Identification of Acetylcholine Receptor Channel-Lining Residues in the M1 Segment of the α-Subunit†

Myles H. Akabas‡ and Arthur Karlin*,§

Center for Molecular Recognition, and Departments of Biochemistry and Molecular Biophysics, Physiology and Cellular Biophysics, Medicine, and Neurology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, New York 10032

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ABSTRACT: The muscle-type acetylcholine (ACh) receptor has the composition $\alpha_2\beta\gamma\delta$. The subunits are arranged quasisymmetrically around a central, ion-conducting, water-filled channel. Each subunit has four membrane-spanning segments, M1−M4, and the channel through the membrane is formed among these segments. Substituting cysteine for each of the residues in and flanking the αM2 segment, we previously found that, at 10 of the 21 mutated positions, the cysteine was accessible to a small, positively charged, sulfhydryl-specific reagent, methanethiosulfonate ethylammonium (MTSEA), and inferred that the residues at these positions are exposed in the channel lumen. We have now applied the substituted-cysteine-accessibility method to αM1. We analyzed 15 consecutive residues, starting at αPro211 at the extracellular end of M1. Wild-type α contains Cys222, which is inaccessible to MTSEA. We mutated each of the other 14 residues to cysteine and expressed the mutant α subunits, together with wild-type β, γ, and δ subunits, in *Xenopus* oocytes. Thirteen of the fourteen mutants gave robust ACh-induced currents. MTSEA irreversibly altered the ACh-induced response of seven cysteine-substitution mutants: αY213C was susceptible to MSTEA added in the presence or the absence of ACh, αP211C, αI215C, αV216C, αN217C, and αI220C were susceptible in the absence of ACh, and αV218C was susceptible in the presence of ACh. These results imply that M1 is exposed in the channel, and its exposure changes during gating or desensitization. From the pattern of exposure of M1 residues, we also conclude that the secondary structure of the extracellular half of M1 is irregular.

The neurotransmitters ACh,[1] serotonin, γ-aminobutyrate, glycine, and glutamate bind to specific receptors that convert this binding into the opening of selective, ion-conducting channels that are integral to these receptors. To understand gating, conduction, and selectivity by these receptors, we must know the structure of the conduction pathway and of the gate. The ACh receptors are composed of five subunits arranged quasisymmetrically around a central channel (Karlin, 1993; Unwin, 1993). These subunits have a characteristic topology: there are four membrane-spanning segments, M1−M4, and both the N-terminus and C-terminus are extracellular. This topology is probably shared by the closely related serotonin, $GABA_A$, and glycine receptor subunits, but not by the glutamate receptor subunits (Karlin, 1993; Hollman et al., 1994). The ion-conduction pathway through the membrane, the channel lumen, must be lined by residues from the hydrophobic membrane-spanning segments.

In the ACh receptor, mutations of residues in and flanking the M2 segments of the different subunits altered channel function (Imoto et al., 1988; Charnet et al., 1990; Villaroel et al., 1991; Imoto et al., 1991; Revah et al., 1991; Cohen et al., 1992; Galzi et al., 1992) and several of these residues were labeled by reactive channel blockers (Hucho et al., 1986; Revah et al., 1990; Pedersen et al., 1992; White & Cohen, 1992). Although five α-helical M2 segments could enclose the channel completely at its narrow cytoplasmic end, other membrane-spanning segments are likely to contribute to the lining of the channel toward its wider extracellular end (Dani, 1989). The M1 segment, which was also specifically labeled by a photoreactive channel blocker (DiPaola et al., 1990), is a likely candidate.

We have developed a systematic approach to the identification of the residues lining ion channels, the substituted-cysteine-accessibility method (SCAM), and have applied it to the ACh receptor (Akabas et al., 1992, 1994a), to the $GABA_A$ receptor (Xu & Akabas, 1993), and to CFTR (Akabas et al., 1994b). It has also been applied to voltage-gated potassium channels (Joho et al., 1995; Lu & Miller, 1995; Pascual et al., 1995) and to a voltage-gated sodium channel (Yang & Horn, 1995). We mutate individually each residue in the membrane-spanning segments to cysteine. If the mutant functions near-normally, we test the accessibility of the new cysteine to small, charged, highly water-soluble, sulfhydryl-specific reagents. The reagents we have used are derivatives of methanethiosulfonate (MTS), namely, the

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* Author to whom correspondence should be addressed.

‡ Center for Molecular Recognition and Departments of Physiology and Cellular Biophysics and Medicine.

