J. Membrane Biol. 182, 233–243 (2001)
DOI: 10.1007/s00232-001-0047-8

The Journal of
Membrane
Biology

The Influence of Surface Charges on Quaternary Ammonium Block of *Shaker* K^+ Channels

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Received: 7 December 2000/Revised: 27 April 2001

Abstract. Block of K^+ channels can be influenced by the ability of charged residues on the protein surface to accumulate cationic blocking ions to concentrations greater than those in bulk solution. We examined the ionic strength dependence of extracellular block of *Shaker* K^+ channels by tetraethylammonium ions (TEA^+) and by a trivalent quaternary ammonium ion, $gallamine^{3+}$. Wild-type and mutant channels were expressed in *Xenopus* oocytes and currents recorded with the cut-open oocyte technique. Channel block by both compounds was substantially increased when the bathing electrolyte ionic strength was lowered, but with a much larger effect for trivalent gallamine. These data were quantitatively well described by a simple electrostatic model, accounting for accumulation of blocking ions near the pore of the channel by surface charges. The surface charge density of the wild-type channel consistent with the results was $-0.1\ e\ nm^{-2}$. *Shaker* channels with T449Y mutations have an increased affinity for both TEA and gallamine but the ionic strength dependence of block was described with the same surface charge density as wild-type channels. Much of the increased sensitivity of *Shaker* K^+ channels to gallamine may be due to a larger local accumulation of the trivalent ion. The negative charge at position 431 contributes to the sensitivity of channels to TEA (MacKinnon & Yellen, 1990). A charge reversal mutation at this location had little effect on the ionic strength dependence of quaternary ammonium ion block, suggesting that the charge on this amino acid may directly affect binding affinity but not local ion accumulation.

Key words: Gallamine — Tetraethylammonium — Surface potential — Ionic strength dependence — Surface charge density — Electrostatic

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Introduction

Different types of K^+ channels vary considerably in their sensitivity to block by external tetraethylammonium (TEA) ions. The effective concentration ranges over more than three orders of magnitude (e.g., *see* Kavanaugh et al., 1991; Grissmer et al., 1994). Much of this difference (MacKinnon & Yellen, 1990) can be attributed to an amino acid in the loop connecting the fifth and sixth membrane spanning domains (position 449 in *Shaker* K^+ channels). A threonine at this position confers a relatively low affinity for TEA and a 50-fold increase in sensitivity is produced by replacement of this amino acid with a tyrosine. However, the identity of the amino acid at this location is not the sole determinant of TEA potency. Both Kv2.1 and Kv3.1 have a tyrosine at the equivalent position and yet the affinity of these channels for TEA differs by more than 30-fold (Taglialatela et al., 1991; Jarolimek et al., 1995). Residues at positions other than 449 may contribute directly to the affinity of the TEA binding site (Pascual et al., 1995). However, it is also possible that charged residues far removed from the binding site may control the apparent affinity by altering the local concentration of the blocking ions.

There are many reports of an electrostatic component in the interaction of toxins with K^+ channels (e.g., Escobar, Root & MacKinnon, 1993; Naini & Miller, 1996; Mullmann et al., 1999), however, there are very few direct demonstrations of an accumulation of ions near the pore entrance by remote surface charges. Moczydlowski et al. (1985) and MacKinnon, Latorre and Miller (1989) presented evidence for negative surface charges on a Ca^{2+}-activated K^+ channel that accumulate permeant K^+ ions near the entrance to the pore. The latter report also showed a role for surface charges in accumulating TEA ions and so affecting the apparent TEA affinity.

The charged groups that contribute to surface

charges on K^+ channels are unknown. A first step toward identifying these charges requires some quantification of their influence on channel behavior. This would allow comparison of surface charge effects in channels of different amino acid structure and a method for assessing the role of candidate amino acids.

We have established a protocol for testing for the contribution of surface charges in block of K^+ channels by quaternary ammonium compounds. We compared the ionic strength dependence of block by monovalent TEA with block by gallamine, a trivalent TEA analog. We found that block of *Shaker* K^+ channels by gallamine was more sensitive to solution ionic strength than was block by the monovalent TEA ion. This was true for channels with either a tyrosine or a threonine at position 449. All the results from both wild-type and T449Y mutant channels could be described by a simple surface charge model with an effective surface charge density of $-0.1\ e\ nm^{-2}$.

MacKinnon and Yellen (1990) suggested an electrostatic role for the aspartate at *Shaker* position 431 in TEA sensitivity making this amino acid a candidate for contributing to the surface charge near the pore. We used the protocol described above and found that reversing the charge at this location produced only a 10% reduction in the effective surface charge density.

Materials and Methods

K^+ Channel Constructs

Several K^+ channel constructs were used in this study. The wild-type channel was the inactivation-deletion version of *Shaker B, ShB* Δ6-46 (Hoshi, Zagotta & Aldrich, 1990). The mutations investigated in this study (D431K, T449Y, and the double mutation D431K T449Y) were introduced into the *ShB* Δ6-46 clone using a two-step PCR protocol and the resulting mutants were analyzed by DNA sequencing.

Oocyte Isolation and Microinjection

Xenopus laevis oocytes were maintained as described by Goldin (1992). Isolated ovarian lobes were rinsed with Ca^{2+}-free OR-2 solution with (in mM): 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$ and 5 HEPES (pH 7.6, NaOH) and then defolliculated by incubation for 60–90 min with 2 mg/ml collagenase Type 1A (Sigma, St. Louis, MO). Cleaned oocytes were transferred and maintained for 2 hours in ND-96 solution with (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES and 2.5 Na-pyruvate (pH 7.6, NaOH) before injection of mRNA coding for the channel of interest. Injected oocytes were transferred to multi-well tissue culture plates and incubated at 18°C in ND-96 solution supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Electrophysiological Recordings

K^+ channel currents were recorded 1 to 5 days after mRNA injection. Recordings were made at room temperature (20–22°C) using the cut-open oocyte voltage clamp apparatus (model CA-1B, DAGAN, Minneapolis, MN). Connections to the different compartments were made with glass capillaries containing 75 μm platinum wires and filled with a 1 M NaCl, 3% agar solution. The experimental chamber (ELV-1, Dagan) was modified to include a low volume (80 μl) chamber insert. The low chamber volume and high flow rates (~1 ml/min.) produced efficient solution exchange. No correction for the junction potential between the 1 M NaCl solution and the experimental solution was made.

