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Acetylcholine Receptor Gating is Influenced by the Polarity of Amino Acids at Position 9′ in the M2 Domain

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Abstract. Ligand-gated ion channels contain a conserved leucine at position 9′ (L9′) in the M2 transmembrane domain. We used multiple substitutions at this position in the γ subunit of the mouse acetylcholine receptor (AChR) (γL9′) to examine the role of residue polarity at this position in the gating process at both the macroscopic and single-channel levels. The midpoint of the macroscopic dose-response relationship (EC_{50}) and the channel closing rate constant, α, decreased as the polarity of the residue at that position increased, suggesting a stabilization of the open state of the channel. Both parameters showed similar dependencies on the polarity of the substituted residue. These data support the notion that during AChR gating, the amino acid at the 9′ position moves into a polar environment, and that interactions between this residue and the polar environment determine the stability of the open state. Since this residue is conserved in all other members of the ligand-gated ion channel family, we suggest that a similar mechanism applies to the other members of the family.

Key words: Ligand-gated ion channels — Site-directed mutagenesis — Conformational changes

Introduction

The nicotinic acetylcholine receptor (AChR) is a member of the so-called "cys-loop" ligand-gated ion channel (LGIC) family, which includes muscle- and neuronal-subtype AChRs, the γ-aminobutyric acid type A ($GABA_A$) receptor, glycine receptor, and the serotonin $5HT_3$ receptor (Karlin & Akabas, 1995; Ortells & Lunt, 1995). Studies from a number of laboratories have identified the second transmembrane (M2) domain as forming most of the lining of the ion channel (Hucho, Oberthur & Lottspeich, 1986; Imoto et al., 1986; Leonard et al., 1988; Giraudat et al., 1989; White & Cohen, 1992; Akabas et al., 1994). A highly conserved leucine (L9′ in the consensus numbering system of Miller (1989), in which position 1′ refers to the cytoplasmic end of the M2 domain) is found in the middle of the M2 domain of the subunits of the members of the LGIC family. Work from several laboratories has implicated this residue in channel gating, but its precise role is unclear. Based on examination of the structure of the *Torpedo* AChR in the closed- and open-channel conformation, Unwin (1995) hypothesized that the L9's from each subunit interact to form a constriction in the closed state of the channel that prevents the flow of ions through the channel. Upon agonist binding, these interactions are destabilized, leading to channel opening. In other words, L9′ forms the "gate" of the channel. Changeux and colleagues carried out experiments on α7 homo-oligomeric AChRs expressed in oocytes in which L9′ was mutated to threonine (L9′T) and found that rate of receptor desensitization was markedly reduced, the macroscopic dose-response relationship was shifted towards lower concentrations, and a new conductance state of the channel appeared (Revah et al., 1991). They postulated that these leucines move into the lumen of the channel to occlude the pore during desensitization, and that replacement of the leucine by the more polar threonine created a conducting desensitized state. Subsequent studies have shown that neither of these two mechanisms is entirely correct.

Cysteine-scanning mutagenesis of the M2 domain of the α subunit of the mouse muscle AChR suggested that the narrowest constriction of the pore is at the cytoplasmic end of the channel, not at the center, where L9′ is located (Akabas et al., 1994; Wilson & Karlin, 1998). This indicates that L9′ cannot serve as the "gate" of the channel. In addition, work from two other laboratories

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in which L9′ was replaced by either threonine (Filatov & White, 1995) or serine (Labarca et al., 1995) in one or more subunits of the mouse muscle AChR showed that the macroscopic dose-response curves shifted progressively towards lower concentrations as more subunits with the L9′T (or L9′S) substitution were introduced into the receptor. In addition, the magnitude of the shift depended only on the number of mutations introduced into the receptor complex, rather than which particular subunits were mutated. Finally, no alteration in conductance state for the receptor was reported by either group. Taken together, these two sets of data indicate that the effects of the mutations were independent and additive, which is not in agreement with predictions from either Unwin's or Changeux's model.