§ Center for Molecular Recognition and Departments of Biochemistry and Molecular Biophysics, Physiology and Cellular Biophysics, and Neurology.

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[1] Abbreviations: ACh, acetylcholine; CFFR, calcium-free frog Ringers; GABA, γ-aminobutyric acid; MTS, methanethiosulfonate; MTSEA, methanethiosulfonate ethylammonium; MTSES, methanethiosulfonate ethylsulfonate; SCAM, substituted-cysteine-accessibility method.

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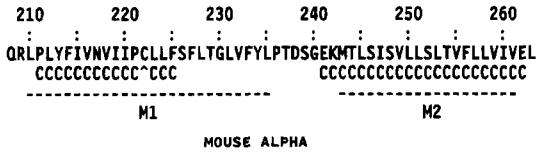

FIGURE 1: M1 and M2 segments of mouse-muscle ACh receptor α subunit. A "C" is under each residue that has been mutated to cysteine. M1 and M2 are underlined.

positively charged MTSethylammonium (MTSEA) and MTSethyltrimethylammonium and the negatively charged MTSethylsulfonate (MTSES) (Stauffer & Karlin, 1994). These highly polar reagents should react with sulfhydryls only at the water-accessible surface of a protein. We assume that, in the membrane-spanning segments, the only water-accessible side chains are those exposed in the channel lumen. The reaction of a MTS reagent with a cysteine attaches $-SCH_2CH_2X$ (X is NH_3^+, $N(CH_3)_3^+$, or SO_3^-) to the cysteine sulfhydryl via a disulfide bond. We assume further that the covalent attachment of any of these groups to a cysteine in the channel will result in altered channel function, which we can detect electrophysiologically.

Using this approach, we previously mutated to cysteine, 22 consecutive residues in and flanking the M2 segment of the mouse ACh receptor α subunit (Figure 1; Akabas et al., 1994a). Twenty-one of these mutants expressed functional receptors, and the ACh-induced responses of 10 of these were irreversibly altered by MTSEA. We inferred that these 10 residues are exposed in the channel, that the M2 segment forms an α-helix interrupted by a short extended link, that the disposition of M2 changes during gating, and that the gate itself is at the cytoplasmic end of the channel. We now report on the application of SCAM to 15 consecutive residues starting at the extracellular end of αM1 (Figure 1). We find that M1 is exposed in the channel and that its exposure changes on the addition of ACh.

EXPERIMENTAL PROCEDURES

The MTS reagents were synthesized by the procedures of Stauffer and Karlin (1994).

cDNAs for the mouse-muscle ACh receptor subunits were a gift from Dr. Toni Claudio. Residues were substituted by cysteine, one at a time, in the M1 segment of the α subunit with the Altered Sites Mutagenesis Kit (Promega). Following mutagenesis, we excised a cassette defined by the restriction enzymes *Dra*III and *Bst*XI and ligated it into wild-type α subunit in the pSP64T plasmid, cut with the same enzymes. The mutation was confirmed by DNA sequencing. Mutants are named as (subunit)(wild-type residue)(residue number)(mutant residue), where the residues are given in the single-letter code.

The *in vitro* transcription of subunit mRNAs, their expression in *Xenopus* oocytes, and two-electrode-voltage-clamp recording of ACh-induced currents were described previously (Akabas et al., 1992). The holding potential was −40 mV. The oocytes were perfused at room temperature at 5 mL/min with Ca^{2+}-free frog Ringers solution (CFFR) (115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 1 μM atropine, 10 mM HEPES, adjusted to pH 7.5 with NaOH). Solution moved past the oocyte at about 40 mm/s.

We assayed the irreversible effects of MTSEA and MTSES by the following protocol: we determined the average of the peak currents evoked by two brief applications of 20 μM ACh, washed with CFFR for 3−5 min, applied the MTS reagent in CFFR in the absence or the presence of ACh (at a concentration at least twice the K_{app}), washed for 5 min, and again determined the average of the peak currents evoked by two brief applications of 20 μM ACh. We averaged the two peak currents evoked by ACh before the MTS reagent was applied and compared this average with the average of the two peak currents evoked by ACh after the MTS reagent was applied. The effect of the MTS reagents was taken as

$$1 - (I_{ACh,after}/I_{ACh,before})$$

MTSEA was applied at a concentration of 2.5 mM and MTSES at a concentration of 10 mM, to compensate for their different rates of reaction with simple thiols in solution (Stauffer & Karlin, 1994).