Recording electrodes were made either of 1 BBL glass with filament (1.5 mm outer diameter) from World Precision Instruments (Sarasota, Fl) or of GC-150F glass (Warner Instruments, Hamden, CT). Electrodes had tip diameters of ~2 μm and were filled with a 3 M KCl solution. Data acquisition was performed using a 12-bit analog/digital converter controlled by a personal computer. Current records were filtered at 5 kHz. The relatively large size of the currents recorded in the cut-open configuration can induce voltage errors if series resistance compensation is not used. The mean value of the compensation used was 0.74 ± 0.05 (SEM) kΩ in normal ionic strength solutions and 2.2 ± 0.11 kΩ in reduced ionic strength solutions ($N = 50$).

Electrical access to the internal compartment was achieved by 0.125% saponin treatment in a solution of (in mM): 100 KCl and 10 HEPES (pH 7.4, NMDG). The external solution was (in mM): 140 NaCl, 2 $CaCl_2$, 10 KCl, 10 HEPES (pH 7.4, NMDG). An external solution of reduced ionic strength was used that consisted of (in mM): 35 NaCl, 0.5 $CaCl_2$, 10 KCl, 10 HEPES (pH 7.4, NMDG). The osmolarity of this solution was matched to that of normal ionic strength by addition of 216 mM dextrose. In order to test for possible effects of dextrose beyond simply maintaining solution osmolarity, we also used mannitol and sucrose. We found that the ionic strength sensitivity of both TEA and gallamine block was the same regardless of which agent was used for osmotic balance. Osmolarity of all solutions was verified using a vapor pressure osmometer (Wescor, Logan, UT). Addition of TEA-Cl was made with substitution for NaCl, which maintained solution ionic strength. Gallamine-I_3 was added to the external solution with no compensation for changes in solution ionic strength.

Oocytes were clamped at a holding potential of −70 mV and 40 msec test pulses were applied to elicit channel current. Channel block by TEA or gallamine was computed by calculating the steady-state current recorded at the end of each pulse as a fraction of the average current recorded before application and following washout of the blocker. Only those results with at least 90% recovery from block were considered. Concentration-response relationships were constructed by plotting the mean ± SEM fraction of current not blocked at a test potential of +40 mV.

Analysis and Modeling

Concentration-response relationships were fit to equation *1* with simplex fitting in Origin (Microcal Software, Northampton, MA) which also provided the error estimates for the fitted parameters. Simulations of the surface charge model required simultaneous solution of Eqs. *1, 2,* and *3.* This was accomplished with a Newton-Raphson iteration technique programmed with the Visual Basic tool within Microsoft Excel (Microsoft, Seattle, WA).

Results

Ionic Strength-dependent Block of *Shaker* Channels by TEA^+ and $Gallamine^{3+}$

TEA block of *Shaker* K^+ channels was greater in solutions of reduced ionic strength as illustrated in Fig. 1.

The first panel of the upper row contains currents recorded from wild-type *Shaker* K channels in a solution of normal ionic strength. The middle panel illustrates block of wild-type channels by 10 mM TEA and the last panel shows that the block was readily reversible.

The lower row of records in Fig. 1*A* illustrates block by the same 10 mM concentration of TEA but in a solution of reduced ionic strength (*see* Methods). It is apparent that TEA was more effective in this low ionic strength solution.

Figure 1*B* illustrates the concentration dependence of TEA block of wild-type *Shaker* channels measured at +40 mV in the normal (●) and the low (□) ionic strength solutions. The dashed lines in this figure are best fits to the data of a standard binding isotherm (*see* Eq. 3). In the normal ionic strength solution, 50% of the channel current was inhibited by a concentration (EC_{50}) of 39 mM, consistent with previous values obtained at this potential (Heginbotham and MacKinnon, 1992). In the low ionic strength solution TEA was approximately twice as effective with an EC_{50} value near 19 mM.

The ionic strength dependence of block by TEA ions seen in Fig. 1 could be due to the presence of an electrostatic component of the binding energy of TEA with its channel receptor. A change in ionic strength would change the electrostatic energy and so alter TEA affinity. Alternatively, the ionic strength dependence of TEA block could be due to the presence of negative surface charges near the entrance to the channel pore. Such surface charges would accumulate cations, including TEA ions, to concentrations above those in bulk solution. A reduction in ionic strength would reduce the "screening" of these charges by ions in the aqueous solution. Consequently, there would be an increase in the local accumulation of TEA resulting in a greater channel block.

It is possible to distinguish between these two mechanisms by comparing the ionic strength dependence of block by monovalent and multi-valent cations. Changes in ionic strength would alter the direct electrostatic component of block independent of the valence of the blocker. In contrast, negative surface charges will preferentially accumulate blockers of higher valence. Thus, we tested for the presence of surface charges affecting TEA block of *Shaker* channels by examining the ionic strength dependence of block by a trivalent TEA analog.

Gallamine is a neuromuscular blocking agent effective at concentrations in the 10s of μM range (e.g., Derkx, Bonta & Lagendijk, 1971). This trivalent cation consists of three tetraethylammonium groups joined (through an O-link) to a phenolic ring (*see* Fig. 2*B*, *inset*) and has been shown to block delayed rectifier K channels in nerve (e.g., Smith & Schauf, 1981) and heterologously expressed *Shaker* channels (Thompson & Begenisich, 2000).

Figure 2 shows that block of *Shaker* channels by this trivalent molecule was much more sensitive to solution ionic strength than was block by monovalent TEA. Fig. 2*A* contains current traces obtained before, during, and following recovery from application of 1 mM gallamine in solutions of normal (upper row) and reduced (lower row) ionic strength. This concentration of gallamine produced only a small block in the normal ionic strength solution but produced substantial block in the solution of reduced ionic strength.

