

State-dependent Accessibility and Electrostatic Potential in the Channel of the Acetylcholine Receptor

Inferences from Rates of Reaction of Thiosulfonates with Substituted Cysteines in the M2 Segment of the α Subunit

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ABSTRACT Ion channel function depends on the chemical and physical properties and spatial arrangement of the residues that line the channel lumen and on the electrostatic potential within the lumen. We have used small, sulfhydryl-specific thiosulfonate reagents, both positively charged and neutral, to probe the environment within the acetylcholine (ACh) receptor channel. Rate constants were determined for their reactions with cysteines substituted for nine exposed residues in the second membrane-spanning segment (M2) of the α subunit. The largest rate constants, both in the presence and absence of ACh, were for the reactions with the cysteine substituted for α Thr244, near the intracellular end of the channel. In the open state of the channel, but not in the closed state, the rate constants for the reactions of the charged reagents with several substituted cysteines depended on the transmembrane electrostatic potential, and the electrical distance of these cysteines increased from the extracellular to the intracellular end of M2. Even at zero transmembrane potential, the ratios of the rate constants for the reactions of three positively charged reagents with α T244C, α L251C, and α L258C to the rate constant for the reaction of an uncharged reagent were much greater in the open than in the closed state. This dependence of the rate constants on reagent charge is consistent with an intrinsic electrostatic potential in the channel that is considerably more negative in the open state than in the closed state. The effects of ACh on the rate constants for the reactions of substituted Cys along the length of α M2, on the dependence of the rate constants on the transmembrane potential, and on the intrinsic potential support a location of a gate more intracellular than α Thr244.

KEY WORDS: conductance • gate • ion selectivity • reaction kinetics • sulfhydryl

INTRODUCTION

Ion channels open, conduct ions selectively, and close. The mechanisms for these functions must reside largely in the residues that line the open channel or obstruct the closed channel. To uncover these mechanisms, we try to determine how the external signals and forces that alter the functional states of an ion channel protein affect both the residues that line the channel and the ions in the channel. Small, charged reagents can serve as surrogates for permeant ions to probe the environment within a channel (Akabas et al., 1992; Stauffer and Karlin, 1994). The rates of reactions of such reagents with cysteines substituted in membrane-spanning segments can be used to identify channel-lining residues, to determine the accessibility of these residues both in the conducting and nonconducting states of the channel, to locate selectivity filters and gates, and

to estimate the electrostatic potential in the vicinity of these residues (Akabas et al., 1994a, 1994b; Akabas and Karlin, 1995; Kurz et al., 1995; Lu and Miller, 1995; Pascual et al., 1995; Kuner et al., 1996; Sun et al., 1996; Xu and Akabas, 1996; Cheung and Akabas, 1997; Liu et al., 1997; Yang et al., 1997; Zhang and Karlin, 1997, 1998). In this paper, we explore the accessibility of channel-lining residues and the electrostatic potential in their vicinity in different functional states of the acetylcholine receptor channel.

The five subunits, $\alpha_2\beta\gamma\delta$, of the muscle-type acetylcholine (ACh)¹ receptor surround the central channel quasi-symmetrically (Unwin, 1993; Galzi and Changeux, 1995; Karlin and Akabas, 1995). The NH₂-terminal half of each subunit is extracellular, and the COOH-terminal half forms three membrane-spanning segments (M1, M2, and M3), a large cytoplasmic loop,

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¹Abbreviations used in this paper: ACh, acetylcholine; AEAETS, 2-aminoethyl-2-aminoethanethiosulfonate; 2-ME, 2-mercaptoethanol; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl-MTS; MTSEH, 2-hydroxyethyl-MTS; MTSET, 2-trimethylammonioethyl-MTS.

a fourth membrane-spanning segment (M4), and a short, extracellular tail. The two ACh binding sites are formed in the extracellular domain in the interfaces between the NH₂-terminal halves of two pairs of subunits, and the channel through the membrane is formed by the membrane-spanning segments of all of the subunits. Residues lining the ion-conducting pathway have been identified on the basis of the functional effects of mutagenesis and by affinity labeling in the M1 segment (DiPaola et al., 1990) and the M2 segment (Huchcho et al., 1986; Imoto et al., 1988; Charnet et al., 1990; Revah et al., 1990; Pedersen et al., 1992) of the different subunits. From the effects of the mutations of charged residues bracketing M2 on rectification and on the sidedness of channel block by Mg²⁺, the NH₂-terminal end of M2 was shown to be intracellular and its COOH-terminal end, extracellular (Imoto et al., 1988).

The systematic identification of all of the channel-lining residues in the M1 and M2 segments of the α and β subunits was approached by the substituted-cysteine-accessibility method (SCAM) (Akabas et al., 1992, 1994a; Akabas and Karlin, 1995; Zhang and Karlin, 1997, 1998). In this method, each residue in the membrane-embedded segments of a channel protein is mutated one at a time to Cys, the mutants are expressed in heterologous cells, and the susceptibility of these substituted Cys to reaction with small, charged, sulfhydryl-specific reagents is determined. If the application of reagent results in an irreversible alteration in the function of the channel, it is inferred that the substituted Cys reacted and, therefore, was exposed in the water-filled lumen of the channel. This inference is based on certain assumptions: in membrane-embedded channel proteins, the sulfhydryl (–SH) group of a native or engineered cysteine residue (Cys) is in one of three environments: in the water-accessible surface, in the lipid-accessible surface, or in the protein interior. We assume that the channel lining is part of the water-accessible surface (Dani, 1989) and further that, in the membrane-spanning domain of the protein, the channel lining is the only water-accessible surface. We assume that hydrophilic, charged reagents will react much faster with sulfhydryls in the water-accessible surface than in the lipid-accessible surface or in the interior of the protein. We synthesized a set of polar sulfhydryl-specific reagents, methanethiosulfonate derivatives, that reacted by the same mechanism and were similar in size, but that differed in their charge (Stauffer and Karlin, 1994). These reagents are directed at water-accessible –SH both because they are polar and because they react at least 5×10^9 faster with dissociated S[–] than with undissociated –SH (Roberts et al., 1986). In the lipid-accessible surface and in the protein interior, ionization of –SH is suppressed because of the low dielectric constant of the environment.

In both M1 and M2 of the ACh receptor, we observed markedly different reactivities in the presence and absence of ACh of several substituted Cys residues to reagents added extracellularly (Akabas et al., 1992, 1994a; Akabas and Karlin, 1995; Zhang and Karlin, 1997, 1998). We ascribed these differences in reactivities to conformational changes concomitant with gating. Because the changes in reactivity were scattered over the length of the channel, with residues affected by ACh near residues not affected by ACh, it was more likely that the reactivities were affected by local conformational changes rather than by a general increase in accessibility due to the opening of a gate closer to the extracellular side than these residues.

In the current work, we determined the rate constants for the reactions of thiosulfonate reagents during brief applications of ACh and in the absence of ACh, with nine susceptible Cys-substituted residues in the M2 segment of the α subunit. We found that the rate constants for the reactions at several, but not all, of the residues were very different in the presence and absence of ACh. In addition, the rates of reaction with positively charged reagents were dependent on the transmembrane holding potential, and this dependence was characteristic of the open state. Even at zero holding potential, the reaction rates of different reagents depended on their charge, indicating that in addition to the extrinsic holding potential there is an intrinsic electrostatic potential in the channel. Furthermore, the profile of intrinsic potential is different in the open and closed states of the channel. The dependence of methanethiosulfonate reaction rates on transmembrane potential was demonstrated in cystic fibrosis transmembrane conductance regulator (Cheung and Akabas, 1997). The intrinsic electrostatic potentials have been estimated from the relative rates of reaction of differently charged methanethiosulfonate reagents in the ACh receptor binding site (Stauffer and Karlin, 1994) and in a vestibule of the Na channel containing residues of the voltage-sensing S4 segment (Yang et al., 1997). A preliminary report of the work in this paper has appeared previously (Pascual and Karlin, 1997b).

METHODS

Mutagenesis and Expression

All mutations were introduced in the M2 segment of the mouse muscle α subunit and capped, runoff cRNA transcripts were obtained for the α -subunit mutants and for wild-type α , β , γ , and δ subunits after linearization of the plasmid cDNA as previously described (Akabas et al., 1994a). cRNAs at a concentration of 1 mg/ml in water were stored at –80°C. They were diluted and mixed for injection at a ratio of 2 α :1 β :1 γ :1 δ . Stage V and VI *Xenopus laevis* oocytes were collected and defolliculated in collagenase following standard procedures (Akabas et al., 1992). Oocytes were injected with 60 nl of cRNA diluted to 1–100 ng/ μ l, de-

pending on desired current expression levels. Cells were kept in culture for 1–10 d before recording.

ACh-induced Current

Currents were recorded under two-electrode voltage-clamp. The oocyte bath solution contained (mM) 115 NaCl, 2.5 KCl, 1.8 MgCl₂, 10 HEPES, pH 7.2, except where indicated otherwise. Solutions flowed at 7 ml/min first through a stainless steel coil immersed in a thermostat at 18.0°C, and then past the oocyte, which was held in a rectangular chamber with a cross-section normal to the direction of solution flow of 4 mm². An agar bridge connected a Ag:AgCl reference electrode to the bath and was placed as close as possible to the oocyte. The bath was clamped at ground potential. We used beveled agarose-cushion (Schreibmayer et al., 1994) glass micropipettes filled with 3 M KCl, resistance ~0.5 MΩ, for both current-passing and voltage-recording electrodes. A few uninjected oocytes from each batch were tested for the presence of endogenous ACh-induced currents, which were never found. The function of wild-type and mutant receptors was assayed as the ACh-induced current elicited by the application of brief (10–20-s) pulses of ACh, at a concentration 10× the EC₅₀, as determined for each mutant, and at a holding potential of -50 mV, except where indicated otherwise. ACh-induced currents ranged from 1 to 25 μA.

Synthesis and Use of Thiosulfonate Derivatives

The positively charged 2-aminoethyl-methanethiosulfonate, CH₃SO₂SCH₂CH₂NH₃⁺ (MTSEA), (Bruice and Kenyon, 1982), and 2-trimethylammonioethyl-MTS, CH₃SO₂SCH₂CH₂N(CH₃)₃⁺ (MTSET), (Stauffer and Karlin, 1994), and the neutral 2-hydroxyethyl-MTS, CH₃SO₂SCH₂CH₂OH (MTSEH), are a set of rapidly reacting, sulfhydryl-specific reagents that differ only in their head groups and whose rates of reaction with a Cys-substitution mutant are readily compared. MTSEA and MTSET were synthesized as previously described and were also purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

MTSEH was newly synthesized by dissolving 40 g of sodium methanethiosulfonate and 41 g of 2-bromoethanol (95%) in acetonitrile. The stirred mixture under argon was refluxed overnight. The mixture was cooled and filtered; the filtrate was concentrated, mixed with methylene chloride, filtered, and concentrated again to yield a yellow oil. 1 ml yellow oil was mixed with 9 ml chloroform, and the small amount of precipitate that formed was removed by centrifugation in a clinical centrifuge. The supernatant was layered on a silica gel (grade 9385, 230–400 mesh; Merck, Darmstadt, Germany) column (32 cm length, 1.9 cm diameter), pre-equilibrated with chloroform (stabilized with 0.75% ethanol). The column was eluted under mild pressure at 3–4 ml/min with 90 ml chloroform and with 260 ml 98% chloroform/2% methanol; the next 100 ml contained the pure product, as determined initially by thin-layer chromatography on silica developed in 98% chloroform/2% methanol. The components were visualized under UV light (254 nm) and by spraying with a mixture containing 1 mM DTNB (5,5'-dithio-bis-2-nitrobenzoate), 0.5 mM dithiothreitol, and trimethylamine in methanol, in which the product gave a white spot against a yellow background. The 100 ml containing the product was reduced to ~10 ml on a rotary evaporator; 20 ml of methylene chloride was added, and the volume was reduced again, first by rotary evaporation, and then on a high vacuum line with liquid nitrogen traps. Approximately 0.6 g of liquid was recovered. By assay with TNB (2-nitro-5-thiobenzoate) (Stauffer and Karlin, 1994), the average purity of three preparations was 98%. The nuclear magnetic resonance

spectrum was consistent with the structure of MTSEH. Mass spectrometry, +FAB ionization, gave MH⁺ 157.

A doubly positively charged thiosulfonate, 2-aminoethyl-2-aminoethanethiosulfonate, NH₃⁺CH₂CH₂SO₂SCH₂CH₂NH₃⁺ (AEAETS), adds the 2-aminoethylthio group to the Cys -SH, just like MTSEA. It was synthesized as previously described (Field et al., 1961, 1964). It was recrystallized by dissolving in methanol, adding about one-third volume of diethyl ether, and storing at 4°C for 2 d. By thin-layer chromatography on cellulose, developed in 60% ethanol/30% 0.1 N HCl/10% t-butanol, the product in methanol gave one ninhydrin-positive spot with an R_f = 0.26. TNB assay gave 95% purity based on a mol wt of 256.9. The melting point was 168–170°C. Mass spectrometry by direct probe electron impact with no solvent gave peaks of 256 and 258, corresponding to the compound with two ³⁵Cl⁻ and to the compound with one ³⁵Cl⁻ and one ³⁷Cl⁻. The nuclear magnetic resonance spectrum was consistent with the structure of AEAETS.

The thiosulfonate reagents are relatively unstable at neutral and alkaline pHs. They hydrolyze to a sulfenic acid (RSOH) and a sulfinate (R'SO₂⁻). In a second step, the sulfenic acid disproportionates to a thiol (RSH) and a sulfinic acid (RSO₂⁻). At pH 7 and 20°C, the half-times for the hydrolysis are 12 min for MTSEA, 11 min for MTSET, and 6 min for AEAETS (Karlin and Akabas, 1998; Stauffer and Karlin, 1994). These reagents are stable for hours, however, in unbuffered water at 4°C. Thus, stocks of the reagents were made daily by dissolving reagent to a concentration of 1–100 mM in water and kept on ice. They were diluted in bath solution just before use. The diluted reagent was placed in a syringe barrel and kept cool by ice-water in a surrounding jacket. The solution was warmed to 18°C as it passed through a thermostated coil just before reaching the oocyte (see above).

The rate constants for the reactions of the thiosulfonates with 2-mercaptoethanol were determined by stopped-flow, rapid-mixing spectrophotometry as previously described (Stauffer and Karlin, 1994). All determinations were at 20°C in 58 mM NaPO₄, 0.1 mM EDTA, pH 7.0, ionic strength 0.130.