We used the SPSS/PC+ (SPSS, Inc.) statistical software to analyze the effects of the MTS reagents by one-way analysis of variance according to the Student-Newman-Keuls test ($P < 0.05$).

Table 1: ACh Dose−Response Characteristics of M1 Cysteine-Substitution Mutants[a]

	K_{app} (μM)	sem	K_{mu}/K_{wt}	nH	sem	$-I_{max}$ (nA)	sem	no. expts
P211C	30.10	1.94	11.60	1.42	0.03	699	230	3
L212C	35.00	0.20	13.40	1.24	0.17	1135	273	3
Y213C	24.80	1.74	9.60	1.49	0.19	1725	325	5
F214C	5.03	0.84	1.90	1.38	0.05	3270	253	3
I215C	16.60	0.55	6.40	1.38	0.01	2435	130	3
V216C	15.00	0.02	5.80	1.66	0.12	2286	593	2
N217C	3.80	0.59	1.40	1.31	0.05	3755	314	3
V218C	9.70	0.87	3.70	1.38	0.09	4470	391	3
I219C	3.60		1.40	1.32		4984	1	
I220C	22.60	2.42	8.70	1.54	0.08	2738	230	4
P221C not expressed								
C222 WT	2.60	0.40	1.00	1.46	0.05	3165	170	12
L223C	1.46	0.21	0.60	1.68	0.13	4128	131	5
L224C	7.39	1.25	2.80	1.59	0.40	3346	363	3
F225C	0.27	0.02	0.10	1.42	0.08	3929	196	3

[a] Dose−response data were obtained as previously described (Akabas et al., 1992).

RESULTS

We infer that an engineered cysteine reacted if the MTS reagent irreversibly altered the ACh-induced current of oocytes expressing the mutant. We require, therefore, that the cysteine-substitution mutants be functional. We found that cysteine was a well-tolerated substitute for every residue from αPro211 to αPhe225, except for αPro221 (Table 1). Wild-type α contains Cys222, which is also present in all of the mutants.

In the absence of MTS reagents, the ACh-induced currents were stable over the duration of an experiment; there was no significant difference in the initial and final average peak currents in oocytes expressing any of the mutants or wild-type receptor. Neither MTSEA nor MTSES, added in the presence or absence of ACh, had significant irreversible effects on wild-type receptor (Figures 2 and 3).

In the absence of ACh, in the closed state of the channel, a 1-min application of 2.5 mM MTSEA had statistically significant effects on αP211C, αY213C, αI215C, αV216C, and αN217C (Figure 2A). We also tested the effects of a 5-min application of MTSEA (Figure 2B). The five residues

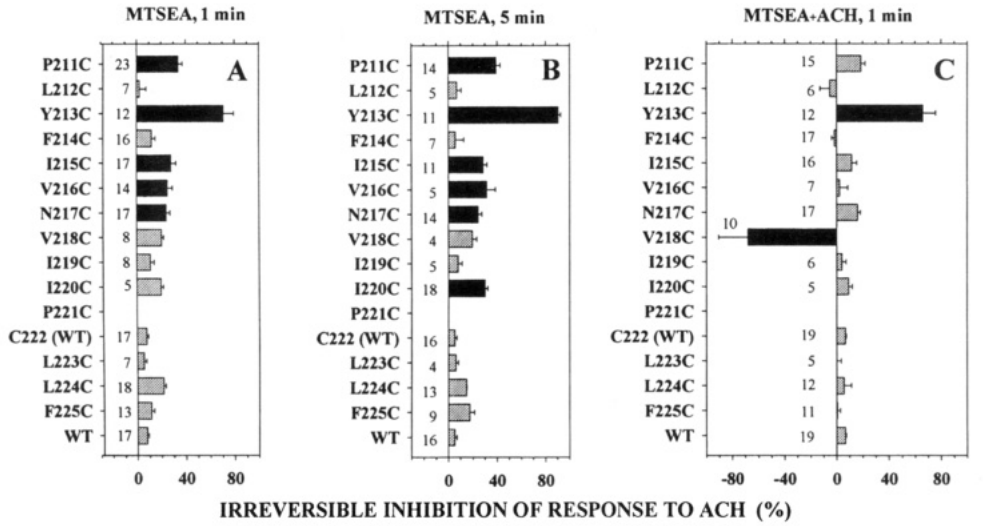

FIGURE 2: Irreversible effects of 2.5 mM MTSEA on the ACh-induced response of wild-type and mutant receptors. The duration of MTSEA application and the coapplication of ACh are indicated above the panels. (A) 1 min, no ACh; (B) 5 min, no ACh; (C) 1 min, ACh. Inhibition is to the right of the axis and potentiation to the left. The means, SEMs, and numbers of oocytes tested are shown. A solid bar indicates that the effect was significantly greater by the analysis of variance ($P < 0.05$) for the mutant than for wild type.