Block by gallamine (Fig. 2*B*) was determined over a range of gallamine concentrations in the normal (●) and the low (□) ionic strength solutions. *Shaker* channel current was blocked in a concentration-dependent manner and block was dependent on ionic strength. The dashed lines are best fits of Eq. *3* to the data with EC_{50} values of 5 and 0.9 mM for normal and low ionic strength conditions. That is, gallamine appeared to be 5 times more effective in the low ionic strength solution than in the normal ionic strength solution—much larger than the two-fold effect for TEA seen in Fig. 1.

The results illustrated in Figs. 1 and 2 show that changes in solution ionic strength had a larger effect on the apparent affinity of trivalent gallamine than monovalent TEA. This result indicates the presence of surface charges near the quaternary ammonium binding site on *Shaker* K channels that are able to produce local accumulations of cations. Another indication of the influence of cation-accumulating surface charges can be seen in the dose-response for gallamine block in the low ionic strength solution (□, Fig. 2*B*). The standard binding isotherm (dashed line) predicts considerably more block at increased gallamine concentrations than is seen from the experimental data. Increased concentrations of gallamine would be expected to produce more block but these trivalent ions also "screen" surface charges and so limit their own local concentration resulting in less than expected channel block. This issue is discussed at some length in MacKinnon et al. (1989).

Thus, the ionic strength sensitivity of block of wild-type *Shaker* K channels by monovalent TEA and trivalent gallamine appears qualitatively consistent with the presence of surface charges producing local accumulations of these ions. However, a more quantitative test is necessary. Such a test will also allow the estimation of the surface charge density and surface electrostatic potential. Such estimates could then be used to compare surface charge densities between channels and to test channel mutants for the identity of the chemical groups providing these charges. Unfortunately, the precise relationship between potentials near a protein surface and bulk ion concentration is unknown. Nevertheless, a