Determination of Reaction Rates in Different Receptor States

The time-course of the reaction of a reagent with a substituted-Cys mutant in the absence of ACh was determined by recording the initial response to ACh and subsequent responses to ACh during several repeats of the following sequence: a short application of reagent, a wash with bath solution, an application of ACh, and another wash. Positively charged ammonium reagents could have either agonist activity or channel blocking activities (Sanchez et al., 1986), and these activities could vary with the mutant. Therefore, the reagents were monitored for such reversible actions on each mutant.

In the presence of ACh, the receptor first opens and then desensitizes in two steps, one fast and one slow (Katz and Thesleff, 1957; Sakmann et al., 1980; Neubig et al., 1982; Heidmann et al., 1983; Hess, 1993). To determine the rate of reaction in the open state, we applied reagent plus ACh for short times (10–20 s), during which the extent of slow desensitization was slight. Slow desensitization of all mutants used in this paper took place at a rate ≤0.005 s⁻¹ (not shown), which was not a significant correction to the decrease in current due to the reaction of the MTS reagents. Therefore, during the first 20 s of application of reagent plus ACh, the reaction was with receptor mainly in the open state and the fast desensitized state.

The reactions of MTSEA and AEAETS with αT244C in the presence of ACh were relatively fast and the end-points were complete inhibition of the ACh-induced current. In these cases, the reactions over intervals as brief as 3 s were detectable. Over this short interval, the amplitude of the current decreased ap-

proximately linearly, and the second-order-rate constant, κ , was estimated by

$$\kappa = (I_2 - I_1) / [xI_1(t_2 - t_1)], \quad (1)$$

where I is current, t is time, x is the concentration of reagent, and subscripts 1 and 2 refer to the beginning and end, respectively, of the measurement interval. In this case, it was possible to change the holding potential in successive reaction intervals, and thus obtain the rate constant as a function of holding potential in a single experiment (first protocol). Each such experiment was repeated on at least three different oocytes.

For more slowly reacting mutants and reagents, a second protocol was used to determine the rate constant for the reaction in the presence of ACh. The following sequence of solutions was applied several times: ACh at $10 \times EC_{50}$ for 10–20 s to test the response, bath solution for 2–4 min, reagent plus ACh for 2–20 s, and bath solution for 3–4 min. The holding potential was fixed during the experiment. We determined the rate constant by fitting the peaks of the test currents to

$$I_t = I_\infty - (I_\infty - I_0) \exp(-\kappa xt), \quad (2)$$

where t is the cumulative time of reagent application, the subscripts refer to the cumulative time at which the current was recorded, x is the concentration of reagent, and κ is the second-order-rate constant. These experiments were repeated on oocytes at different holding potentials.

In the presence of ACh, MTSET increased the rate of desensitization so that the decrease in the amplitude of the current in the presence of MTSET had both an irreversible component due to the reaction and a reversible component due to desensitization. We estimated the rate constant for the reaction of MTSET with the open state using the second protocol. We fit the following equation (see APPENDIX B for derivation) to the test currents:

$$\kappa x = (I_{\text{PRE}}/Q_{\text{DUR}}) (1 - Q_{\text{POST}}/Q_{\text{PRE}}), \quad (3)$$

where κ is the second-order rate constant, x is the concentration of reagent, I_{PRE} is the peak current induced by ACh at $10 \times EC_{50}$ obtained before the reaction, Q_{DUR} is the total charge flow (current integrated over time) during the reaction with MTSET in the presence of ACh, Q_{POST} is the total charge flow during the test response to ACh at $10 \times EC_{50}$ after the reaction, and Q_{PRE} is the total charge flow during the test response before the reaction. In practice, MTSET and ACh were applied several times to each oocyte, each preceded and followed by test responses so that the test response after one application of MTSET was the test response preceding the next application. The rate constant for the reaction occurring during each MTSET application was calculated by Eq. 3. Several rate constants were thus obtained from each experiment, and these were averaged using each one to recalculate the degree of reaction in each time period, $\exp(-\kappa xt)$, and from these we calculated the cumulative extent of reaction as a function of the cumulative time of exposure to MTSET. These points were then fit by Eq. 2. This averaging procedure gave the most weight to the first rate constant, involving the greatest change in response, and the least weight to the last, involving the smallest change in response.

A third protocol was used to determine the rate constants for reactions in the absence of ACh, which was identical to the second protocol except that reagent was added in the absence of ACh. The data were fit by Eq. 2.

All oocytes were tested for stability of responses to ACh before any reagents were applied by three to five applications of ACh over a period of 5–15 min. The criterion for acceptable stability was that the peak currents varied $<3\%$ from each other. Thus, run-down or run-up of the responses was $<3\%/5$ min or $0.0001/s$.

RESULTS

Protocols for Determining Rates

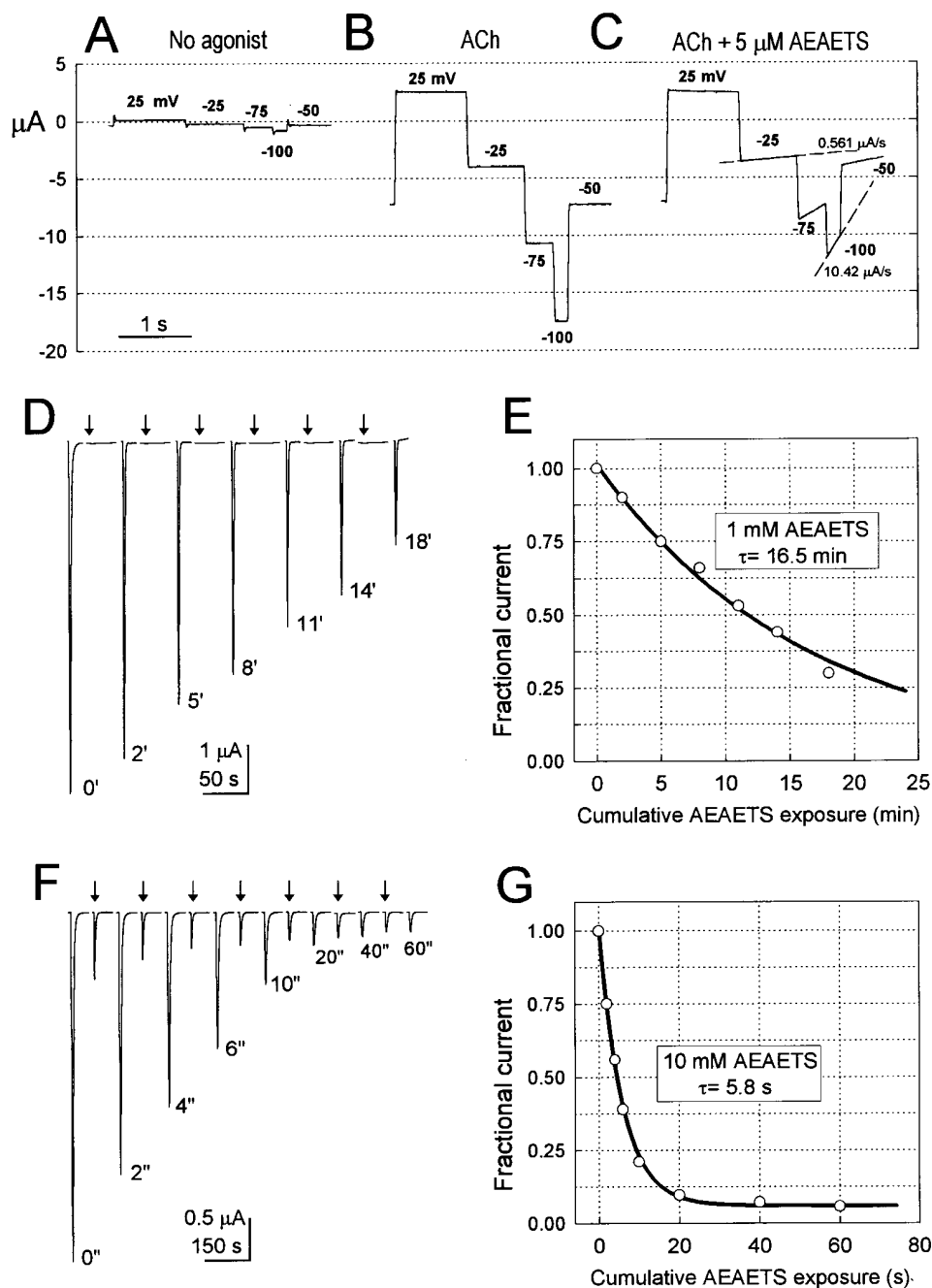
We used three different protocols (see METHODS) to determine the rate constants for the reactions of the thiosulfonate reagents with the Cys-substituted mutants in the presence and absence of ACh and as a function of holding potential. We applied the first protocol to reactions with large rate constants and large effects, in which case short applications of low concentrations of reagent had readily measurable functional effects. In this protocol, we applied the thiosulfonate and ACh continuously for a few seconds, during which time we stepped the membrane potential to four different values. As a control for this protocol, we tested for voltage-gated channels in the oocyte that might respond to the jumps in holding potential and found none (Fig. 1 A). We also found that during the brief applications of ACh, slow desensitization was negligible, and the current was constant (Fig. 1 B). During brief applications of ACh and reagent, the current magnitude declined linearly, as illustrated by the effect of the reaction of $\alpha T244C$ with $5 \mu M$ AEAETS in the presence of $60 \mu M$ ACh (Fig. 1 C). In other experiments, we found that the change in current was irreversible (data not shown). The rate constant at each holding potential was estimated using Eq. 1.

In the second protocol, which we applied to slower reactions, the membrane potential in each experiment was fixed, and we repeatedly applied the sequence: ACh, wash, reagent plus ACh, and wash. This protocol is illustrated for the reaction of $\alpha S248C$ with 10 mM AEAETS plus $100 \mu M$ ACh (Fig. 1 F). In this case also, slow desensitization was negligible during each reaction period (2–20 s). The rate constant was determined by an exponential fit (Eq. 2) to the test ACh responses as a function of the preceding cumulative duration of exposure to reagent (Fig. 1 G).

The third protocol was used for reactions in the absence of ACh. In each experiment, the reagent was applied several times (at a fixed holding potential), interspersed as in the second protocol, with washes and test responses. This is illustrated for the reaction of 1 mM AEAETS with $\alpha T244C$ (Fig. 1 D). The rate constant was determined by fitting Eq. 2 to the test responses as a function of the preceding cumulative duration of the reaction (Fig. 1 E).

Dependence of Rate Constants on Functional State

We determined the rate constants for the reactions of MTSEA with nine substituted Cys in and bracketing $\alpha M2$, previously found to be accessible (Akabas et al., 1994a). The mutants were $\alpha E241C$ at the intracellular end of the channel, $\alpha T244C$, $\alpha L245C$, $\alpha S248C$, $\alpha L251C$, $\alpha S252C$, $\alpha V255C$, $\alpha L258C$, and $\alpha E262C$ at the extracel-



induced current, plotted against the cumulative time of exposure to AEAETS. The solid line is a single exponential fit (see METHODS). (F–G) Reaction of 10 mM AEAETS with α S248C in the presence of ACh. (F) An oocyte was alternatively exposed to 25 μ M ACh in the absence and presence of AEAETS and allowed to recover for 4 min after each application. The following sequence of applications was repeated seven times: ACh for 10 s, wash 4 min, ACh plus AEAETS for 2–20 s (beginning at arrows), and wash 4 min. ACh for 10 s was added again at the end. The clamp potential was -50 mV. The cumulative duration of exposure to AEAETS before each ACh-induced response is indicated next to the peak of current. (G) As in E, normalized peak currents were plotted against the cumulative duration of exposure to AEAETS (\circ).

lular end of the channel. The rate constants were determined for the reactions in the absence and presence of ACh (Fig. 2, Table I).

For the reactions in the absence of ACh, the rate constants ranged from $0.21 \text{ M}^{-1} \text{ s}^{-1}$, for the reaction with

α L258C, to $480 \text{ M}^{-1} \text{ s}^{-1}$, for the reaction with α T244C, a range of 2,300-fold. For the reactions in the presence of ACh, the rate constants ranged from $2.2 \text{ M}^{-1} \text{ s}^{-1}$, for α L245C, to $16,800 \text{ M}^{-1} \text{ s}^{-1}$, for α T244C, a range of 7,600-fold. Both in the absence and presence of ACh,

FIGURE 1. Determination of the rate constants for the reactions of thiosulfonates with substituted cysteines. (A–C) Reaction of AEAETS with α T244C in the presence of ACh. (A) Current recorded in the absence of ACh while clamping an oocyte expressing α T244C receptor to 25 mV for 1 s, -25 mV for 0.8 s, -75 mV for 0.4 s, and -50 mV for 0.25 s. (B) Currents induced by 60 μ M ACh under the same voltage protocol as in A. (C) Currents in the presence of 5 μ M AEAETS plus 60 μ M ACh under the same voltage protocol as in A. Reagent and ACh were added before the start of the first voltage step. The time course of inhibition was approximately linear, and the pseudo-first order reaction rate constant at each voltage was estimated by the slope divided by the current amplitude at the beginning of each voltage interval. Linear fits and slope values are shown for the reactions at -25 and -100 mV. The records in A–C were obtained from the same cell. (D–E) Reaction of AEAETS with α T244C in the absence of ACh. (D) ACh-induced currents before and after applications of 1 mM AEAETS in the absence of ACh. The following sequence of applications was repeated five times: 60 μ M ACh for 10 s, wash 4 min, AEAETS for 2–4 min (beginning at arrows), and wash 3 min; ACh was applied for 10 s to obtain the final response. The cumulative duration of exposure to AEAETS before each ACh-induced response is indicated next to the peak of current. The clamp potential was -50 mV. (E) Peak ACh-induced currents in D (\circ), normalized to the initial ACh-

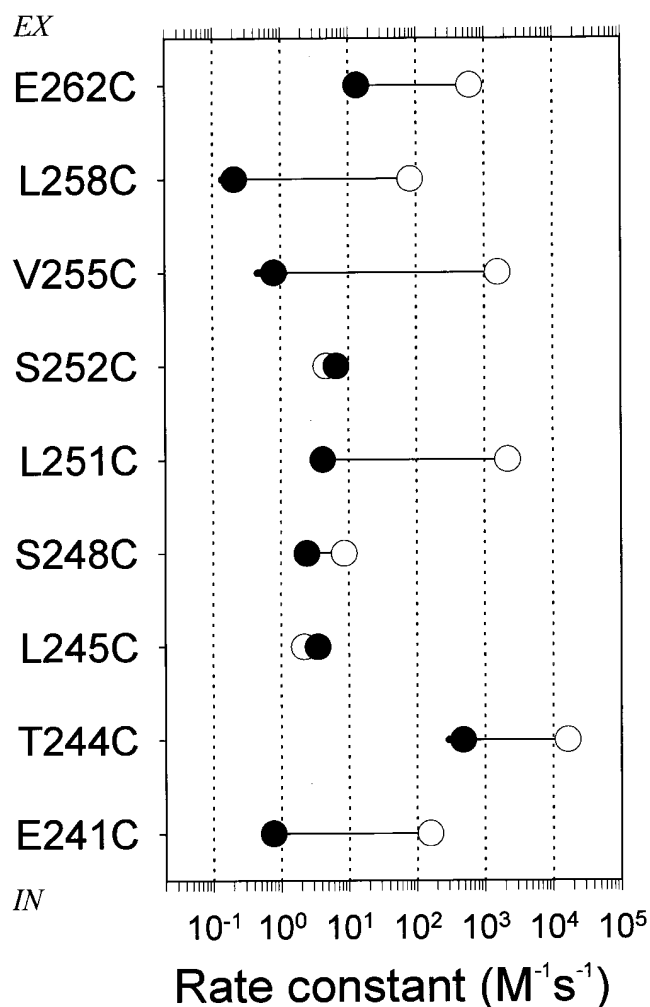


FIGURE 2. Rate constants for the reaction of MTSEA with substituted Cys mutants in the absence (●) and presence (○) of ACh. When reagent was added with ACh, the ACh concentration was $10\times$ the EC_{50} for the mutant. The protocols are given in METHODS. MTSEA concentrations ranged from 2.5 μ M to 5 mM. Horizontal lines connect the mean rate constants in the two conditions. Each symbol is the mean of three to seven independent determinations. Thick lines represent the SEM where it extends beyond the symbol. The holding potential was -50 mV.

extracellularly applied MTSEA reacted fastest with α T244C, close to the intracellular end of the channel.

