for each methylene group seen here for increases in tail length and seen in experiments on model compounds. Of course, each mutation is present in all four subunits of the channel protein, and the alkyl tail of the blockers may have the opportunity to interact with more than one subunit at once.

Substitution of this position with isoleucine, which is bulkier than the wild-type threonine residue, improves the binding of C_8-TEA more than that of C_{10}-TEA. This result may be analogous to Armstrong's (1971) finding that the affinity of C_{12}-TEA for squid axon K^+ channels is lower than that of C_9-TEA. Perhaps there is a steric conflict between the longer side chains of isoleucine and the long alkyl tail of C_{10}-TEA blocker.

Substitutions at the 469 position clearly do not affect the tail binding site exclusively. All substitutions at this position produce small changes in TEA affinity and affect the single-channel conductance (Table 5). Nevertheless, the far larger changes in affinity of the long-chain alkyl-TEA derivatives, which are well correlated with the side chain at position 469, make attractive the hypothesis that this site contributes to the hydrophobic binding pocket for long-chain blockers.

Experimental Procedures

Mutagenesis and RNA Synthesis

The site-specific mutations were introduced into the *Sh*H4 K^+ channel cDNA (Kamb et al., 1988) in a Bluescript vector (Stratagene). We deleted the coding sequence for amino acids 6–46 to remove fast inactivation (Hoshi et al., 1990; Choi et al., 1991). Oligonucleotide-directed mutagenesis followed the *dut⁻ ung⁻* selection scheme of Kunkel (1985). The mutated region of the plasmid was sequenced to confirm the success of the mutation (Sanger et al., 1977). The plasmid containing cDNA encoding for wild-type or mutant channels was linearized using EcoRI. The mRNAs were synthesized in vitro using T7 polymerase as described (MacKinnon and Yellen, 1990).

Expression in Oocytes

The oocytes were harvested from Xenopus laevis (Xenopus I, Michigan) that were previously injected with human chorionic gonadotropin. The follicle layers of oocytes were removed by incubation of dissected sacs of oocytes in collagenase (type 1A, Sigma) at 1 mg/ml in Ca^{2+}-free Ringer's solution for 2 hr. Each oocyte was injected with approximately 50 nl of mRNA. The oocytes were kept at 18°C in the modified frog Ringer's solution containing sodium pyruvate, theophylline, penicillin, and streptomycin as previously described (MacKinnon and Miller, 1988).

Synthesis of n-Alkyl-TEA Compounds

The n-alkyl-TEA salts $RNEt_3^+Br^-$ (where R is propyl, butyl, hexyl, octyl, or decyl) were prepared by methods described by Kellett and Doggett, 1966. The 3-oxa-octyl derivative (melting point 110°C–112°C) was prepared by O-alkylation of N,N-diethylethanolamine (NaH; n-pentyl bromide) followed by quaternization with ethyl bromide. All compounds gave satisfactory physical and spectral data.

Patch Recordings

Excised patches were recorded 2–7 days after mRNA injection. They were voltage clamped and currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Burlingame, CA). The currents were sampled at either 5 or 10 kHz and filtered at 3 kHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA).

The recordings were performed with the following solutions: internal (160 mM KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 10 mM HEPES [pH 7.4]) and external (5 mM KCl, 155 mM NaCl, 3mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES [pH 7.4]). The specified amount of alkyl-TEA was added to the internal solution; for recording external TEA blockade, TEA-Cl was added as replacement for NaCl.

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