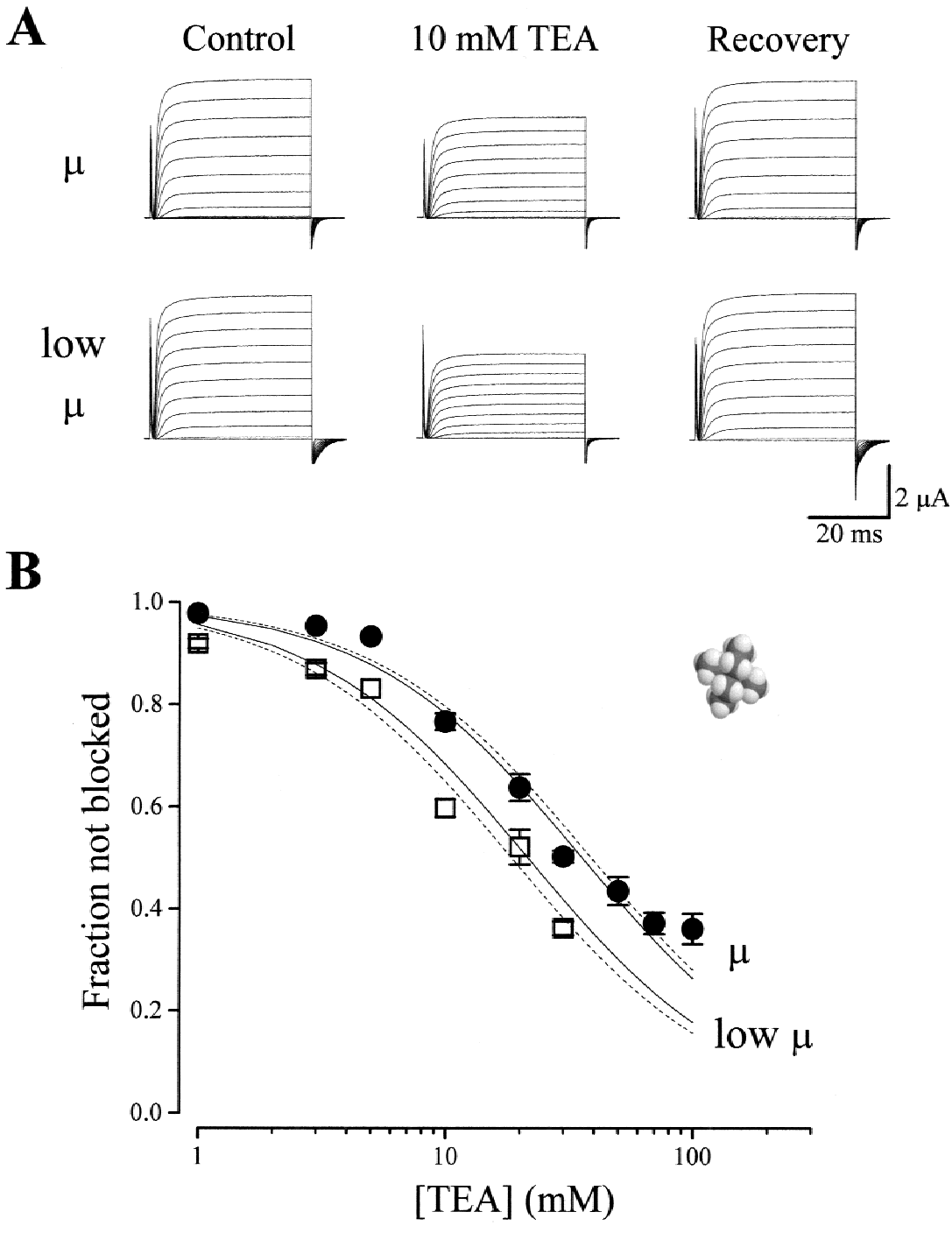

Fig. 1. Ionic-strength dependence of TEA block of wild-type (WT) *Shaker*. (*A*) *Upper panel:* Typical channel currents from oocytes expressing the WT *Shaker* channels. Currents were recorded at voltages ranging from −70 mV to +50 mV in 10 mV increments before, during, and following recovery from application of 10 mM TEA in the normal ionic strength solution. *Lower panel:* Currents from same oocyte before, during and following recovery from block in reduced ionic strength solution. (*B*) Fraction of current not blocked at the indicated TEA concentrations in normal (●) and reduced (□) solution ionic strength. Steady-state currents were measured at the end of 40 msec pulse to +40 mV. Error bars associated with the symbols are SEM values from 3–13 measurements at each concentration and are shown whether smaller or larger than the symbol. Symbols without error bars represent single observations. Dashed lines represent fits of the data to a standard binding isotherm (Eqn. *3*). TEA blocked WT channels with an EC_{50} value of 39 mM in normal ionic strength and 19 mM in reduced bulk solution ionic strength. Solid lines are the predictions of the surface charge model summarized by Eqns. *1, 2* and *3. Inset:* Space-filling representation of the structure of TEA in an aqueous environment.

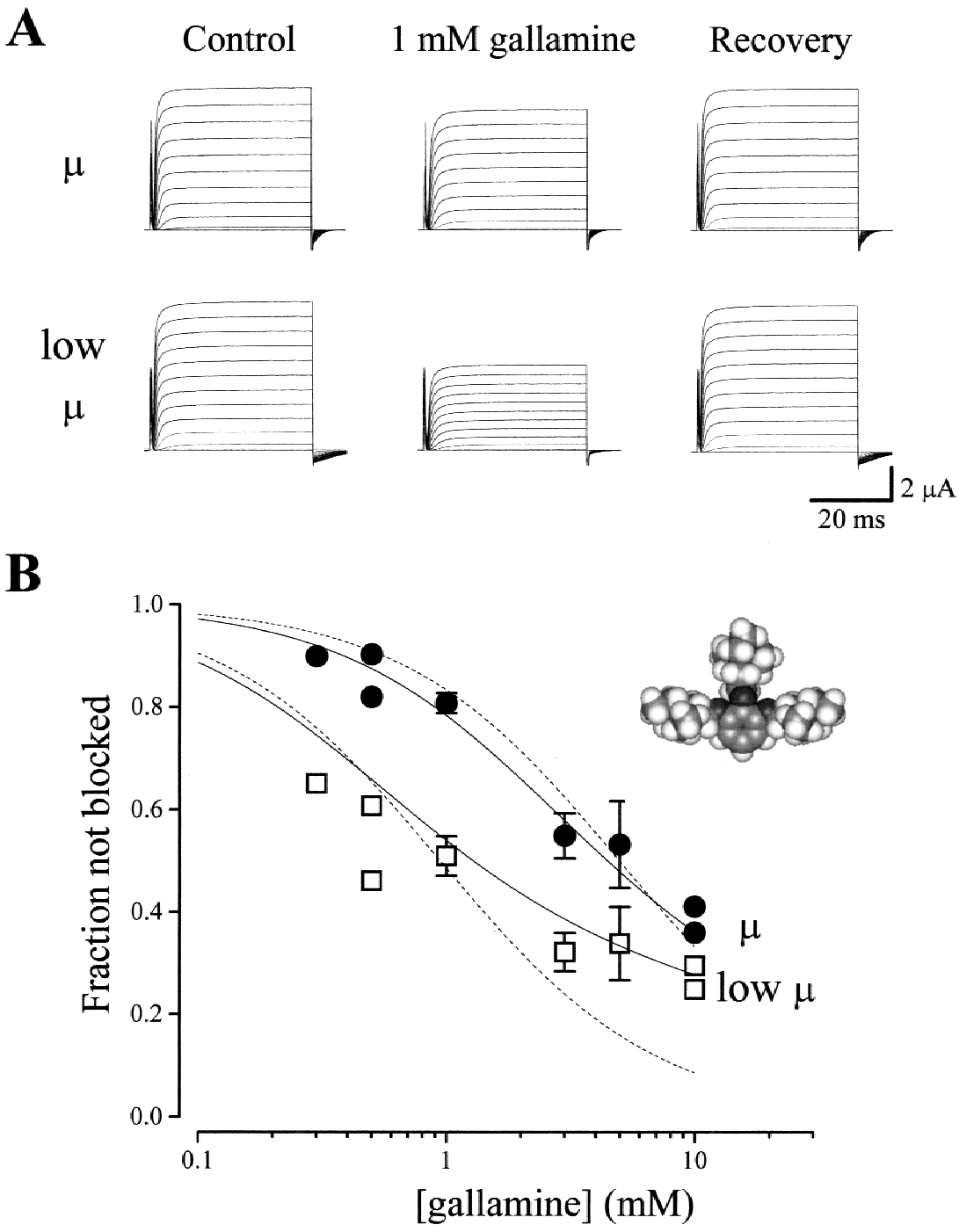

Fig. 2. Ionic-strength dependence of channel block by gallamine. (*A*) *Upper panel:* Typical channel currents from oocytes expressing the WT *Shaker* channels at voltages ranging from −70 mV to +50 mV in 10 mV increments before, during and following recovery from application of 1 mM gallamine in normal ionic-strength solution. *Lower panel:* Block by 1 mM gallamine in reduced ionic-strength solution. (*B*) Fraction of current not blocked at the indicated gallamine concentrations in normal (●) and reduced (□) ionic-strength solution. Error bars are SEM values from 3–13 measurements at each concentration and are shown whether smaller or larger than the symbol. Symbols without error bars represent single observations. Dashed lines represent fits of the data to the standard binding isotherm (Eqn. *3*). Solid lines are predictions of the surface charge model. *Insets:* Space-filling representation of the structure of gallamine in an aqueous environment.

simple first-order approximation of this relation can provide the predictions necessary for a quantitative test of the presence of surface charges and for the estimation of surface charge density and surface potential.