MTSET was tested only on α T244C and α S248C. Both in the presence and absence of ACh, it reacted more slowly than MTSEA with α T244C, and its rate of reaction with α S248C was too slow to be measured (Fig. 3, Table I).

The rate constants for the reactions of AEAETS with α T244C, α S248C, α L251C, and α L258C were all smaller than those for MTSEA in the absence of ACh and were all larger than those for MTSEA in the presence of ACh (Fig. 3). (The reaction of AEAETS in the absence of ACh with α S248C was too slow to be measured.)

The rate constants for the reactions of the uncharged MTSEH was fast enough to be determined only with α T244C, α L251C, and α L258C. MTSEH reacted more slowly than the other reagents both in the presence and absence of ACh, except with α L258C in the absence of ACh, where all reagents reacted very slowly (Fig. 3).

As a rule, the charged reagents reacted faster than the uncharged MTSEH. Among the charged reagents, size and charge both influenced the rates of reaction. MTSEA (+1) is smaller than MTSET (+1), which is smaller than AEAETS (+2). In the absence of ACh, size appears to be more important than charge; in the presence of ACh, both charge and size were important (Fig. 3).

In most cases, the rate constants for the reactions of the thiosulfonates with substituted Cys in the channel were orders of magnitude slower than the rate constants for their reactions with 2-mercaptoethanol in homogeneous solution (Table I). Even the fastest reaction of MTSEH in the channel, with α L251C in the presence of ACh, was $1,200\times$ slower than the reaction of MTSEH with 2-mercaptoethanol in solution. The fastest reactions of MTSEA and MTSET, with α T244C in the presence of ACh, were only four times slower than the reactions with 2-mercaptoethanol, but the reactions with α T244C in the absence of ACh were five to six orders of magnitude slower than the reactions with 2-mercaptoethanol. As discussed later, the reactions in the channel can be slowed by low accessibility to the Cys, steric hindrance around the Cys, and suppressed ionization of the Cys $-SH$. These retarding influences can be partly compensated by the electrostatic potential in the channel.

The reactions of MTSEA with six of the nine substituted Cys were much faster in the presence of ACh than in its absence (Fig. 2). The rate constants were larger by factors ranging from 35 for α T244C to 1,970 for α V255C (Table I). For MTSET and AEAETS, also, the rate constants for the reaction with α T244C in the presence of ACh were much larger than in the absence of ACh (Fig. 3). For AEAETS, the rate constant was larger by a factor of 51,000 (Table I). Also, AEAETS reacted much faster with α S248C, α L251C, and α L258C in the presence of ACh than in its absence.

MTSET evoked a small current in α T244C in the absence of ACh. Similarly, AEAETS evoked a small current in α L258C in the absence of ACh. Even in these two cases, where the reagents themselves evoked a detectable current, the reactions were still far faster in the presence of ACh, when the current was large, than in the absence of ACh, when the current was small (Fig. 3).

Not all reactions were accelerated by the addition of ACh. For the reactions of α L245C and α S252C with MTSEA, the rate constants in the presence and absence of ACh were barely distinguishable, and, for the reac-

TABLE I
Rate Constants for the Reactions of the Thiosulfonates with Substituted Cysteines

Mutants		MTSEH	MTSEA	MTSET	AEAETS
αE262C	-ACh		13 ± 3 (4)		
	+ACh		620 ± 120 (3)		
	+ACh/-ACh		47		
αL258C	-ACh	0.31 ± 0.05 (3)	0.21 ± 0.07 (4)		0.21 ± 0.10 (2)
	+ACh	3.7 ± 0.7 (3)	83 ± 4 (5)		146 ± 22 (4)
	+ACh/-ACh	12	395		695
αV255C	-ACh		0.80 ± 0.30 (3)		
	+ACh		1580 ± 350 (4)		
	+ACh/-ACh		1970		
αS252C	-ACh		6.6 ± 1.1 (4)		
	+ACh		4.7 ± 0.7 (4)		
	+ACh/-ACh		0.71		
αL251C	-ACh	0.24 ± 0.003 (3)	4.2 ± 0.5 (3)		0.87 ± 0.20 (3)
	+ACh	7.9 ± 2.4 (3)	1690 ± 180 (3)		9900 ± 5300 (4)
	+ACh/-ACh	33	402		11400
αS248C	-ACh	0 (3)	2.4 ± 0.3 (6)	0 (4)	0 (4)
	+ACh	0 (3)	8.6 ± 1.3 (9)	0 (4)	19.8 ± 2.9 (3)
	+ACh/-ACh		3.6		
αL245C	-ACh		3.5 ± 0.3 (4)		
	+ACh		2.2 ± 0.2 (4)		
	+ACh/-ACh		0.6		
αT244C	-ACh	0.22 ± 0.02 (3)	480 ± 190 (4)	2.6 ± 0.3 (4)	1.25 ± 0.29 (4)
	+ACh	0.25 ± 0.02 (9)	16800 ± 3600 (9)	600 ± 180 (6)	63600 ± 13100 (6)
	+ACh/-ACh	1.1	35	230	51000
αE241C	-ACh		0.77 ± 0.21 (6)		0 (3)
	+ACh		158 ± 17 (4)		0 (3)
	+ACh/-ACh		205		
HOCH ₂ CH ₂ SH		9530 ± 230	76300 ± 4300	212000 ± 8000	261000 ± 6000

The rate constants for the reactions with the mutants were determined at a holding potential of -50 mV. Rate constants are second-order with units, 1 M/s. Mean rate constants ± SEM (number of experiments) are presented. Rate constants for reactions that were slower at the highest concentration of reagents tested than the run down of the oocytes (0.0001/s) are given a value of 0. Rate constants for the reactions with 2-mercaptoethanol were determined as described in METHODS and are the average of at least two independent experiments with five runs each.

tion of MTSEA with αS248C, the rate constants differed only by a factor of 3.6 (Fig. 2). Also, the rate constant for the reaction of the uncharged MTSEH with αT244C was unchanged by the addition of ACh. There is no obvious correlation between the distance of a substituted Cys from the extracellular end of the channel and the effects of ACh on the rate constant. If there were a gate in the middle of M2, then the opening of this gate should have increased the rates of reaction of all substituted Cys distal to it. No such simple pattern of effects of ACh is apparent.

Reaction Rates in the Desensitized State

In the presence of ACh, the receptor opens and also undergoes transitions in two steps, fast and slow, to desensitized states (Katz and Thesleff, 1957; Sakmann et al., 1980; Neubig et al., 1982; Heidmann et al., 1983;

Hess, 1993). To estimate the rate constant for the reaction of MTSEA with αT244C in the slow desensitized state, we applied 60 μM ACh for several minutes until the current had decreased to ~20% of its peak value. At this point, at least 80% of the receptors were in the desensitized state and some fraction of the remainder were in the open state. After a wash of 15 s, brief compared with the half-time of ~1 min for recovery from desensitization, we added 85 μM MTSEA for 30 s (Fig. 4). Based on the estimates of the rate constants above, this application of MTSEA would have modified over 99% of receptors if ACh had been added simultaneously with MTSEA and 25% of receptors in the absence of ACh. After the MTSEA application, the responses to brief applications of ACh recovered to 75% of the initial amplitude; i.e., there was 25% irreversible inhibition, as would be expected in the absence of ACh. A subsequent application of MTSEA in the absence of

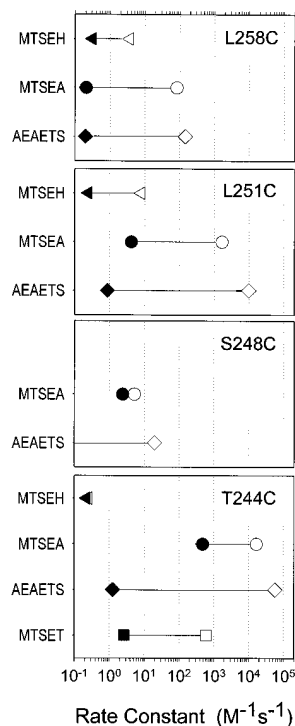


FIGURE 3. Comparison of the rate constants for the reactions of AEAETS (diamonds), MTSEA (circles), MTSET (squares), and MTSEH (triangles) with substituted Cys mutants in the absence (filled symbols) and presence (open symbols) of ACh. Thick lines indicate the SEMs of the rate constants where larger than the symbol. The reactions of MTSET and MTSEH with α S248C, both in the presence and absence of ACh, were undetectable. For α L258C, the rate constant for the reaction with AEAETS in the absence of ACh is an upper limit because AEAETS induced some current. The holding potential was -50 mV. Each symbol is the average of two to seven independent measurements.

ACh caused a similar irreversible inhibition of the response. In five similar experiments using different MT-SEA concentrations, we estimated the rate constants for the reaction with the desensitized state from the extent of irreversible inhibition due to a 30-s application of 2.5 or 85 μ M MTSEA to mostly desensitized receptors. The rate constant for the reaction with the desensitized state was 110 ± 26 $M^{-1} s^{-1}$, compared with 480 ± 190 $M^{-1} s^{-1}$ in the absence of ACh, and $16,800 \pm 3,600$ $M^{-1} s^{-1}$ during brief applications of ACh. The difference between the first two rate constants is not statistically significant.

Dependence of the Reaction Rates on the Transmembrane Electrostatic Potential

The electrostatic potential at each point in the channel is a sum of an intrinsic electrostatic potential, ψ_s , due to charges in the surrounding protein and in the channel, and of a fraction, δ , of the extrinsic, transmembrane potential, ψ_M . We now consider the effect of ψ_M on the rate constants of the reactions of the thiosulfonates with the various substituted Cys. As a first approximation (see DISCUSSION), we characterize these effects in terms of the equation, $\kappa = \kappa_0 \exp(-z\delta\beta\psi_M)$, where κ_0 is the effective rate constant at zero holding potential, z is the algebraic charge on the reagent, δ is the electrical distance from the extracellular medium to the probed residue, and β is $F/(RT)$.

In the absence of ACh, the rate constants for the reactions of α T244C with AEAETS, MTSEA, and MTSEH

were not significantly dependent on ψ_M in the range of -100 to 0 mV (Fig. 5). The least-squares fit of the above equation yields $z\delta$ equal to 0.04 ± 0.02 , 0.05 ± 0.05 , and 0.008 ± 0.04 , respectively. The addition of these reagents caused no detectable increase in leak current, and hence the channel remained predominantly closed. The reaction with MTSET, however, was significantly dependent on holding potential, and the least-squares fit of κ versus ψ_M yielded $z\delta$ equal to 0.38 ± 0.02 . In this case, however, MTSET (7.5 μ M) induced a small current, $\sim 5\%$ as large as that induced by 60 μ M ACh, and it is likely that the voltage dependence of the rate constant was characteristic of the open state in which the reaction was predominantly occurring.

In the presence of ACh, reactions of AEAETS and MTSET with α T244C were dependent on the holding potential (Fig. 6). The least-squares fit of κ versus ψ_M yielded $z\delta$ equal to 0.33 ± 0.05 for AEAETS and equal to 0.42 ± 0.06 for MTSET. (These values are based on the simple equation above; the values of $z\delta$ given in the legend to Fig. 6 are based on the more complicated Eq. 7 in the DISCUSSION.) The reaction rates of MTSEA and MTSEH, however, were not significantly dependent on ψ_M , with $z\delta$ equal to -0.008 ± 0.04 and -0.09 ± 0.12 , respectively.

That the rate of reaction of MTSEH was independent of holding potential is consistent with its neutrality. The absence of voltage dependence of the reaction of MTSEA, however, was unexpected. One possibility is that MTSEA could enter the channel and react as a deprotonated, uncharged amine. MTSEA is partly deprotonated at pH 7.2, the pH of the bath solution. Because MTSEA hydrolyzes rapidly at alkaline pH, it is difficult to obtain a titration curve, but from the initial part of such a curve, we could estimate that the pK_a is no lower than 8.5. MTSEA would be 5% deprotonated if the pK_a of the amine were 8.5. Lowering the pH of the bath solution to 6.5, however, which decreased the fraction of deprotonated MTSEA fivefold, did not alter the rate of reaction of MTSEA with α T244C in the absence of ACh, at either -100 or -50 mV (Fig. 5, unfilled hexagons with dot). Thus, it was predominantly the charged form of MTSEA that reacted with α T244C, and the apparent absence of voltage dependence of the reaction rate was not due to the fraction of uncharged MTSEA. We will argue below that the lack of voltage dependence of MTSEA is likely a result of its permeability through the open channel.