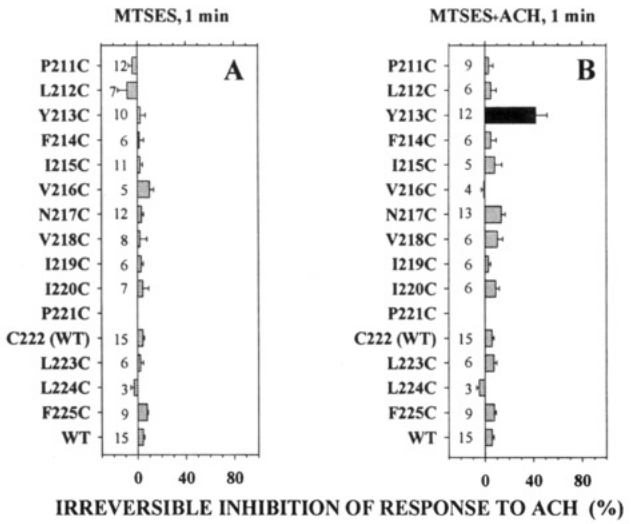

FIGURE 3: Irreversible effects of 10 mM MTSES on the ACh-induced response of wild-type and mutant receptors either in the absence (A) or in the presence of ACh (B). The means, SEMs, and numbers of oocytes tested are shown. A solid bar indicates that the inhibition was significantly greater ($P < 0.05$) for the mutant than for wild type.

inhibited by a 1-min application were not further inhibited by a 5-min application; apparently the reaction had gone to completion. One mutant, αI220C, which was not significantly inhibited after a 1-min application of MTSEA, was significantly inhibited after a 5-min application.

In the presence of ACh, when the channels visit the open, desensitized, and closed states, a 1-min application of 2.5 mM MTSEA had statistically significant effects on αY213C and αV218C (Figure 2C). The effect on αV218C was a potentiation of subsequent ACh-induced current. MTSEA added in the presence of ACh had a similar potentiating effect on αS252C in M2, which was correlated with a decrease in the K_{app} for ACh and was rationalized as a stabilization of the open state relative to the closed state, superimposed on a decrease in conductance (Akabas et al., 1994a). In oocytes expressing αV218C, the addition of 2.5 mM MTSEA for 5 min in the absence of ACh did not prevent the potentiation due to the subsequent addition of MTSEA for 1 min in the presence of ACh. It appears that MTSEA did not react with αV218C in the absence of ACh.

The negatively charged MTSES added in the absence of ACh had no effect on any of the mutants (Figure 3A). Added in the presence of ACh, 10 mM MTSES inhibited only αY213C (Figure 3B).

DISCUSSION

We interpret the above results to indicate that the N-terminal third of αM1 contributes to the lining of the channel. The only prior indication of this was the specific photolabeling of the extracellular end of αM1 in *Torpedo* ACh receptor by the noncompetitive inhibitor quinacrine azide (DiPaola et al., 1990; Karlin, 1991). Other evidence indicated that some residues in the middle of M1 are accessible to highly hydrophobic reagents, possibly reaching M1 from the lipid bilayer (Marquez et al., 1989; Blanton & Cohen, 1994). From the effects of the mutation of one of these residues, the cysteine in γM1 aligned with αCys222, Lo et al. (1991) concluded that it is not exposed in the channel. Our results are consistent with the exposure in the channel of the third of αM1 closest to the extracellular side of the membrane and the lack of exposure in the channel of the middle third, including αCys222.