A Simple Surface Charge Model

Gouy (1910) and Chapman (1913) considered an infinite, planar surface with a uniform surface charge density bordering an aqueous solution with mobile ions. The relationship between the surface charge density σ (electronic charges/nm^2) and surface potential ψ_0 (in mV) is given by the Grahame (1947) equation:

$$\sigma = \frac{1}{2.7}\left[\sum_i c_i\left(\exp\frac{-z_i\Psi_0}{25} - 1\right)\right]^{\frac{1}{2}} \quad (1)$$

where c_i is the bulk concentration (in M) of the i^{th} ion of valence z_i.

The presence of a negative surface charge accumulates the positively charged blocking ions according to:

$$[X]_o = [X]_b \exp\left(\frac{-z\Psi_0}{25}\right) \quad (2)$$

where $[X]_o$ is the concentration of blocking ions at the channel surface and $[X]_b$ is the bulk concentration. Thus, the surface concentration of the blocking ions must be taken into account in computing channel block:

$$\text{Fraction not Blocked} = \frac{1}{1+\frac{[X]_o}{K_d}} \quad (3)$$

To predict quaternary ammonium ion block of channels in solutions of different ionic strength, we solved the set of Eqs. *1*, *2*, and *3* using the ionic composition of our experimental solutions (*see* Methods).

There are considerable limitations to this simple approach to surface electrostatics. These have been extensively discussed (e.g., Davies & Rideal, 1963; McLaughlin, Szabo & Eisenman, 1971; Aveyard & Haydon, 1973; Begenisich, 1974). Nevertheless, this simple theory captures much of the essence of surface phenomena and has been used in other similar studies (e.g., MacKinnon et al., 1989). One of the important assumptions of this simple double-layer theory is that the ions in solution are considered to be point charges. This assumption may not be too unreasonable for TEA ions but may present a more significant problem when applied to the larger gallamine ion used in this study (*compare* insets of Figs. 1 and 2). Stern (1924) has shown how to modify the double-layer theory to take into account the finite size of the ions. However, the simple version of this theory exemplified by Eqs. *1–3* was successful in quantitatively describing all our results so this type of modification of the theory was unnecessary—at least for the purposes of this type of study.

The simple surface charge theory was able to quantitatively reproduce the ionic strength dependence of block by TEA shown in Fig. 1*B* (solid lines). A negative surface charge density of −0.1 e nm^{-2} was able to account for block of wild-type channels by TEA in the normal and reduced ionic strength solutions with a model K_d of 70 mM. This latter value is about a factor of 2 larger than that determined from the bulk solution concentration in normal ionic strength (39 mM) owing to the accumulation of the blocker by the negative surface potential. The surface potentials (ψ_0 in Eq. 1) were computed to have values of −17 mV in normal solution ionic strength and −29 mV in reduced solution ionic strength. The increased amount of block in the low ionic strength solution is entirely accounted for by the predicted increased surface potential leading to increased local accumulation of TEA.

This same surface charge density was able to quantitatively account for the ionic strength sensitivity of block of *Shaker* channels by gallamine. The solid lines in Fig. 2*B* represent the amount of block predicted by the surface charge model in the normal and low ionic strength solutions. For these simulations a model K_d affinity of 25 mM was used for both the normal and low ionic strength solutions. The model K_d value is 5-fold larger than the bulk EC_{50} value of 5 mM for the normal ionic strength condition and 25 times larger than the EC_{50} value of 0.9 mM for the low ionic strength solution. The much higher apparent affinity for gallamine in the low ionic strength solution is, in the context of the surface charge model, a direct result of the increased accumulation of this trivalent ion by local surface charges.

The model K_d values for block of wild-type channels by gallamine and TEA were 25 mM and 70 mM, respectively. These values are surprisingly similar considering the 8 to 20-fold differences in EC_{50} values obtained from bulk solution concentration. However, this result is expected from the preferential accumulation of the trivalent gallamine at the channel surface relative to the monovalent TEA. According to the surface charge model, there is very little difference in the intrinsic affinity of the channel for these two compounds. Rather, the difference appears mostly to be due to their differential accumulation by surface charges on the channel.

Owing to its trivalent nature, gallamine not only blocks the channel but also screens the surface charges resulting in computed surface potentials (in the presence of 3 mM gallamine) of −15 mV and −22 mV in normal and reduced solution ionic strength compared to values of −17 and −29 mV, respectively, determined from the TEA data.