The reactions of AEAETS in the presence of ACh with substituted Cys closer to the extracellular end of the channel than α T244C were also voltage dependent (Fig. 6). The value of $z\delta$ decreased as the distance from the extracellular end of the channel decreased. The fit of κ versus ψ_M yielded $z\delta$ equal to 0.21 ± 0.04 for α S248C, 0.17 ± 0.04 for α L251C, and 0.007 ± 0.01 for α L258C. Thus, the reactions of AEAETS with α T244C,

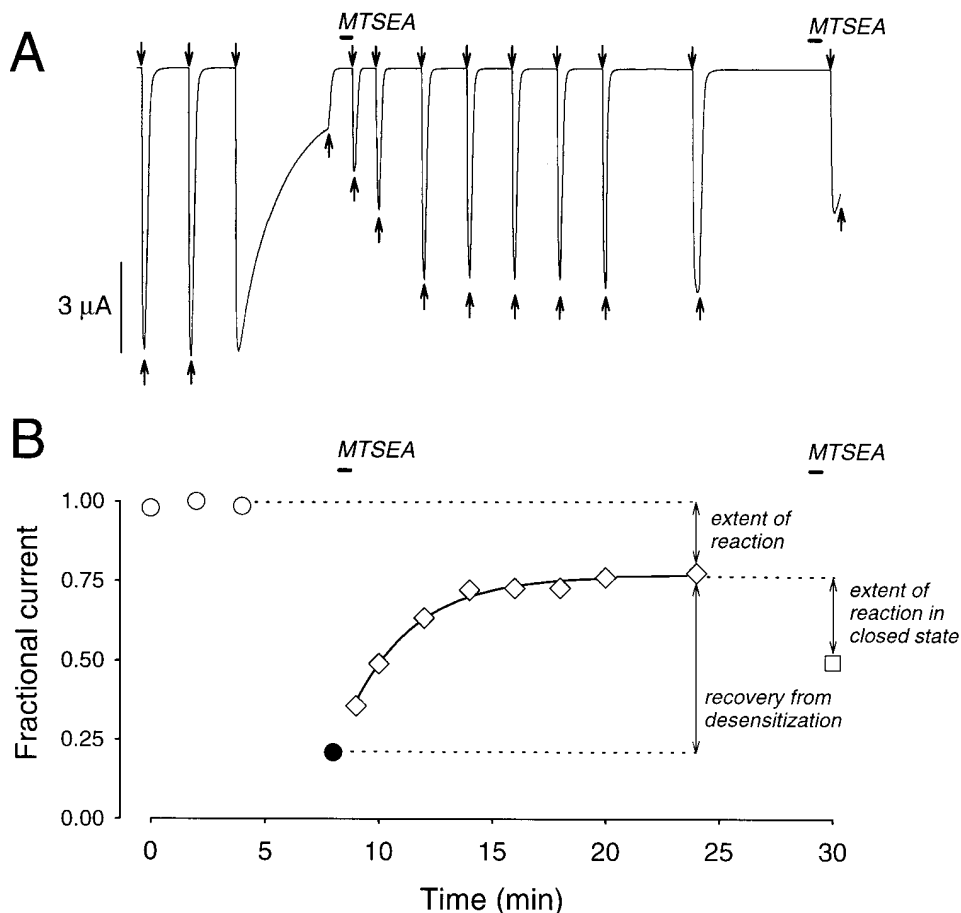


FIGURE 4. Reaction of α T244C in the desensitized state. (A) Experimental protocol for determining the extent of MTSEA reaction with α T244C in the desensitized state. Applications of 60 μ M ACh are indicated by downward arrows, of bath solution by upward arrows, and 85 μ M MTSEA by horizontal bars. The time scale is given as the abscissa in B. Two initial responses elicited by 10-s applications of ACh were followed by a 4-min application of ACh, during which the current declined to 20% of its peak value by desensitization. Bath solution was applied for 15 s, and then MTSEA was applied for 30 s. After a 15-s wash, ACh was re-applied several times and the responses increased as the receptors recovered from desensitization. MTSEA was applied again for 30 s to the receptors in the resting state, and the effect of this application was assayed by a final application of ACh. The holding potential was -50 mV. (B) The inhibition, $1 - I_t/I_0$, due to both desensitization and the reaction of MTSEA is plotted as a function of the recording time, corresponding to the experiment in A. The recovery from desensitization was fit by a single exponential function with a time constant of 106 ± 27 s ($n = 5$), characteristic of the mutant α T244C.

α S248C, and α L251C were significantly dependent on ψ_M . For MTSEA, $z\delta$ was equal to -0.08 ± 0.09 at α S248C and 0.04 ± 0.008 at α L251C. There was no significant dependence of the reactions of MTSEA on membrane potential.

DISCUSSION

Controlling the States of the Receptor and the Channel

We have determined the rates of reaction of substituted Cys in the channel in the presence and absence of ACh, and we would like to associate these rates with the open and closed states of the channel. Neither in the presence nor absence of ACh, however, is the receptor and its channel in a single state. Four different principal functional states of the receptor have been characterized, resting, active, fast desensitized, and slow desensitized (Katz and Thesleff, 1957; Sakmann et al., 1980; Neubig et al., 1982; Heidmann et al., 1983; Hess,

1993). The channel is open only in the active state and is closed in the other three states. Immediately upon binding ACh, the receptor undergoes a sub-millisecond transition from the resting state to the active state. In the continued presence of ACh for hundreds of milliseconds, the receptor enters the fast desensitized state and then, in tens of seconds, the slow desensitized state. The occupied receptor reaches an equilibrium distribution among the different states, which favors the desensitized state.

Could the reaction rates measured in the absence of ACh be due to reaction occurring during spontaneous openings? In the absence of ACh, wild-type receptor opens spontaneously, but with a probability $<10^{-5}$ (Jackson, 1989). One of the mutants used here, α L251C, however, has a higher spontaneous open probability than wild type, although the open probability of unliganded receptor is still orders of magnitude lower than the open probability of doubly liganded receptor (Auerbach et al., 1996). We did not detect any difference between leak

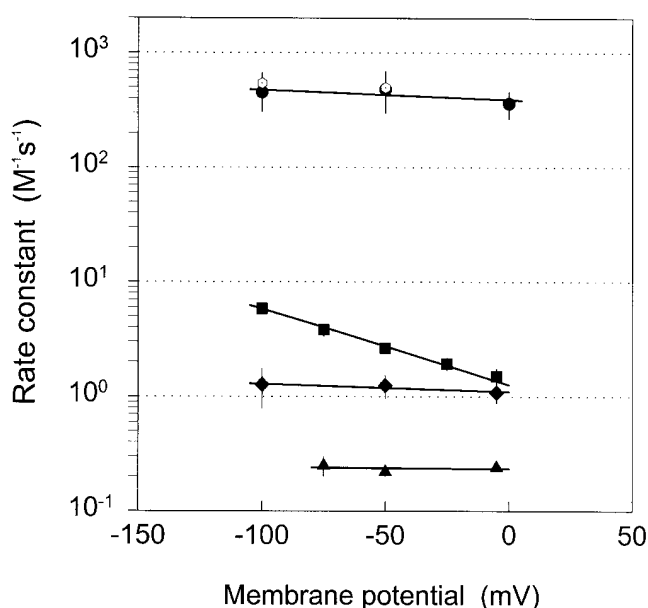


FIGURE 5. Rate constants for the reactions of thiosulfonate derivatives with α T244C in the absence of ACh as a function of membrane potential. Second-order rate constants for reactions with AEAETS (filled diamonds), MTSEA (filled circles), MTSEA at pH 6.5 (unfilled hexagons with dot), MTSET (filled squares), and MTSEH (filled triangles) are shown as a function of membrane potential. Nonlinear-least-squares fit of the data by Eq. 7 yielded the following parameters (parameters without errors were assumed): for MTSEA, $C = 390 \pm 55$, $D = 0$, $z\delta = 0.05 \pm 0.05$; for AEAETS, $C = 1.10 \pm 0.06$, $D = 0$, $z\delta = 0.040 \pm 0.021$; for MTSET, $C = 1.28 \pm 0.08$, $D = 0.01$, $z\delta = 0.40 \pm 0.02$. These parameters were used to generate the curves. For MTSEH, for which $z = 0$, a line of zero slope was drawn at the mean κ .

currents in uninjected oocytes and leak currents in oocytes expressing either wild-type receptor or any Cys-substituted mutant. The leak current was often $\sim 0.5\%$ of the ACh-induced current. We also did not detect any change in leak current with any mutant when we added

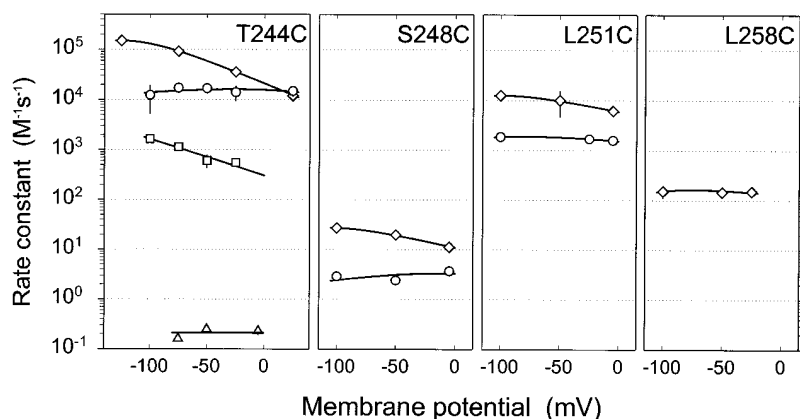


FIGURE 6. Rate constants for the reactions of Cys-substituted mutants in the presence of ACh with AEAETS (\diamond), MTSEA (\circ), MTSET (\square), and MTSEH (\triangle) as a function of membrane potential. Means and SEM for three to seven determinations are shown. Nonlinear least squares fit of the data by Eq. 7 yielded the following parameters (parameters without errors were assumed), which were used to generate the curves: for α T244C and MTSEA, $C = 46,900 \pm 4,190$, $D = 2$, $z\delta = 0.38 \pm 0.04$; for α T244C and AEAETS, $C = 21,200 \pm 410$, $D = 0.007 \pm 0.0004$, $z\delta = 0.54 \pm 0.009$; for α T244C and MTSET, $C = 307 \pm 60$, $D = 0.01$, $z\delta = 0.44 \pm 0.06$; for α S248C and MTSEA, $C = 6.6 \pm 1.8$, $D = 1$, $z\delta = 0.28 \pm 0.12$; for α S248C and AEAETS, $C = 11 \pm 0.5$, $D = 0.007$, $z\delta = 0.31 \pm 0.02$; for α L251C and MTSEA, $C = 1,680 \pm 16$, $D = 0.09 \pm 0.003$, $z\delta = 0.15$; for α L251C and AEAETS, $C = 6,060 \pm 270$, $D = 0.007$, $z\delta = 0.26 \pm 0.01$; for α L258C and AEAETS, $C = 131 \pm 130$, $D = 0.007$, $z\delta = 0.12 \pm 0.04$. For α T244C and MTSEH, $z = 0$, and the overall mean of the mean κ at the three ψ_M , 0.21 ± 0.05 , is plotted with zero slope.

the open-channel blocker QX-314. Therefore, the fraction of receptors that was open in the absence of ACh was very small compared with the fraction that was open immediately after adding ACh.

The reagents themselves could activate the receptor, as do high concentrations of some other amines (Sanchez et al., 1986). The quaternary ammonium MTSET does act as a low affinity agonist of the receptor, and AEAETS is a weak agonist of the mutant α L258C. In these cases, reagent-induced current is readily detected. In no other cases, however, did we detect a reagent-induced increase in current. Also, the second-order rate constant for the reaction of MTSEA with α T244C in the absence of ACh was independent of MTSEA concentration, which would not be the case if MTSEA were both activating the receptor and reacting with it. Furthermore, all of the accessible Cys mutants from α E241C to α V255C are protected against reaction with MTSEA by the open-channel blocker QX-314 in the presence of ACh, but not in the absence of ACh (Pascual and Karlin, 1997a). This is evidence that the reaction in the absence of ACh is predominantly with the closed state of the channel and not with a spontaneously open state or with a reagent-induced open state. We will also argue below that the dependence of reaction rates on the transmembrane potential is a characteristic of the open state and not of the closed states, and we observed voltage dependence of the rates only when we also detected receptor-mediated currents.

After brief exposure to ACh, the receptors are distributed among the resting, active, and desensitized states. This distribution, which is dependent on the kinetics of the transitions between states, could vary somewhat among the mutants, although, from the maximum currents obtained, none of the mutants appeared to have a low open probability. Nevertheless, some of the variation among the mutants in the effects

of ACh on reaction rates could be partly due to differences in gating kinetics.

For one mutant, α T244C, MTSEA reacted 35 \times faster during a brief exposure to ACh than in the absence of ACh. To get an estimate of the rate in the desensitized state, we carried out the same reaction after the mutant receptor was \sim 80% (or more) desensitized (Fig. 4). The rate constant in this largely desensitized state was no faster than that in the resting state and much slower than that in the open state. We do not know the rate constant for the reaction in the fast desensitized state. It is likely, however, that those reactions in the presence of ACh that were dependent on holding potential or that were retarded by open channel blockers were predominantly with the open state and not with any of the closed states, including the fast desensitized state.

Factors Determining the Reaction Rates

The rate constants for the reactions of the thiosulfonates with the substituted Cys depend on properties of the channel pathway to the Cys, properties of the Cys, and properties of the reagent. Obviously, changes in the structure of the channel underlying changes in its functional state could affect both access to a Cys residue and its local environment.

The overall rate of reaction cannot be faster than the rate of passage of the reagent from the bath to the vicinity of the $-SH$. We assume that the pathway is the water-filled channel. For a number of substituted Cys, the voltage sensitivity of the reaction rate and protection against the reaction by open-channel blockers support this assumption. The rate of passage through the channel from the extracellular medium to the Cys could be very different in the open and closed states of the channel because of differences in the structure of the lining or of water in the channel (Green and Lu, 1995). In addition, the movement of charged reagents to the target Cys could be affected by the electrostatic field along the pathway (see below).

The reactivity of a target Cys is largely determined by its local environment. A major factor in the reactivity is the pK_a of the $-SH$, because thiosulfonates react 9–10 orders of magnitude faster with a deprotonated $-S^-$ than with a protonated $-SH$ (Roberts et al., 1986). A Cys facing the water-filled channel should react faster than one facing other residues or lipid, both because the ionization of the $-SH$ is more likely in the environment with the higher dielectric constant and because there is more room to form an activated complex. The positions and configurations of the residues surrounding the channel are likely to be fluctuating and, therefore, so are the extent of exposure and the degree of steric hindrance to the formation of an activated complex. The reactions with Cys $-SH$ exposed in rare or short-lived fluctuations should be much slower than re-

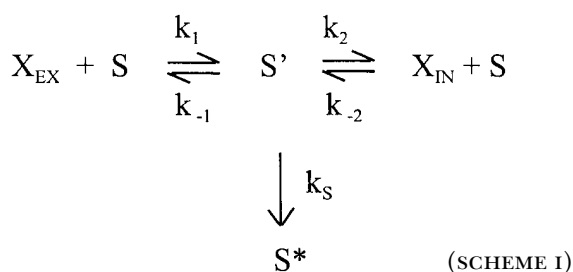
actions with residues exposed most or all of the time. The wide range of rate constants for the reactions with the set of substituted Cys that we consider exposed could be due in part to differences in their pK_a and in part to their degrees of exposure and steric hindrance. These factors also could account for the Cys in the channel reacting much slower, in most cases, than 2-mercaptoethanol in solution (Table I).