Our interpretation is based on a number of assumptions. We assume that the MTS reagents react at an appreciable rate only with water-accessible cysteines for two reasons: MTSEA is three orders of magnitude more soluble in water than in *n*-octanol (Akabas et al., 1992). Furthermore, the reaction of the MTS reagents with sulfhydryls, as exemplified by the reaction of methylmethanethiosulfonate with 2-mercaptoethanol, is 10 orders of magnitude faster with the ionized thiolate than with the unionized thiol (Roberts et al., 1986), and only water-exposed cysteine thiols are likely to ionize. We also assume that the only water-accessible surface in the membrane-spanning domain of the receptor is the lining of the channel. Given the topology of the receptor, M1 spans the membrane, but the limits of the membrane-spanning portion are not known. The predicted length of M1 is 27 residues, but minimally about 20 residues in an α-helical conformation and about 10 residues in a β

strand conformation are needed to span a bilayer. Conceivably, the N-terminal third of M1 might not be within the membrane-spanning domain and might be exposed at an extracellular water-accessible surface of the protein different from the channel lining. Six of the seven cysteine-substituted residues that react with the positively charged MTSEA, however, do not react with the negatively charged MTSES, and this charge-selectivity is consistent with these residues being in the cation-specific channel.

Because we use an engineered cysteine as a reporter of the accessibility of the corresponding wild-type residue, we need to consider whether the cysteine side chain has the same exposure as the wild-type side chain it replaced. We assume that if the function of the mutant receptor is similar to that of wild-type receptor, then the structures are also similar. As we found in the M2 segment of the ACh receptor (Akabas et al., 1994a), the mutation to cysteine was well-tolerated. Of the 14 mutants in M1, only αP221C failed to yield functional receptors. [The mutation of the aligned proline to glycine in the neuronal α7 subunit also resulted in nonfunctional receptors (Dang & Patrick, 1993).] The K_{app} characterizing the response of the 13 functional mutants ranged from 13 times greater to 10 times smaller than the K_{app} of wild-type receptor (Table 1). [For one mutant in M2, αL251C, which was analyzed by single-channel recording, the 20-fold decrease in K_{app} was due to changed gating kinetics (Sigurdson et al., 1994).] In M1, the Hill coefficients of the responses of the mutants were little different than the Hill coefficient for the response of wild-type receptor. Because the functional properties of the mutants were only slightly different than those of wild-type receptor, we assume that the structures of the mutants were little different from the structure of wild type. The K_{app} obtained by us for wild-type receptor was 2.6 μM, compared to 11 μM obtained by Tomaselli et al. (1991) using fast application of ACh and patch clamp recording. Fast desensitization, which limited the peak currents we observed, accounts for this difference.

We infer that a residue substituted by cysteine is accessible in the channel if the response of the mutant to ACh is irreversibly altered by an MTS reagent. Although we do not know the detailed mechanism of the alterations, whether on the conductance or on the kinetics of channel opening and closing, the alteration itself allows us to infer that the reagents reacted with the substituted cysteines. The magnitudes of statistically significant[2] alterations range from 24% inhibition of αN217C by 1 min of MTSEA (Figure 2A) to 91% inhibition of αY213C by 5 min of MTSEA (Figure 2B). The small extents of inhibition of αI215C, αV216C, and αN217C following a 1-min reaction with MTSEA were not reflections of incomplete reactions, since the extents of inhibition are not much different after a 5-min reaction. There are clear differences, however, between the susceptibility of these three mutants to MTSEA in the presence and absence of ACh: only in the absence of ACh does MTSEA have a significant effect (Figure 2). In contrast to statistically significant effects on current, which we can interpret to be the result of covalent modification and, hence, to be indicative of the accessibility of the modified cysteine in the channel, the lack of a statistically significant effect does not necessarily indicate that no reaction has occurred. The covalent modification could have little effect on function, or it could have canceling effects, for example, by decreasing conductance and increasing channel open time. Nevertheless, our finding that αCys222 and αLeu223 are unaffected by the highly hydrophilic MTSEA is consistent with the labeling of these residues by highly hydrophobic labels (Marquez et al., 1989; Blanton & Cohen, 1994). The screening approach we have used identifies many residues at the water-accessible surface but not necessarily all such residues.