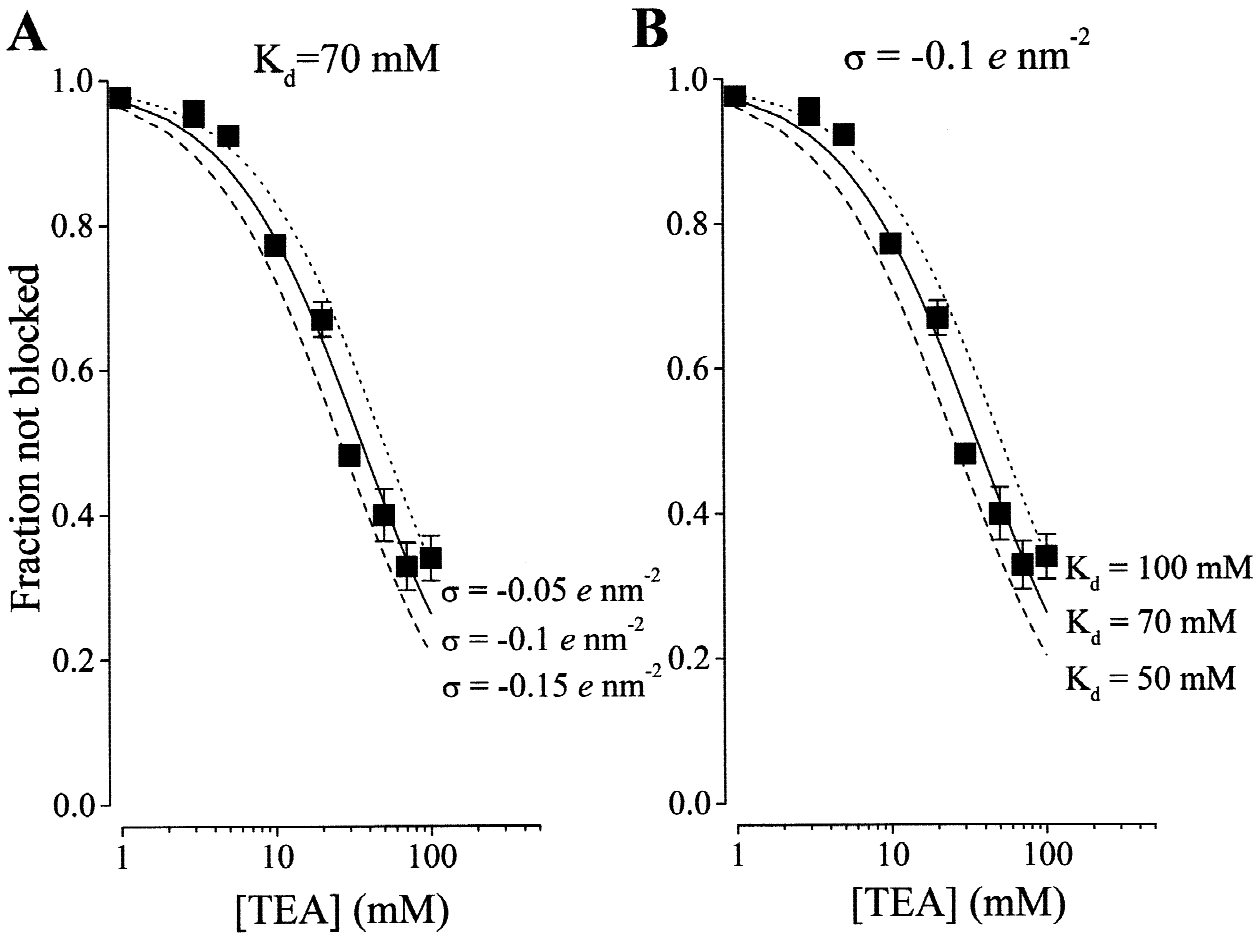

Fig. 3. Sensitivity of model to changes in surface charge density and affinity of blocker. Example of model sensitivity using data from TEA block of wild-type channel in normal ionic-strength solution (Fig. 1*B*). Various values of σ and K_d were used to create the simulations as indicated.

Sensitivity of Model Prediction to Model Parameters

Figure 3 illustrates the sensitivity of the model predictions to changes in surface charge density and model K_d values. The data points are block of wild-type channels by TEA in the normal ionic strength solution from Fig. 1*B*. The solid lines reproduce the model simulation from Fig. 1*B* and represents a surface charge density of −0.1 *e* nm^{-2} and a model K_d value of 70 mM. Fig. 3*A* shows that changing the surface charge density to −0.05 or −0.15 *e* nm^{-2} (at a fixed model K_d value of 70 mM) predicts measurably different relations. Part *B* of this figure shows that model K_d values of 50 or 100 mM predicts (with a fixed surface charge density of −0.1 *e* nm^{-2}) dose-response relations that are distinguishable from that with a value of 70 mM.

Gallamine Block of Channels with High TEA Sensitivity

Channels with a tyrosine (Y) at position 449 have a substantially higher affinity for extracellular TEA than do wild-type channels (MacKinnon & Yellen, 1990). We examined gallamine block of T449Y mutant channels and the ionic strength sensitivity of this mutant channel. Figure 4*A* shows that block of high affinity T449Y mutant channels by TEA was, like wild-type channels, dependent on solution ionic strength. Since the T449Y mutation does not involve a change of charged residues, it is unlikely to alter local surface charge density. Thus, we would expect the surface charge model, with the wild-type channel surface charge density of −0.1 *e* nm^{-2}, to be able to simulate the ionic strength sensitivity of TEA block of T449Y mutant channels with a model K_d appropriate for this higher affinity mutant. The solid lines in Fig. 4*A* show that the surface charge model was able to reasonably reproduce TEA block of T449Y mutant channels in normal (■) and low (□) ionic strength solutions with a model K_d value of 2.2 mM.

The T449Y mutation increased the channel affinity for gallamine analogous to its effect on TEA affinity. Fig. 4*B* illustrates gallamine block of T449Y mutant