Although the thiosulfonate reagents used here have a common reaction mechanism, there are differences in size and charge. We observed in some mutants that the addition of ACh had different effects on the reactions of the different reagents (Fig. 3). For example, at α T244C, the rate constants for MTSEA, MTSET, and AEAETS were all much larger in the presence of ACh than in its absence, whereas for MTSEH the rate constants in the presence and absence of ACh were the same. Size differences cannot explain these results because MTSEH is the same size as MTSEA. Also, the rate constant for MTSEH is much smaller than the rate constant for MTSEA both in the presence and absence of ACh. That MTSEH is uncharged must be a major factor in its smaller rate constant and in the lack of effect on the rate constant of channel opening. At α S248C, by contrast, the size of the reagents and local steric hindrance around the $-SH$ must play an important role in the reactions, because MTSET, with a relatively bulky trimethylammonium head-group, did not react with α S248C, even though it can pass this position to react with α T244C. MTSEA and AEAETS, with unsubstituted ammonium head groups, did react with α S248C. At α L251C, the rate constants for the reactions of MTSEH, MTSEA, and AEAETS are all larger in the presence of ACh than in its absence (Fig. 3), and the qualitative similarity of the effects of ACh on the rates is consistent with an increase in the exposure of this residue to all three reagents or an increase in the pK_a of the Cys. That the effect is 10,000-fold for AEAETS, 200-fold for MTSEA, and 40-fold for MTSEH indicates that factors in addition to changes in exposure or pK_a affect these rates.

Electrostatics and the Kinetics of Reactions in the Channel

Uncharged MTSEH reacted much more slowly than positively charged MTSEA, MTSET, and AEAETS with α T244C, especially in the open state (Fig. 3). This result and others discussed below suggest that charge and, therefore, electrostatic potential play important roles in the reactions of these reagents in the channel. The electrostatic potential sensed by a charged reagent in the channel is the electrical distance times the extrinsic transmembrane potential, $\delta\psi_M$, plus the intrinsic electrostatic potential, ψ_S . The intrinsic potential in the channel arises from permanent charges in the surrounding protein, from the difference in the dielectric constants of the channel and the surrounding protein,

and from other ions and water in the channel (Dani and Eisenman, 1987; Green and Andersen, 1991; Konno et al., 1991; Green and Lu, 1995; Eisenberg, 1996). We present a simple model for the kinetics of a reaction in a channel and for the dependence of the reaction rate on electrostatic potential. This model places the target Cys in a site in the channel, with a barrier on either side, and the rate constants for crossing these barriers are treated according to absolute-reaction-rate theory (Woodhull, 1973; Dani and Eisenman, 1987; Hille, 1992). The advantages and limitations of this simple approach to ion permeation have been discussed elsewhere (Dani and Levitt, 1990; Hille, 1992). We assume that the jumps to and from the site, but not the reaction itself, depend on the electrostatic potential. The electrostatic contribution to the heights of the barriers involves both ψ_M and ψ_S , and the kinetic equations based on the model allow us to estimate the electrical distance, δ , to the site of reaction, and the intrinsic electrostatic potential, ψ_S , at the site. The kinetic steps are indicated in Scheme I.



X_{EX} is the reagent in the extracellular medium, X_{IN} is the reagent in the intracellular medium, S is the unoccupied site with an unreacted Cys, S' is the site reversibly occupied by the reagent, and S^* is the site with the Cys covalently modified by the reagent. The rate constants for the jumps (associations and dissociations) of X are k_1 from the extracellular medium to S , k_{-1} from S to the extracellular medium, k_2 from S to the intracellular medium, and k_{-2} from the intracellular medium to S . k_S is the pseudo-first-order rate constant for the covalent reaction of X and the Cys in the complex S' .

The concentration of unreacted sites (and Cys) is $s_0 - s^*$, where s_0 is the initial and total concentration of sites and s^* is the concentration of modified sites. Experimentally, the concentration of unreacted Cys is estimated from the current, I , elicited by ACh before the addition of X and after the reaction with X (and removal of unreacted X) as

$$(s_0 - s^*)/s_0 = (I - I_\infty)/(I_0 - I_\infty), \quad (4)$$

where I_∞ is the current after reaction of all channels and I_0 is the current before any reaction (see Eq. 2). The solution of the differential equations corresponding to Scheme I is

$$(s_0 - s^*)/s_0 = \exp(-\lambda t), \quad (5)$$

where λ is a combination of the rate constants and concentrations of reactants (APPENDIX A). When the reagent is applied just from the extracellular side, and $x_{\text{IN}} = 0$, then $\lambda = \kappa x_{\text{EX}}$, where κ is the effective second-order rate constant for the reaction and

$$\kappa = k_S k_1 / (k_{-1} + k_2 + k_S). \quad (6)$$

Applying absolute-reaction-rate theory to the movement of X to and from the site, we obtain expressions for the rate constants that depend on free energy differences between ground and transition states at the peaks of the barriers. For X with charge z , these free energies contain electrostatic terms that depend on z . The electrical distances to the barriers are taken to be midway between the site and the medium on either side; i.e., the electrical distances from the extracellular medium to the barriers are $\delta/2$ for barrier 1 and $(1 + \delta)/2$ for barrier 2 (Woodhull, 1973). We assume that k_S is independent of ψ_M and ψ_S ; i.e., that any separation of charge that might occur in the formation of the activated complex between X and the Cys is over too short a distance to be influenced by the gradients in ψ_M and ψ_S at S . (The possible effect of the electrostatic potential on the ionization of the Cys $-SH$ is discussed below.) We obtain

$$\begin{aligned}
 \kappa = k_S (k_1^0/k_{-1}^0) \exp(-z\beta\delta\psi_M) / \\
 [1 + (k_2^0/k_{-1}^0) \exp(-z\beta\psi_M/2)], \quad (7)
 \end{aligned}$$

where k_1^0 , k_{-1}^0 , and k_2^0 are the rate constants at $\psi_M = 0$, and $\beta = F/(RT)$. Eq. 7 has three independent parameters, $C = k_S k_1^0/k_{-1}^0$, $D = k_2^0/k_{-1}^0$, and δ .

For a relatively impermeant reagent that can jump from the extracellular medium to S , but not from S to the intracellular medium, $D = k_2^0/k_{-1}^0 \ll 1$. Therefore, $\ln \kappa = \ln C - z\beta\delta\psi_M$, and the slope of $\ln \kappa$ vs. ψ_M is $-z\beta\delta$ (see Woodhull, 1973). For a permeant reagent, however, the slope will be smaller in magnitude than $z\beta\delta$. For example, for a positive reagent that can leave S at the same rate to either side of the membrane (i.e., $k_2^0 = k_{-1}^0$), Eq. 7 predicts that the slope of $\ln \kappa$ vs. ψ_M would be small and negative or even positive (apparent $\delta < 0$) for negative ψ_M and would approach $-z\beta\delta$ only for large, positive ψ_M . A constant slope over the entire range of ψ_M is characteristic either of an impermeant reagent ($k_2^0 = 0$) or of the condition, $k_2^0 \gg k_{-1}^0$ (in which case the apparent $\delta = \delta - 0.5$).

The electrical distance, δ , refers to the location(s) in the transmembrane electric field of the reagent charge(s) when the reagent is in a position to react with the probed Cys. The charged ammonium group in MTSEA and MTSET is separated by $\sim 5 \text{ \AA}$ from the sulfur atom that reacts with the Cys $-S^-$. In AEAETS, each of the two charges is $\sim 5 \text{ \AA}$ from the reactive sulfur. The charge(s) of these reagents could be as much as one

helical turn above or below the reacting Cys. Neither the distributions of configurations and orientations of the reagents in the vicinity of the probed Cys nor the orientations that can lead to reaction are known. We therefore take δ to be the mean of the δ s of the charge(s) of the reagent in its productive configurations and orientations. We assign this mean δ to the probed residue, even though it could refer to a location as much as 5 Å away. We assume that $z = 1$ for MTSEA and MTSET and that $z = 2$ for AEAETS.²

Under certain conditions, the intrinsic electrostatic potential at S, ψ_S , can be estimated from the ratio of the effective rate constants, ${}^1\kappa$ and ${}^2\kappa$, for two reagents, 1X and 2X , with unequal charges, z_1 and z_2 (Stauffer and Karlin, 1994). We take the ratio of the effective rate constants extrapolated to $\psi_M = 0$. From Eq. 7 and the expression of the rate constants in terms of the barrier heights (APPENDIX A), we obtain at $\psi_M = 0$.

$$\begin{aligned} {}^1\kappa^0/{}^2\kappa^0 &= ({}^1k_S/{}^2k_S) ({}^1g_1/{}^1g_{-1}) ({}^2g_{-1}/{}^2g_1) \\ &\quad \{ \exp [-(z_1 - z_2) \beta \psi_S] \} \\ &\quad (1 + {}^2k_2^0/{}^2k_{-1}^0) / (1 + {}^1k_2^0/{}^1k_{-1}^0), \end{aligned} \quad (8)$$

where ${}^1g_1/{}^1g_{-1}$ and ${}^2g_1/{}^2g_{-1}$ are the nonelectrostatic contributions to the "affinity constants," ${}^1k_1^0/{}^1k_{-1}^0$ and ${}^2k_1^0/{}^2k_{-1}^0$, of 1X and 2X , for the site.

The extraction of ψ_S from Eq. 8 requires its simplification. Three conditions would allow this simplification. One condition is that the ratio of the rate constants for the reactions of the two reagents with the target Cys in the channel, ${}^1k_S/{}^2k_S$, is close to the ratio of the rate constants for the reaction of the two reagents with a small thiol such as 2-mercaptoethanol (2-ME); i.e., ${}^1k_S/{}^2k_S = {}^1k_{ME}/{}^2k_{ME}$ (Stauffer and Karlin, 1994). The rate constants themselves for the reactions of a given reagent with Cys in the channel and 2-mercaptoethanol in solution are likely to be very different because of differences in thiol pK_a and steric hindrance in the two environments. Nevertheless, we assume that these factors affect both reagents more or less equally so that the ratio of their rate constants with Cys in the channel and 2-mercaptoethanol in solution are approximately equal. (With regard to steric hindrance, this condition is more likely with reagents of similar size, such as MTSEA and MTSEH, than with reagents that differ in size, such as AEAETS and MTSEH.)

A second condition that would simplify Eq. 8 is that the nonelectrostatic contributions to the affinity constants of the two reagents for the site are approximately

equal; i.e., $({}^1g_1/{}^1g_{-1}) ({}^2g_{-1}/{}^2g_1) \approx 1$. There is no sign that the reagents actually bind anywhere in the channel,³ so that these ratios involve mainly the rate constants of ingress to and egress from the site rather than specific association and dissociation. Thus, the assumption of this condition is plausible if the reagents differ in charge but are similar in size.

A third simplifying condition is that $(1 + {}^2k_2^0/{}^2k_{-1}^0) / (1 + {}^1k_2^0/{}^1k_{-1}^0) \approx 1$. This would be the case if the reagents were relatively impermeant, because then ${}^1k_2^0/{}^1k_{-1}^0$ and ${}^2k_2^0/{}^2k_{-1}^0 \ll 1$. These inequalities may be valid for permeant reagents as well,⁴ because the narrowest part of the ACh receptor channel, presumably its highest barrier, is at the intracellular end and distal to the substituted Cys tested here (Imoto et al., 1991; Villarroel et al., 1991; Cohen et al., 1992). Also, in the fits of a two-barrier-one-site model to the current-voltage relationships for inorganic cations (Dani and Eisenman, 1987) and for organic cations (Sanchez et al., 1986), the inner barrier was higher than the outer barrier. Even if one of the reagents were quite permeant, however, and $k_2^0/k_{-1}^0 \approx 1$, ignoring $(1 + {}^2k_2^0/{}^2k_{-1}^0) / (1 + {}^1k_2^0/{}^1k_{-1}^0)$ would introduce only a small error [$(1/\beta) \ln 2 = 17$ mV] in the estimate of ψ_S .

We form the ratio, ρ_0 , of the effective rate constants for the reactions of the two reagents with the target Cys at zero transmembrane potential divided by the ratio of the rate constants for the reactions of the two reagents with 2-ME.

$$\rho_0 = ({}^1\kappa^0/{}^2\kappa^0) / ({}^1k_{ME}/{}^2k_{ME}) \quad (9)$$

and, from Eq. 8 and the three above conditions,

$$\rho_0 \approx \exp [-(z_1 - z_2) \beta \psi_S]. \quad (10)$$

As with δ , ψ_S should be considered the weighted mean of the ψ_S of the charge(s) of the reagents over their distributions of reactive configurations. The mean ψ_S , however, could be different for two charged reagents. As with δ , we assign ψ_S to the probed Cys, although it could refer to a location as much as 5 Å away.

²We assume that AEAETS has a charge close to 2. If, for example, the pK_a of each amino group were the same as the minimum for MTSEA, 8.5, and they did not interact, then the average charge of AEAETS at pH 7.2 would be 1.9. If the ammonium groups did interact and, for example, if the first proton dissociated with a pK_a of 8 and the second with a pK_a of 9, then, at pH 7.2, the average charge would be 1.86.

³The affinities of the thiosulfonates for the open channel of wild-type receptor are certainly lower than 10^2 M⁻¹, because millimolar concentrations resulted in no detectable reversible block of ACh-induced current. We do not know the affinity of any of the reagents for the "site" around a substituted Cys, but it is unlikely to be any greater than the affinity of the open wild-type channel.

⁴The permeabilities of MTSEA and MTSET relative to K⁺ through the open ACh receptor were calculated from the shift in the reversal potential when the Na⁺ in oocyte bath solution was substituted by these reagents (Akabas et al., 1994a). The relative permeabilities were ~ 0.6 . Also, these reagents carried considerable inward current. By comparison, the permeabilities relative to Na⁺ of some organic cations of radii comparable to the radii of the cross-sections of MTSEA and MTSET are 0.36 for Tris, 0.87 for ethanolammonium, and 0.36 for N,N-dimethylethanolammonium (Cohen et al., 1992).