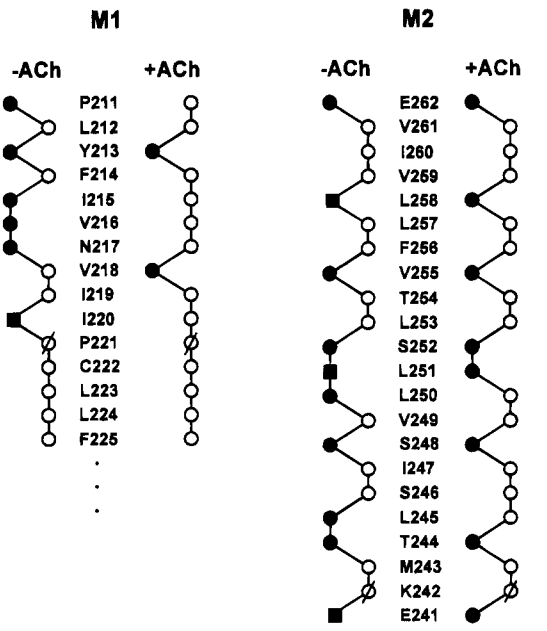

FIGURE 4: ACh-induced changes in the exposure in the channel of residues in M1 and M2. Exposure in the channel is based on the functional effect on cysteine-substituted residues of 2.5 mM MTSEA added for 1 min (filled circles) or for 5 min (filled squares) in the absence (left) or presence (right) of ACh. Residues unaffected by 5 min of MTSEA in the absence of ACh or by 1 min of MTSEA in the presence of ACh are indicated by open circles. αP221C and αK242C did not give functional channels (slash through circle).

In the M1 segment, the pattern of accessibility of the substituted cysteines is not compatible with the exposure of a stripe of regular secondary structure, either α-helix or β-strand. (Figure 4). The extracellular half of M1 might have an irregular secondary structure, or the M1 segments in the two α subunits might have different structures or exposures in the channel. From the pattern of labeling by a hydrophobic reagent, Blanton and Cohen (1994) concluded that the region of αM1 from αCys222 to αLeu228 also did not form a regular secondary structure. In the M2 segment, however, the pattern of accessibility was consistent with an exposed stripe of an α-helix, interrupted by a three-residue stretch of extended structure (Akabas et al., 1994a).

Whatever the structure of M1 is, this structure or its location relative to other membrane-spanning segments is

[2] To determine whether a mutant reacted with an MTS reagent, we determined whether the response of the mutant was altered significantly more than the response of wild type. We determined the statistical significance of these differences by the analysis of variance, using the Student-Newman-Keuls criterion, as previously described (Akabas et al., 1994a). Using this criterion, the responses of αP211C, αY213C, αI215C, αV216C, and αN217C were significantly altered by a 1-min application of MTSEA in the absence of ACh. By a less stringent criterion, for example, least significant difference, the response of αL224C was also significantly altered, and, conversely, by a more stringent criterion, Tukey, only the responses of αP211C, αY213C, and αI215C were significantly altered. Thus, for those mutants where the effects of MTSEA are small, our conclusion about whether or not a mutant reacted with MTSEA depends on the stringency of the statistical test used.

different in the absence and in the presence of ACh (Figure 4). In the absence of ACh, the channel is predominantly closed. In wild-type receptor, the spontaneous open probability is 10^{-5} (Jackson, 1986). For none of the M1 or M2 cysteine-substitution mutants was there a greater baseline leak than for wild-type receptor, indicating that the spontaneous open probabilities are not markedly increased. In the presence of ACh, the receptors spend time in the the open, closed, and desensitized states. During the 1 min of application of MTS reagent in the presence of ACh, there is substantial current (microamperes), and thus some of the receptors are open, but a large fraction is desensitized [see Figure 2B in Tomaselli et al. (1991)]. Thus, the difference in the exposure of M1 and M2 in the absence and in the presence of ACh correlates with the difference between the closed state and the open or desensitized states.

In neither M1 nor M2 does the ACh-induced change in exposure arise from a rotation of these segments around their axes, because in neither segment is a new stripe of residues exposed in the presence of ACh. One possibility is that, in the ring of five subunits surrounding the channel, the M1 segments and the M2 segments alternate and that during gating these segments move relative to one another (Akabas et al., 1994a), increasing the exposure in the channel of αLeu258, αVal255, and αLeu251 in M2 and decreasing the accessibility of αPro211, αIle215, αVal216, and αAsn217 in M1 (Figure 4). The movements of M1 and M2 could flip a gate possibly formed by the cytoplasmic loop between them.

The negatively charged MTSES reacted only with αY213C and only in the presence of ACh. This result is similar to the susceptibility of αV255C in M2 to MTSES only in the presence of ACh (Akabas et al., 1994a). Both αV255C and αY213C were accessible to MTSEA both in the presence and in the absence of ACh. ACh apparently induces a subtle change in the environment of these residues that permits access by an anionic reagent in an otherwise cation-selective channel. The inhibition resulting from the reactions of the oppositely charged MTSEA and MTSES with αY213C and αV255C cannot be due to solely an electrostatic interaction with cations conducted by the channel.

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