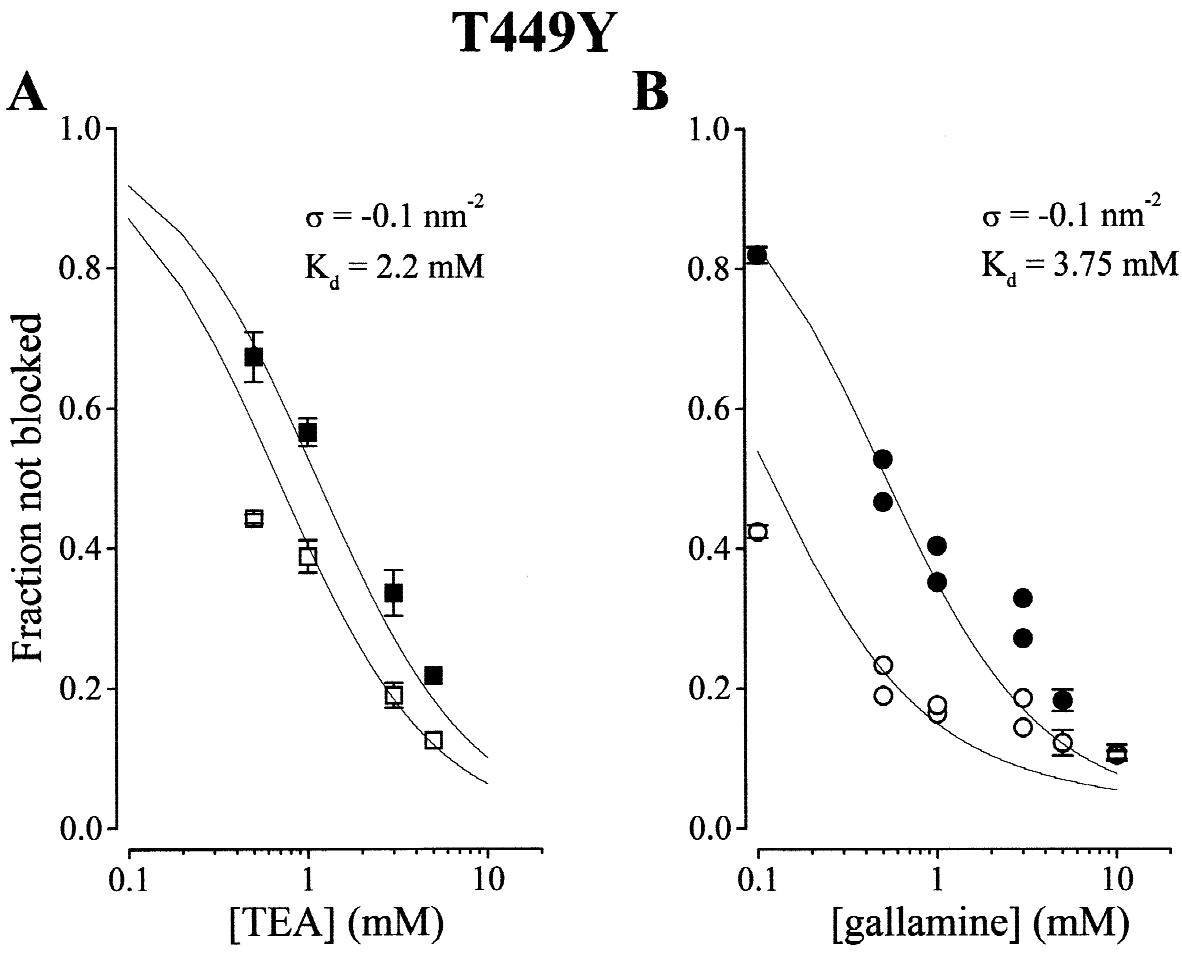

Fig. 4. Ionic-strength dependence of TEA and gallamine block of a highly TEA sensitive channel. Fraction of current not blocked, calculated from steady-state current at +40 mV, at indicated concentrations of (*A*) TEA and (*B*) gallamine in normal (●) and reduced (□) solution ionic strength. Lines are the computations from the electrostatic model with values of σ and K_d as indicated. Error bars are SEM values from 3–5 measurements at each concentration and are shown whether smaller or larger than the symbol. Symbols without error bars represent single observations.

channels in normal (●) and low (○) ionic strength solutions. These data could be simulated by the surface charge model with a model K_d value of 3.75 mM and the same −0.1 e nm^{-2} surface charge density.

Contribution of Aspartate 431 to Surface Potential

As indicated in the Introduction, aspartate 431 has been proposed to play an electrostatic role in TEA block of *Shaker* channels (MacKinnon and Yellen, 1990). We mutated this negatively charged aspartate to a positively charged lysine—a net charge reversal of +8 in the homotetrameric channel. If the negative charge of the side chain of this amino acid contributed significantly to the surface potential, we would expect to find a significantly reduced sensitivity of channel block to solution ionic strength. This would be especially true for gallamine block since this trivalent compound will be particularly sensitive to changes in surface potential.

Thus, we tested for ionic strength dependence of TEA and gallamine block of D431K channels in wild-type and T449Y backgrounds. We found that the D431K mutation had little effect on the ionic strength sensitivity of block by TEA or gallamine. In agreement with the results of MacKinnon and Yellen (1990), we found that this mutation produced a reduced sensitivity to TEA. The sensitivity to gallamine was likewise reduced. The surface charge model provided a good description of the mutant channel data with a surface charge density (−0.09 e nm^{-2}) only 10% less than the value that was able to account for the wild-type and T449Y channels with the aspartate at position 431.

These results are summarized in Table 1. Shown are the observed fractions of current not blocked by TEA

Table 1. Tests of the surface potential model with the D431K mutation in a wild-type and a high-affinity T449Y channel backbone

Channel	Blocker	Normal ionic strength		Low ionic strength	
		Predicted	Observed	Predicted	Observed
D431K WT	TEA, 20 mM	0.73	0.73 ± 0.02 (10)	0.63	0.58 ± 0.02 (10)
	Gallamine, 3 mM	0.72	0.72 ± 0.03 (5)	0.56	0.53 ± 0.04 (5)
D431K/T449Y	TEA, 1 mM	0.72	0.72 ± 0.02 (6)	0.62	0.58 ± 0.04 (6)
	Gallamine, 3 mM	0.74	0.74 ± 0.02 (6)	0.58	0.57 ± 0.03 (6)

The Table summarizes D431K WT (wild-type) and D431K/T449Y (high-affinity) channel block (expressed as fraction of current not blocked) by TEA and gallamine (at indicated concentrations) in normal and reduced ionic-strength solutions. Fraction of current not blocked was measured at the end of 40 msec pulses to +40 mV. Observed values are means ± SEM, *n* values are indicated in parentheses. The ionic-strength dependence of block was examined by comparing the observed values with those predicted by the surface charge model. Model predictions of channel block were generated with a surface charge density of $-0.09\ e\ nm^{-2}$ and K_d values for TEA and gallamine of 100 and 40 mM for D431K channels and 4.7 and 44 mM for D431K/T449Y channels, respectively.

and gallamine for D431K channels in both wild-type and T449Y backgrounds in normal and low ionic strength solutions. Also shown are the effects of these compounds predicted by the surface charge model with a surface charge density of $-0.09\ e\ nm^{-2}$. In all cases, the model with this surface charge density was able to accurately reproduce the experimental observations. Thus, the negative charge of D431 appears to contribute little, if at all, to the negative surface charge near the *Shaker* channel pore.