The rate constants for the reactions of AEAETS with α T244C, α S248C, and α L251C in the presence of ACh vary with the transmembrane potential (Fig. 6). The fits of Eq. 7 to these data yield estimates of δ . The estimates obtained, however, depend on whether or not AEAETS is taken to be permeant through the open channel. If AEAETS is not permeant, $k_2^0/k_{-1}^0 (= D)$ in the denominator of Eq. 7 is zero, and Eq. 7 reduces to the simpler equation used in RESULTS. This equation yields estimates of δ of 0.16 for α T244C, 0.10 for α S248C, and 0.08 for α L251C, assuming $z = 2$ (see Footnote 2). If D is allowed to be a free parameter in the fit of Eq. 7 to the κ for α T244C, then we estimate $\delta = 0.27 \pm 0.004$, $D = 0.007 \pm 0.0004$, and $C = 21,200 \pm 410$. The fitted curve generated by Eq. 7 with these parameters is shown in Fig. 6. Taking D for AEAETS to be 0.007, we estimate $\delta = 0.16$ for α S248C, 0.13 for α L251C, and 0.06 for α L258C (Fig. 7). Thus, if a reagent is permeant through the channel, the slope of $\ln \kappa$ versus ψ_M is smaller in magnitude than $|z\beta\delta|$, where δ is the actual electrical distance to the Cys.

For MTSET, the fit of Eq. 7 with $D = 0$ yields $\delta = 0.42$ and with $D = 0.01$, $\delta = 0.44$. The curve corresponding to the latter parameters is shown in Fig. 6.

The rate constants for the reactions of MTSEA do not appear to be dependent on the transmembrane potential (Fig. 6). Nevertheless, the predominant form of MTSEA at pH 7.2 is the protonated, charged form, and the rate constant for its reaction was unaffected by lowering the pH, increasing the proportion of charged MTSEA, and decreasing fivefold the proportion of the minor uncharged form. Thus, it seems likely that it was the charged ammonium form of MTSEA that reacted in the channel. The lack of dependence on ψ_M can be

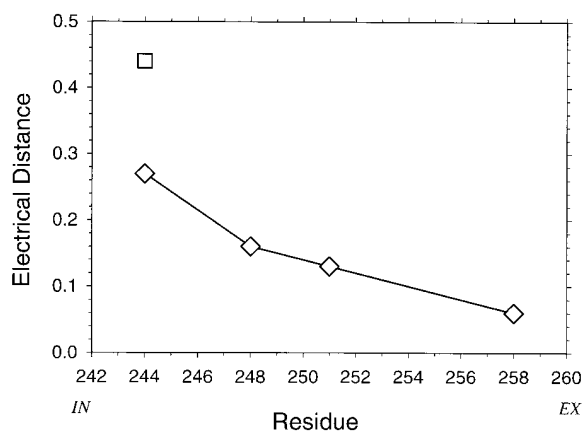


FIGURE 7. Electrical distance, δ , from the extracellular medium to the probed Cys, determined in the presence of ACh. The δ were obtained from the fits in Fig. 6, assuming $z = 1$ for MTSET (□) and $z = 2$ for AEAETS (◇).

rationalized in terms of Eq. 7 if $D > 0$ (see Footnote 4). The curves shown in Fig. 6 for α T244C, α S248C, and α L2251C were generated with $D = 2, 1$, and 0.09 , and with $z\delta = 0.38, 0.28$, and 0.15 , respectively. (All fitting parameters and errors are shown in Fig. 6, legend.) Thus, it is possible to fit the κ for MTSEA with electrical distances similar to those derived from the AEAETS and MTSET data. (The fits of the MTSEA data, however, are not unique and do not determine δ for the probed Cys.)

The electrical distances inferred from the reactions of AEAETS and MTSET are smaller than those inferred from the voltage dependence of open-channel block. The electrical distance from the extracellular medium to the binding site for the open-channel blocker QX-222, a trimethylammonium lidocaine derivative, is 0.78 (Neher and Steinbach, 1978) and for QX-314, a triethylammonium lidocaine derivative, the electrical distance is 0.35 (Pascual, J.M., and A. Karlin, manuscript in preparation). (The electrical distances obtained with different ACh receptor channel blockers cover a wide range; Sanchez et al., 1986.) The quaternary heads of these blockers are likely to bind around α S248 (Charney et al., 1990; Pascual and Karlin, 1997a), which the fit of the κ for AEAETS indicated has a δ of 0.15 (Fig. 7). Either the open channel blockers bind more deeply than α Ser248 or our analysis of κ versus ψ_M underestimates δ . We would be underestimating δ if the effective charge of AEAETS in the channel were less than the assumed 2 or if D were larger than 0.007. These factors could also account for the smaller δ estimated for AEAETS than for MTSET at α T244C. Also, if MTSET were oriented in the channel predominantly with its charge toward the intracellular side, it would be sampling δ 5 Å closer to the intracellular end than α T244C, the likely mean position of the two charges of AEAETS. Given the uncertainties in the effective z , in the locations of the charge(s), and in D , the absolute value of δ for the reaction at each Cys is perhaps less informative than the voltage dependence itself and the monotonic increase in δ from the extracellular to the intracellular end of the open channel.

The closing rate (but not the opening rate) of the channel is voltage dependent (Anderson and Stevens, 1973; Neher and Steinbach, 1978; Auerbach et al., 1996), and it is conceivable that such voltage dependence of the transitions between functional states, in conjunction with different rates of reaction of the Cys in the different states, could lead to voltage dependence of the rates of thiosulfonate reactions. This mechanism for voltage dependence, however, should affect the reactions of all thiosulfonates and of all substituted Cys, the reaction rates of which are strongly state dependent, and this dependence should be independent of the permeability of the reagents. On the contrary, although the reac-

tions of AEAETS and of MTSET were voltage dependent, the reactions of MTSEH and of MTSEA were not voltage dependent at any residue. Furthermore, although the reaction of AEAETS at α T244C was voltage dependent, the reaction at α L258C was not voltage dependent (Fig. 6), despite the larger rate constants in the presence of ACh for the reaction of AEAETS at both residues (Fig. 2). Also, because the rates of reaction of all reagents would be affected, voltage dependence of the pK_a of the target Cys is an unlikely explanation for the voltage dependence of the rates of reaction. The voltage dependence of the movement of the reagents to and from the site of reaction, as in the model, is a better explanation for the observed voltage dependence of the overall rate constants.

In the absence of ACh, the rates of reaction of α T244C with AEAETS, MTSEA, and MTSEH are all independent of ψ_M (Fig. 5). In the closed state, we expect k_2 and, hence, D in Eq. 7 to be zero. Thus, for the first two reagents, this independence is consistent with δ equal to zero at α T244C in the closed state. Thus, in the nonconducting channel, there is little drop in transmembrane potential between the extracellular medium and α T244C, and the transmembrane potential drops entirely over a high-resistance gate (Finkelstein and Peskin, 1984) that lies intracellular to α T244 (Akabas et al., 1994a; Wilson and Karlin, 1998; Zhang and Karlin, 1998).

The Intrinsic Electrostatic Potential in the Channel

Even at $\psi_M = 0$, the charges of the reagents appear to be important factors in their rates of reaction. Thus, if we compare the rate constants for the reactions of AEAETS and of MTSEH with α T244C in the open channel and with 2-mercaptoethanol in solution, we find that AEAETS reacted $100,000\times$ faster than MTSEH with α T244C, but only $27\times$ faster with 2-mercaptoethanol. The ratio of these ratios, ρ_0 , is 3,700 (Fig. 8 A); AEAETS reacted 3,700 times faster than MTSEH with α T244C, after factoring out the difference in their intrinsic reactivities. We ascribe this enhancement to an intrinsic electrostatic potential, ψ_S , acting on the charges of AEAETS. According to Eq. 10, $\psi_S = -104$ mV (Fig. 8 B, \diamond). Similarly, at α T244C in the presence of ACh, ρ_0 for MTSEA and MTSEH yields $\psi_S = -230$ mV (Fig. 8 B, \circ), and ρ_0 for MTSET and MTSEH, $\psi_S = -105$ mV (Fig. 8 B, \square). The mean of the three estimates is -146 ± 43 mV.

At α L251C, the ρ_0 for AEAETS and MTSEH implies $\psi_S = -42$ mV, and ρ_0 for MTSEA and MTSEH implies $\psi_S = -80$ mV. The mean is -61 ± 21 mV. At α L258C, the two pairs yield -6 and -26 mV, respectively; the mean is -16 ± 10 mV. Thus, ψ_S increases from ~ -150 mV close to the intracellular end of the channel to ~ -15 mV close to the extracellular end (Fig. 8 B).

We can also calculate ψ_S from ρ_0 for the pairs AEAETS and MTSET and for AEAETS and MTSEA. At α T244C,

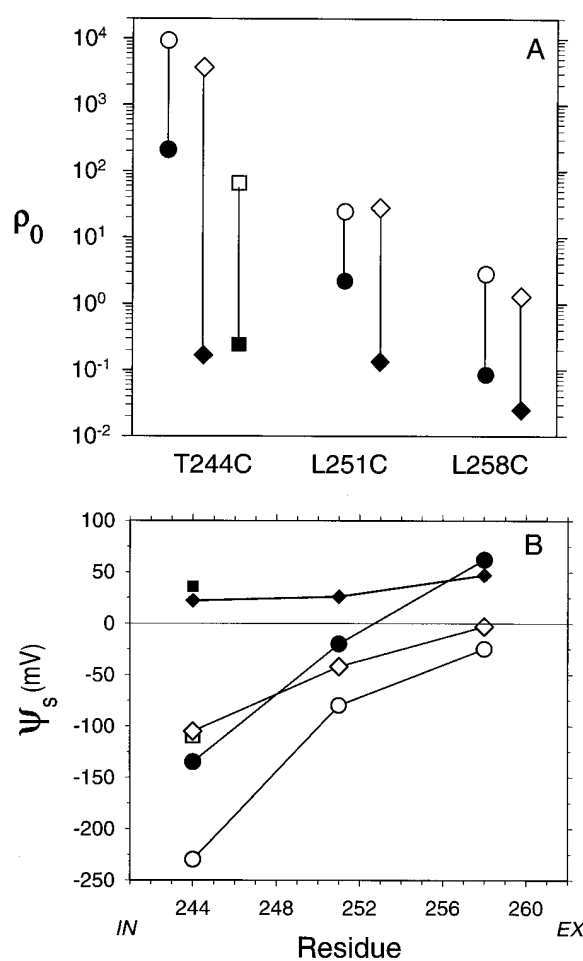


FIGURE 8. The relative rates of reaction and the intrinsic electrostatic potential in the channel at α T244C, α L251C, and α L258C. (A) The rate constants for the reactions of MTSEA (circles), AEAETS (diamonds), and MTSET (squares) with substituted Cys in α M2 were determined at various membrane potentials (Fig. 6), and the rate constants at zero membrane potential were determined by interpolation or extrapolation. Filled symbols are for reactions in the absence of ACh and open symbols are for reactions in the presence of ACh. These rate constants were divided by the rate constants for the reactions of MTSEH with the same substituted Cys, to obtain relative rates of reaction with the Cys in the channel. Also, the rate constants for the reactions of each of the charged thiosulfonates with 2-mercaptoethanol in solution was divided by the rate constant for the reaction of MTSEH with 2-mercaptoethanol to obtain relative rates of reaction with a simple thiol in solution. The relative rate constants for the reactions with receptor were divided by the relative rate constants for the reactions with 2-mercaptoethanol to obtain ρ_0 for each mutant and reactant, both in the presence and absence of ACh. (B) The intrinsic potentials, ψ_S , were calculated from Eq. 10. Symbols are as in A.

ρ_0 for AEAETS and MTSET is 56 and ψ_S is -101 mV, in agreement with $\psi_S = -104$ mV inferred from the pair AEAETS and MTSEH. Thus, the large negative electrostatic potential at α T244C does not depend on the use of MTSEH as one of the pair. On the other hand, ρ_0 for the pair AEAETS and MTSEA implies a small positive

potential, and ρ_0 for MTSEA and MTSET is >1 , although ρ_0 would be 1 if only the charges of the reagents mattered. The relative rate of reaction of MTSEA in the channel compared with the other charged reagents was greater than would be predicted by its relative rate of reaction with 2-mercaptoethanol in solution. One possibility is that the relatively small and flexible MTSEA is less sterically hindered in its reaction with Cys in the channel than are MTSET and AEAETS. In that case, the assumption that ${}^1k_S/{}^2k_S = {}^1k_{ME}/{}^2k_{ME}$ in the derivation of Eq. 10 underestimates the advantage of MTSEA.

In the absence of ACh, in the predominantly closed state of the channel, the mean intrinsic potentials at α T244C, α L251C, and α L258C are -26 , $+3$, and $+54$ mV, respectively (Fig. 8 B). As in the open state, the reaction of MTSEA in the closed channel appears anomalously high, and ψ_S based on MTSEA and MTSEH is more negative at α T244C and α L251C than ψ_S based on AEAETS and MTSEH.

The profile of intrinsic potentials estimated on the basis of Eq. 10 should be viewed as a qualitative picture. No pair of the reagents are perfectly matched, and the errors in the approximation of Eq. A24 by Eq. 10 are unknown. Nevertheless, in all cases, the estimated intrinsic potential is 60–120 mV more negative in the open state than in the closed state, and the slopes of the intrinsic potential are consistent (Fig. 8 B).

The more negative electrostatic potential in the open state than in the closed state should enhance the rates of reaction of the charged reagents with all substituted Cys in the open state compared with the closed state. The differences in the rate constants between the open and closed states of the reactions of MTSEA with the substituted Cys (Fig. 2), however, do not correlate simply with the intrinsic potential profile (Fig. 8 B). The rate constants k_S for the reaction of the substituted Cys in the two states and the nonelectrostatic affinity constants, g_i/g_{-1} , in the two states are also factors. By normalizing the rate constant for the reaction of a charged reagent by the rate constant for the reaction of MTSEH, we can plausibly eliminate these factors. We could, however, determine the rate constant for the reaction of MTSEH in the presence and absence of ACh only at three residues; at the other residues the reactions with MTSEH were too slow to analyze. We could not, therefore, estimate the separate effects of intrinsic potential and non-electrostatic effects on the rate constants at these other residues.

Electrostatic potentials that could enhance transport of charged substrates have been modeled or measured in channel vestibules and in binding sites. In the “charged-channel model” of the ACh receptor channel, fixed charges were postulated to produce potentials of the order of -50 mV in the inner vestibule (Dani and Eisenman, 1987). The electrostatic potential

in the ACh binding site of the ACh receptor was estimated as -30 mV (at ionic strength 0.135) (Stauffer and Karlin, 1994). By a similar approach, the electrostatic potential near one S4 residue exposed in an outer vestibule of the sodium channel was estimated to be -46 mV (Yang et al., 1997). Calculations based on the crystal structure of acetylcholinesterase indicated that there is a strong electrostatic dipole aligned with a gorge leading to the active site that could serve to attract ACh (Ripoll et al., 1993), and there are many other examples of intrinsic electrostatic fields guiding enzyme–substrate, protein–protein, and protein–nucleic acid interactions (Honig and Nicholls, 1995).

It is likely that a considerable part of the intrinsic potential in the ACh receptor channel arises from four aligned glutamates, two in α (Glu241) and one each in β and δ , that form the “intermediate ring of charge” and play a major role in the conductance and selectivity of the open channel (Imoto et al., 1988; Konno et al., 1991).⁵ In a three-barrier–two-site model of conductance through the ACh receptor channel, the effects of altering the charge in the intermediate ring was fit by slightly shifting the electrical distance of the central barrier and by altering the barriers and wells by free energies of 1 kT or less, equivalent to 25 mV or less (Konno et al., 1991). In the homomeric neuronal ACh receptor formed by five $\alpha 7$ subunits, neutralization of the intermediate ring was necessary but not sufficient to switch the charge selectivity from cationic to anionic (Galzi et al., 1992).

The total electrostatic potential at each point in the channel is the intrinsic potential superimposed on the transmembrane potential, $\psi_S + \delta\psi_M$ (Fig. 9). The magnitude and sign of the contribution of the total electrostatic potential to the free energy of a permeating ion depend on z , the charge of the ion. Potentials in the range of -50 to -200 mV at the barriers and sites in the channel certainly would have a strong influence on both the conductance and charge selectivity of the channel, rejecting anions and producing quite different current–voltage relationships for monovalent and divalent cations.

Location of the Gate

One strategy to locate a gate is to use substituted-cysteine-accessibility method to identify as near as possible

⁵The reaction of the thiosulfonates with Cys in the channel, just as with thiols in solution, is with the negatively charged thiolate. This negative charge itself generates a negative electrostatic potential in the channel, which contributes to κ for the reaction of charged reagents. The ratio ρ , however, contains κ/k_{ME} for the charged reagent, in which the contribution of the Cys thiolate to ψ_S and κ should be approximately canceled by the contribution of the 2-mercaptoethanol thiolate to k_{ME} .

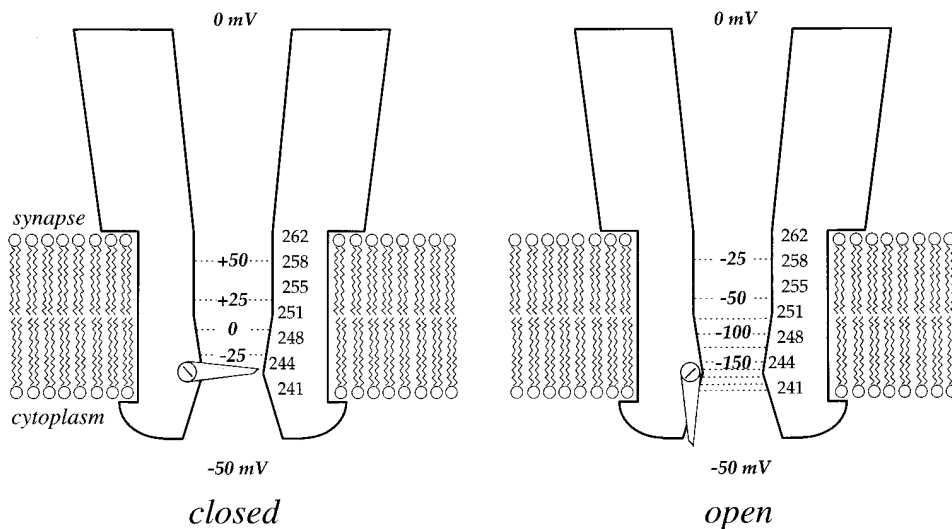


FIGURE 9. Scheme of the electrostatics and the gate of the ACh receptor channel in the open and closed states. The sum of $\delta\psi_M + \psi_S$ is indicated by a contour plot through the channel with equipotentials every 25 mV. For the open state, δ is from Fig. 7, $\psi_M = -50$ mV, ψ_S is the average of the values at each residue in Fig. 8 B; for the closed state, $\delta = 0$ and ψ_S are the average of the values in Fig. 8 B. In the open state, $\delta\psi_M + \psi_S$ extrapolates to ~ -250 mV at $\alpha E241$, the last contour shown.

to the closed gate the channel-lining residues on its two sides (Wilson and Karlin, 1998). In the closed state, only residues on the extracellular side of the gate should be accessible to charged reagents added to the extracellular medium, and only residues on the intracellular side of the gate should be accessible to charged reagents added to the intracellular medium. We previously observed that MTS reagents, applied extracellularly in the absence of agonist, were able to react with residues as close to the cytoplasmic end of the channel as $\alpha T244C$ (Akabas et al., 1994a) and at the aligned $\beta G255C$ (Zhang and Karlin, 1998). We inferred that the gate was at least as close to the cytoplasmic end of the channel as these residues. The results of the application of the MTS reagents from the extracellular and intracellular sides of the membrane in whole-cell, patch-clamped HEK 293 cells were consistent with the location of the activation gate in the region of the channel between $\alpha E241$ and $\alpha T244$ (Wilson and Karlin, 1998).

Similarly, in the homologous GABA_A receptor, the residue aligned with ACh receptor $\alpha Thr244$, $\alpha_1 Val257$, was accessible to *p*-chloromercuribenzenesulfonate in the closed state of this channel, implying that the gate was located close to the intracellular end of the channel (Xu et al., 1995; Xu and Akabas, 1996).

An assumption in this approach is that the reagents reach a substituted Cys via the water-filled channel and do not bypass a closed gate. MTSEA, however, can permeate through lipid membranes (Holmgren et al., 1996) and was found to react with $\alpha G240C$ at the intracellular end of the closed channel (Wilson and Karlin, 1998). This reaction in the closed state was prevented by the addition of 10 mM free cysteine to the cytoplasm. MTSEA also reacted with $\alpha G240C$ in the open state of the channel, but this reaction was not affected

by 10 mM cysteine in the cytoplasm. (The rate constant in the open state was three orders of magnitude faster than in the closed state.) Thus, in the closed state, MTSEA reached $\alpha G240C$ via the bilayer and the cytoplasm and, in the open state, MTSEA reached $\alpha G240C$ principally via the open channel. MTSEH, we must assume, can also permeate the bilayer, since it is less polar than MTSEA.

The reactions in the closed state of MTSEA and MTSEH with $\alpha T244C$, however, do not appear to be due to their bypassing a gate by penetrating the bilayer, for the following reasons. MTSEA penetrates the bilayer as an unprotonated amine, but its rate of reaction with $\alpha T244C$ in the closed state was unaffected by our lowering of the pH of the medium, decreasing the concentration of unprotonated amine fivefold (Fig. 5). Furthermore, the reactions of both MTSEA and MTSEH with $\alpha T244C$ in the open state were completely blocked by the open channel blocker QX-314. QX-314 likely binds between $\alpha S248$ (Charnet et al., 1990) and $\alpha V255$, so that protection of $\alpha T244C$ is by block of the channel rather than occlusion of $\alpha T244C$ itself (Pascual and Karlin, 1997a). Thus, MTSEA and MTSEH did not bypass the channel blocker to react at a detectable rate with $\alpha T244C$, which it would have done had it reached $\alpha T244C$ through the bilayer.

The results of this paper support the previous conclusion. Not only did MTSEA and AEAETS react with $\alpha T244C$ in the absence of ACh, but the rate constants for these reactions were higher than the rate constants for the reactions of these reagents at any of the tested substituted Cys closer than $\alpha T244C$ to the extracellular end of the channel (Figs. 2 and 3). Also, the addition of ACh, which induced opening of the channel, barely had an effect on the rate constants for the reaction of MTSEH with $\alpha T244C$ or for the reactions of MTSEA

with α L245C and α S248C. As discussed above, the current carried by open ACh receptor channels in the presence of ACh was vastly greater than the current carried due to spontaneous openings in the absence of ACh. If the reaction of MTSEA with α T244C in the absence of ACh only occurred during the occasional spontaneous openings, the difference in rates in the presence and absence of ACh would have been far greater than the 35-fold difference observed (Table I). Thus, the effects of ACh on the rates of reaction of the thiosulfonates with the substituted Cys in M2 is not consistent with a gate between the extracellular end of the channel and α T244, such as one postulated at the level of α L251 (Unwin, 1995).

The difference in the presence and absence of ACh in the electrical distances of the substituted Cys in M2 also are not consistent with a gate more extracellular than α T244. The electrical distance of α T244C, α S248C, and α L251C were greater than zero in the presence and zero in the absence of ACh. This suggests that in the closed channel there is a gate closer to the intracellular end of the channel than α T244C, and that most of the transmembrane potential drops across this gate. The difference in the intrinsic potential in the presence and absence of ACh (Fig. 9) is also consistent with a gate between α E241 and α T244, which in the closed state shields the residues more extracellular than it from the electric field arising from the glutamates in the intermediate ring.

Conclusions

The accessibility and electrostatic potential in the open and closed channel of the ACh receptor were probed from the extracellular side of the membrane with one neutral and three positively charged thiosulfonate reagents. The rate constants of the reactions of these reagents with Cys substituted for nine residues in the M2 segment of the α subunit were variably affected by the presence of ACh and by the transmembrane electrostatic potential. These results were rationalized in terms of differences in the structure and in the electrostatic potential in the open and closed channel. In the region between the extracellular end of the channel and α T244 close to the intracellular end of the channel, ACh induced local changes in structure but no general increase in accessibility consistent with a gate in this region. The electrical distance of the residues in the open channel increased from zero at its extracellular end to ~ 0.4 near the intracellular end; by contrast, in the closed channel the electrical distance was zero at α T244, consistent with a gate closer to the intracellular end than α T244. Even at zero transmembrane potential, the much greater rates of reaction in the open channel of positively charged reagents compared with the neutral MTESH was consistent with the existence of an intrinsic electrostatic potential of ~ -150 mV near

α T244, which decreased in magnitude to ~ -25 mV at α L258. In the closed channel, the intrinsic potential was 60–120 mV more positive at each probed residue. The strongly negative intrinsic potential in the open channel might arise largely from the ring of four aligned glutamates, including α E241, at the intracellular end of the channel. In the closed channel, these glutamates might be screened by a gate between the α E241 and α T244. The intrinsic electrostatic potential could strongly influence the conductance and charge selectivity of the channel.

APPENDIX A

Kinetic Equations Governing Reactions in the Channel

The equations corresponding to Scheme I are:

$$ds'/dt = (k_1x_{\text{EX}} + k_{-2}x_{\text{IN}})s - (k_{-1} + k_2 + k_S)s', \quad (\text{A1})$$

$$ds^*/dt = k_Ss', \quad \text{and} \quad (\text{A2})$$

$$s_0 = s + s' + s^*, \quad (\text{A3})$$

where the lower case x is the concentrations of X in the locations indicated by the subscripts, s , s' , and s^* are the concentrations of the site (and of the Cys) in the unoccupied, reversibly occupied, and irreversibly reacted states, and s_0 is the initial and total concentration of sites (and Cys).

The solution of Eqs. A1, A2, and A3 is

$$s_0 - s^* = s_0 \exp(-\lambda t), \quad (\text{A4})$$

where the effective rate constant

$$\lambda \cong k_S A / (A + B), \quad (\text{A5})$$

where

$$A = k_1x_{\text{EX}} + k_{-2}x_{\text{IN}}, \quad \text{and} \quad (\text{A6})$$

$$B = k_{-1} + k_2 + k_S. \quad (\text{A7})$$

Eq. A5 is an approximation for λ that depends on the condition that $k_S A / (A + B)^2 \ll 1$. This inequality holds for the reaction conditions used here, because in all cases $\lambda = k_S A / (A + B) < 0.2 \text{ s}^{-1}$ and $A + B \gg 1$. The quantity, $s_0 - s^*$ is the concentration of unreacted Cys after time t and equals the concentration of the unreacted, unoccupied site, s , after unreacted reagent is removed, when $s' = 0$.

Applying absolute-reaction-rate theory to the movement of X to and from the site, we obtain expressions for the rate constants that depend on free energy differences between ground states and transition states at the peaks of the barriers. For X with charge z , these free energies contain electrostatic terms that depend on z . We will explicitly state these electrostatic-energy terms and lump together all other free-energy terms and the preexponential factors. Thus,

$$k_1 = g_1 \exp[-z\beta(\delta\psi_M/2 + \psi_1)], \quad (\text{A8})$$

$$k_{-1} = g_{-1} \exp[-z\beta(-\delta\psi_M/2 + \psi_1 - \psi_S)], \quad (\text{A9})$$

$$k_2 = g_2 \exp\{-z\beta[(1 - \delta)\psi_M/2 + \psi_2 - \psi_S]\}, \text{ and} \quad (\text{A10})$$

$$k_{-2} = g_{-2} \exp\{-z\beta[-(1 - \delta)\psi_M/2 + \psi_2]\}. \quad (\text{A11})$$

The g_1 , g_{-1} , g_2 , and g_{-2} are each the product of a constant and $\exp(-\Delta G_{\text{NE}}^{\pm}/RT)$. The $\Delta G_{\text{NE}}^{\pm}$ are the non-electrostatic standard free energy differences, $G_{1,\text{NE}}^{\pm} - G_{\text{EX,NE}}^0$, $G_{1,\text{NE}}^{\pm} - G_{\text{S,NE}}^0$, $G_{2,\text{NE}}^{\pm} - G_{\text{S,NE}}^0$, and $G_{2,\text{NE}}^{\pm} - G_{\text{IN,NE}}^0$, respectively, where the subscripts EX, IN, 1, and 2 refer to the extracellular medium, the intracellular medium, barrier 1, and barrier 2, respectively, and NE indicates nonelectrostatic (i.e., independent of the charge of X). The algebraic charge of X is z , $\beta = F/RT$, δ is the electrical distance from the extracellular medium to the site S, ψ_M is the membrane electrostatic potential difference (intracellular minus extracellular), and ψ_1 , ψ_2 , and ψ_S are the intrinsic electrostatic potentials at barrier 1, barrier 2, and S, respectively. The electrical distances to the barriers are taken as midway between the site and the medium on either side; i.e., the electrical distances from the extracellular medium to the barriers are $\delta/2$ for barrier 1 and $(1 + \delta)/2$ for barrier 2 (Woodhull, 1973). We assume that k_S is independent of ψ_M and ψ_S ; i.e., that any separation of charge that might occur in the formation of the activated complex between X and the Cys is over too short a distance to be influenced by the gradients in ψ_M and ψ_S at S.