The model surface charge densities and the model K_d values for TEA and gallamine for wild-type, T449Y, D431K, and T449Y/D431K channels are summarized in Table 2. The D431K in the wild-type T449 background produced the same modest reduction in affinity for TEA and gallamine (a factor of 1.4 and 1.6, respectively). In the T449Y background, the D431K mutation reduced the affinity of the channel for TEA also by a relatively modest value of 1.9. In contrast, the D431K mutation in the T449Y background reduced the affinity for gallamine by more than 7-fold. That is, the D431K/T449Y double mutation had a disproportionate effect on gallamine affinity, suggesting some cooperative action of the amino acids at both 431 and 449 in defining the affinity of the pore for gallamine.

Table 2. Summary of channel characteristics predicted by surface charge model

			WT	T449Y	D431K	D431K/T449Y
	σ (e nm^{-2})		−0.1	−0.1	−0.09	−0.09
TEA	K_d (mM)		70	2.2	100	4.7
	Ψ	μ	−17	−17	−15	−15
	(mV)	$\mu/4$	−29	−29	−17	−17
Gallamine	K_d (mM)		25	3.75	40	44
	Ψ	μ	−15	−15	−14	−14
	(mV)	$\mu/4$	−22	−22	−20	−20

Shown are computed values of surface charge density, σ (e nm^{-2}), apparent K_d (mM) and surface potential, Ψ (mV) in both normal (μ) and reduced ($\mu/4$) ionic strength solutions. Values are reported for the wild-type and mutant channels in the presence of either TEA or gallamine.

Discussion

There has been a number of studies investigating the existence of surface charges in a variety of K^+ channels. However, most of these have focused on surface charges that affect voltage-dependent gating (e.g., Elinder, Madeja & Århem, 1996; Elinder & Århem, 1999). Much less effort has gone into examining the influence of surface charges on channel permeation and pharmacology. Moreover, there is little or no information on the molecular identity of any surface charges.

Negative surface charges on the external face of the channel could accumulate cationic blocking ions to levels above the concentration in bulk solution. Changes in solution ionic strength would, by "screening" these surface charges, alter the local concentration of the blocking ion. This ionic strength effect would be expected to be larger for ions of greater valence.

We found that block of *Shaker* K^+ channels by TEA ions was sensitive to solution ionic strength. Block by gallamine, a trivalent TEA analog, was more sensitive to ionic strength than monovalent TEA. A simple surface charge model based on the Grahame (1947) equation could quantitatively account for the ionic strength dependence of block of wild-type channels by both TEA and gallamine with a single, effective surface charge density of $-0.1\ e\ nm^{-2}$. According to this analysis, the apparently higher affinity of the pore for gallamine (based on bulk concentrations) is almost entirely accounted for by the much larger accumulation that occurs with this trivalent ion.

As discussed above, there are considerable inadequacies in applying this simple theory to proteins with

specific, discrete charges. Thus, the particular surface charge density represents an effective, average value. However, this simple theory has often been successfully applied and, at the very least, provides the means for quantitatively comparing surface charge effects in different channels.

MacKinnon et al. (1989) examined the ionic strength sensitivity of block of Ca^{2+}-activated K^+ channels by several charged blocking molecules including TEA. Their results could be described by the Grahame (1947) equation with a negative surface charge density of -0.06 to -0.12 e nm^{-2} near the pore in this K^+ channel, quite similar to our model value for *Shaker* channels. The similarity of the findings from these two types of K^+ channels suggests some common organization of electrostatic elements near the mouth of the pore.

Shaker K^+ channels with a tyrosine (Y) at position 449 have a substantially higher affinity for extracellular TEA than do wild-type channels (MacKinnon and Yellen, 1990). We tested the applicability of the simple surface charge model to T449Y mutant channels. The surface charge model with the same surface charge density determined from wild-type channels was able to quantitatively describe block by both TEA and gallamine (Fig. 4, lines).

MacKinnon and Yellen (1990) found that the affinity of *Shaker* K^+ channels for TEA correlated with changes in the charge of the amino acid at position 431. This could occur either through changes in the local concentration of the blocking ion or through a direct electrostatic contribution of the charge at this site to TEA binding. Measurements of the ionic strength sensitivity of TEA and gallamine block of D431K mutant channels can distinguish between these two possibilities. The ionic strength dependence of block by TEA and gallamine was only slightly affected as if the surface charge density was reduced by 10%. Since the D431K mutation in the homotetrameric channel (a change from four negative to four positive charges) only reduces the apparent surface charge density by 10%, the negative charge on this residue can account for no more than 5% of the total surface charge density on the wild-type channel. Thus, the charge on the amino acid at position 431 affects quaternary ammonium block of *Shaker* channels by directly altering the intrinsic affinity—not by changes in the local concentration of the blocker.

The ionic strength sensitivity of monovalent and trivalent cation block of *Shaker* K^+ channels suggests the presence of surface charges near the pore that are capable of producing local accumulations of cations to concentrations considerably larger than those in bulk solution. We showed that these results can be quantitatively described by a simple surface charge model. Even the modest model surface charge density of -0.1 e nm^{-2} (producing a predicted surface potential near -25 mV) accumulates monovalent cations almost 3-fold above bulk level, divalent cations by more than 7-fold, and trivalent cations by 20-fold.

Thus, the presence of charges, as yet unidentified, on the surface of K^+ channels will have a significant influence on the permeability and pharmacological properties of K^+ channels. K^+ channels certainly differ in their sensitivity to blocking ions because of differences in receptor structure but may also differ because of differences in surface charges near these receptors. The results of the present study establish a simple method for assessing the presence of surface charges near the outer entrance to the pore in K^+ channels. This approach combined with site-specific mutagenesis should allow the molecular identification of these surface charge structures.

We thank Jill Thompson for molecular biological and other technical assistance, for helpful discussions and for critically reading this manuscript. We are grateful to Robert Dirksen and Kevin Gingrich for constructive comments on an earlier version of this manuscript. This work was supported in part by grants from the National Institutes of Health (NS-14138) and the National Science Foundation (IBN-0090662).

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