Hereafter, we will consider only conditions in which $x_{\text{IN}} = 0$ and, therefore, in which $A = k_1 x_{\text{EX}}$, and k_{-2} does not enter the equations. Under this condition,

$$\lambda = k_S k_1 x_{\text{EX}} / (k_1 x_{\text{EX}} + k_{-1} + k_2 + k_S). \quad (\text{A12})$$

Furthermore, for concentrations, x_{EX} , that are well below saturation of the sites, $k_{-1} + k_2 + k_S \gg k_1 x_{\text{EX}}$, and the effective rate constant, λ , is proportional to x_{EX} ; i.e.,

$$\lambda = k_S k_1 x_{\text{EX}} / (k_{-1} + k_2 + k_S). \quad (\text{A13})$$

We define κ as the effective second-order-rate constant, where $\lambda = \kappa x_{\text{EX}}$, and

$$\kappa = k_S k_1 / (k_{-1} + k_2 + k_S). \quad (\text{A14})$$

To examine the dependence of κ on ψ_M , we will first rewrite Eqs. A8–A10:

$$k_1 = k_1^0 \exp(-z\beta\delta\psi_M/2), \quad (\text{A15})$$

$$k_{-1} = k_{-1}^0 \exp(z\beta\delta\psi_M/2), \text{ and} \quad (\text{A16})$$

$$k_2 = k_2^0 \exp[-z\beta(1 - \delta)\psi_M/2], \quad (\text{A17})$$

where k_1^0 , k_{-1}^0 , and k_2^0 are the rate constants at $\psi_M = 0$.

Substituting Eqs. A15–A17 into A14, we obtain

$$\begin{aligned} \kappa = & k_S (k_1^0/k_{-1}^0) \exp(-z\beta\delta\psi_M/2) / \\ & [1 + (k_2^0/k_{-1}^0) \exp(-z\beta\psi_M/2) + \\ & (k_S/k_{-1}^0) \exp(-z\beta\delta\psi_M/2)]. \end{aligned} \quad (\text{A18})$$

We obtain δ from the slope of $\ln(\kappa)$ as a function of ψ_M .

$$\begin{aligned} d\ln(\kappa)/d\psi_M = & \\ -z\beta \{ & \delta - (1/2) [(k_2^0/k_{-1}^0) \exp(-z\beta\psi_M/2) + \\ & (k_S/k_{-1}^0) \delta \exp(-z\beta\delta\psi_M/2)] / [1 + (k_2^0/k_{-1}^0) \exp \\ & (-z\beta\psi_M/2) + (k_S/k_{-1}^0) \exp(-z\beta\delta\psi_M/2)] \}. \end{aligned} \quad (\text{A19})$$

From Eq. A19, we see that the slope is constant and equal to $-z\beta\delta$ only when $k_{-1}^0 \gg k_2^0$ and $k_{-1}^0 \gg k_S$. (The exponential factors are <10 for values of z , δ , and ψ_M considered here.) If the rate constants for the jumps of the reagents between the extracellular medium and the site are similar to those for inorganic cations, then k_{-1}^0 should be at least 10^7 s^{-1} , and even if the local concentration of X in the occupied site were 1 M, the pseudo-first-order rate of reaction of X with the Cys, $k_S < 2 \times 10^5 \text{ s}^{-1}$, based on the highest rate constant for the reaction of the thiosulfonates with 2-mercaptoethanol in solution. We will, therefore, neglect the term $(k_S/k_{-1}) \exp(-z\beta\delta\psi_M/2)$ in the denominator of Eqs. A18 and A19, recognizing that this approximation could contribute to an underestimation of δ .

We can rewrite Eq. A18 as

$$\kappa = C \exp(-z\beta\delta\psi_M) / [1 + D \exp(-z\beta\psi_M/2)], \quad (\text{A20})$$

which has three independent parameters, $C = k_S k_1^0 / k_{-1}^0$, $D = k_2^0 / k_{-1}^0$, and δ .

Under certain conditions, the intrinsic electrostatic potential at S, ψ_S , can be estimated from the rate constants. We take the ratio of the rate constants, ${}^1\kappa$ and ${}^2\kappa$, for two reagents, ${}^1\text{X}$ and ${}^2\text{X}$, with unequal charges, z_1 and z_2 (Stauffer and Karlin, 1994). For reagent ${}^1\text{X}$, Eqs. A18 and A8–A10 give

$$\begin{aligned} {}^1\kappa = & {}^1k_S ({}^1g_1/{}^1g_{-1}) \exp[-z_1\beta(\delta\psi_M + \psi_S)] / \\ & \{1 + ({}^1g_2/{}^1g_{-1}) \exp[-z_1\beta(\psi_M/2 + \psi_2 - \psi_1)]\}, \end{aligned} \quad (\text{A21})$$

and a similar equation for ${}^2\text{X}$. As discussed above, we neglect the term $k_S \exp(-z\beta\delta\psi_M/2)$ in the denominator of Eq. A18. The ratio of the observed rate constants is

$$\begin{aligned} {}^1\kappa/{}^2\kappa = & ({}^1k_S/{}^2k_S) ({}^1g_1/{}^1g_{-1}) ({}^2g_{-1}/{}^2g_1) \{ \exp[-(z_1 - z_2) \\ & \beta(\delta\psi_M + \psi_S)] \} \{ 1 + ({}^2g_2/{}^2g_{-1}) \exp \\ & [-z_2\beta(\psi_M/2 + \psi_2 - \psi_1)] \} / \{ 1 + ({}^1g_2/{}^1g_{-1}) \exp \\ & [-z_1\beta(\psi_M/2 + \psi_2 - \psi_1)] \}. \end{aligned} \quad (\text{A22})$$

We assume that the ratio of the rate constants for the reactions of the two reagents with a substituted Cys in the channel, ${}^1k_s/{}^2k_s$, is close to the ratio of the rate constants for the reaction of the two reagents with the small thiol, 2-ME in solution; i.e., ${}^1k_s/{}^2k_s = {}^1k_{ME}/{}^2k_{ME}$ (Stauffer and Karlin, 1994).

We form the ratio, ρ , of the effective rate constants for the reactions of the two reagents with the substituted Cys divided by the ratio of the rate constants for the reactions of the two reagents with 2-ME:

$$\rho = ({}^1\kappa/{}^2\kappa) / ({}^1k_{ME}/{}^2k_{ME}). \quad (\text{A23})$$

Let ρ_0 be ρ at $\psi_M = 0$; i.e., for ${}^1\kappa$ and ${}^2\kappa$ determined at, or extrapolated to, $\psi_M = 0$.

$$\begin{aligned} \rho_0 = & ({}^1g_1/{}^1g_{-1}) ({}^2g_{-1}/{}^2g_1) \{ \exp[-(z_1 - z_2) \beta \psi_S] \} \\ & \{ 1 + ({}^2g_2/{}^2g_{-1}) \exp[-z_2 \beta (\psi_2 - \psi_1)] \} / \\ & \{ 1 + ({}^1g_2/{}^1g_{-1}) \exp[-z_1 \beta (\psi_2 - \psi_1)] \}. \end{aligned} \quad (\text{A24})$$

In Eq. A24, ${}^1g_1/{}^1g_{-1}$ and ${}^2g_{-1}/{}^2g_1$ are the nonelectrostatic contributions to the affinity constants for the site, ${}^1k_1^0/{}^1k_{-1}^0$ and ${}^2k_1^0/{}^2k_{-1}^0$. Also, $({}^1g_2/{}^1g_{-1}) \exp[-z_1 \beta (\psi_2 - \psi_1)] = {}^1k_2^0/{}^1k_{-1}^0$, and $({}^2g_2/{}^2g_{-1}) \exp[-z_2 \beta (\psi_2 - \psi_1)] = {}^2k_2^0/{}^2k_{-1}^0$; i.e., the parameter D in Eq. A20.

Eq. A24 can be approximated by a simpler expression with two further assumptions (see DISCUSSION): (a) the nonelectrostatic contributions to the affinity constants for the two reagents are similar, and (b) for both reagents, $k_2^0/k_{-1}^0 \ll 1$; i.e., the jump of reagent from the site of reaction to the extracellular side is much faster than the jump from the reaction site to the intracellular side.

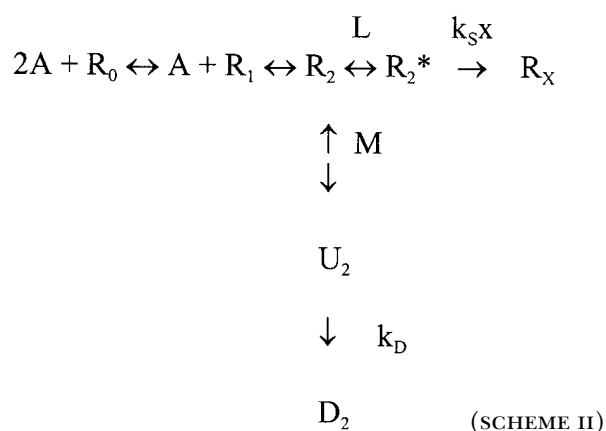
$$\rho_0 \cong \exp[-(z_1 - z_2) \beta \psi_S]. \quad (\text{A25})$$

APPENDIX B

Rate of Reaction of MTSET with Receptor in the Open State in the Face of Desensitization

We consider the following kinetic scheme (Scheme II), where A is agonist, R_0 , R_1 , and R_2 are receptors with 0, 1, and 2 molecules of A bound, R_2^* is the doubly occupied, open state, L is the isomerization constant for opening, R_X is the product of the reaction of R and X, x is the concentration of X, $k_s x$ is the pseudo-first-order rate constant for the reaction, U_2 is the fast desensitized state, M is the isomerization constant for fast desensitization, k_D is the rate constant for slow desensitization, and D_2 is the doubly occupied, slow desensitized state.

We assume that during the short period of the application of the reagent X and ACh, the double-arrow steps are at equilibrium and the single-arrow steps are irreversible. During the long wash period, however, all desensitization is reversed. The rate constant for the re-



action of X with the open state is much greater than the rate constants for the reaction with other states. ACh is applied at a saturating concentration so that only doubly liganded receptor species need be considered. Thus, the sum of the concentrations of all receptor species that are not negligible is

$$w = r_2 + r_2^* + r_x + u_2 + d_2, \quad (\text{B1})$$

where the concentrations are represented by lower-case letters. Furthermore, the sum of the concentrations of the species that are in reversible equilibrium is

$$y = r_2 + r_2^* + u_2. \quad (\text{B2})$$

The equilibrium isomerizations are governed by

$$r_2/u_2 = M, \text{ and} \quad (\text{B3})$$

$$r_2/r_2^* = L. \quad (\text{B4})$$

Thus,

$$r_2 = y/B, \quad (\text{B5})$$

$$r_2^* = y/(LB), \text{ and} \quad (\text{B6})$$

$$u_2 = y/(MB), \quad (\text{B7})$$

where $B = (1 + 1/L + 1/M)$.

The rate of decrease in y due to both reaction and slow desensitization is given by

$$dy/dt = -k_D u_2 - k_s x r_2^* = -[k_D/(MB) + k_s x/(LB)] y \quad (\text{B8})$$

and, therefore,

$$y = y^0 \exp\{-[k_D/(MB) + k_s x/(LB)] t\}. \quad (\text{B9})$$

The mean current at a given holding potential carried by the fraction of open ACh receptors is

$$I = A r_2^* = Ay/(LB), \quad (\text{B10})$$

where A is a coefficient that depends on the holding potential.

In the experimental protocol, ACh-induced current was recorded before (I_{PRE}) and after (I_{POST}) application of MTSET. Also, the current was recorded during the application of MTSET plus ACh (I_{DUR}). All applications

were separated by 5-min washes, during which all receptors not covalently modified returned to the resting state. The holding potential was the same throughout. This series was repeated several times so that the I_{POST} after one application of MTSET plus ACh served as I_{PRE} preceding the next application of MTSET plus ACh.

During the first ACh-induced current,

$$y = w \exp \{-[k_{\text{D}} / (MB)] t\} \quad (\text{B11})$$

and, therefore,

$$I_{\text{PRE}} = [A / (LB)] w \exp \{-[k_{\text{D}} / (MB)] t\}. \quad (\text{B12})$$

During the coapplication of MTSET and ACh,

$$I_{\text{DUR}} = [A / (LB)] w \exp \{-[k_{\text{D},\text{X}} / (MB) + (k_{\text{S},\text{X}}) / (LB)] t\}, \quad (\text{B13})$$

where the rate constant for slow desensitization in the presence of both ACh and MTSET, $k_{\text{D},\text{X}} > k_{\text{D}}$, the rate constant for slow desensitization in the presence of ACh alone.

During the postreaction ACh-induced current,

$$I_{\text{POST}} = [A / (LB)] (w - r_{\text{X}}') \exp \{-[k_{\text{D}} / (MB)] t\}, \quad (\text{B14})$$

where r_{X}' is the concentration of product of the reaction of R and X formed during the τ' seconds of prior application of MTSET and ACh. From Eqs. B12 and B14,

$$r_{\text{X}}' = w (1 - I_{\text{POST}} / I_{\text{PRE}}), \quad (\text{B15})$$

where I_{PRE} and I_{POST} are taken at the same time after adding ACh. For greater precision, we integrate the

currents recorded over the duration, τ , of the pre- and postreaction applications of ACh to obtain the total charge that flowed, Q_{PRE} and Q_{POST} . Integration of Eqs. B12 and B14 shows that $Q_{\text{POST}} / Q_{\text{PRE}} = I_{\text{POST}} / I_{\text{PRE}}$ and, therefore,

$$r_{\text{X}}' = w (1 - Q_{\text{POST}} / Q_{\text{PRE}}). \quad (\text{B16})$$

Since $dr_{\text{X}}' / dt = k_{\text{S},\text{X}} r_{\text{2}}^*$,

$$r_{\text{X}}' = k_{\text{S},\text{X}} \int_0^{\tau} r_{\text{2}}^* dt = k_{\text{S},\text{X}} \int_0^{\tau} (I_{\text{DUR}} / A) dt = k_{\text{S},\text{X}} Q_{\text{DUR}} / A. \quad (\text{B17})$$

Combining Eqs. B16 and B17:

$$k_{\text{S}} = [Aw / (xQ_{\text{DUR}})] (1 - Q_{\text{POST}} / Q_{\text{PRE}}). \quad (\text{B18})$$

From Eq. A13,

$$Aw = I_{\text{PRE}} LB / \exp \{-[k_{\text{D}} / (MB)] t\}, \quad (\text{B19})$$

we take

$$Aw \approx I_{\text{PRE}} \quad (\text{B20})$$

and

$$k_{\text{S}} \approx [I_{\text{P}} / (xQ_{\text{DUR}})] (1 - Q_{\text{POST}} / Q_{\text{PRE}}). \quad (\text{B21})$$

The fractional error in this approximation is

$$\epsilon = (Aw - I_{\text{PRE}}) / I_{\text{PRE}} \approx L (1 + 1/M + k_{\text{D}} t / M). \quad (\text{B22})$$

For $L \approx 0.1$, $M \approx 1$, $k_{\text{D}} \approx 0.005 \text{ s}^{-1}$, and $t = 10 \text{ s}$, $\epsilon \approx 0.2$; i.e., the estimate is low by 20%. In general, the rate constants will be underestimated by Eq. B21.

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