In an earlier study, we hypothesized that the role of the conserved leucine is to set the mean open time of the channel through interactions with other, polar, regions of the receptor when the channel is in the OPEN state (Filatov & White, 1995). When L9′ is replaced by a polar residue such as theonine, these interactions are strengthened, resulting in a stabilization of the open state of the channel, and the concomitant longer mean open time and a leftward shift in the dose-response curve.

In this study, we have tested this notion by carrying out multiple substitutions at L9′ in the γ subunit (γL9′) of the mouse muscle AChR which encompass a wide range of residue polarities, as measured using the Eisenberg hydrophobicity scale (Eisenberg et al., 1984). Two different measures of gating, the midpoint of the macroscopic dose-response curve (EC_{50}) and the channel closing rate (α) were determined for receptors comprised of wild-type α, β, and δ subunits and a mutant γ subunit. Both the EC_{50} values and the channel closing rate constant decrease as the polarity of the residue at γL9′ is increased. The results support the notion that L9′ interacts with a polar environment in the OPEN state of the channel, and that these interactions determine the stability of the open channel.

Materials and Methods

Molecular Biology and Cell Transfection

Subunits of the mouse muscle AChR in the mammalian expression vector pRBG4 were used for all expression studies. Site-directed mutants were created using either the Altered States (Promega, Madison, WI) or the QuickChange (Stratagene, La Jolla, CA) mutagenesis systems. Mutant γ subunits are described as "γL9′X", where "X" represents the amino acid replacing L9′.

Receptors were expressed in tsA201 cells, a derivative of the widely used HEK 293 cell line (obtained from R. Horn, Thomas Jefferson University). Cells were transfected using the calcium phosphate precipitation technique using a total of 1.25 μg AChR cDNAs in a subunit stoichiometry of 2:1:1:1 (α:β:γ:δ) per 35 mm dish. Cells were incubated with the DNA-calcium phosphate precipitate for 10 hr, and then fresh medium was added. Cells were used 24–36 hr after transfection. In some cases, cells were cotransfected with a vector encoding the CD8 antigen (Margolskee, McHendry-Rinde & Horn, 1993), and then transfected cells were identified using adherence of microspheres containing an anti-CD8 antibody (Dynal A.S., Oslo, Norway (Jurman et al., 1994)). Cells with at least three beads attached were then used for recording.

Electrophysiology and Data Analysis

Whole cell recording was carried out at a holding potential of −70 mV using an Axopatch 200A (Axon Instruments, Burlingame, CA) with an extracellular solution containing (in mM): 140 NaCl, 5 KCl, 1.7 $MgCl_2$, 1.8 $CaCl_2$, 25 HEPES, pH 7.4 and an intracellular solution containing 145 KCl, 5 NaCl, 1 EGTA, 25 HEPES, pH 7.4. After obtaining the whole-cell configuration, cells were lifted off of the bottom of the dish and ACh was delivered to the cells using a Warner SF 77 Fast Step perfusion system (Warner Instruments, Hamden, CT) under computer control. Under these conditions, the solution around the cell could be completely exchanged within 10–15 msec. Due to the large size of some of the currents, series resistance compensation (90–95%) was used.

Dose-response curves from individual cells were normalized to the maximum current and fit to Eq. 1 using a Levenberg-Marquardt algorithm in a commercially available analysis package (Igor Pro, WaveMetrics, Lake Oswego, OR):

$$\theta = \left[1 + \left(\frac{EC_{50}}{[A]}\right)^{n}\right]^{-1} \quad (1)$$

where θ is the normalized current, [*A*] is the ACh concentration, EC_{50} is the concentration of ACh required to obtain the half-maximum current, and *n* is the apparent Hill coefficient. Only those cells that had stable current amplitudes (<10% variation with repeated application of a test dose throughout the construction of the dose-response curve) were used.

Single-channel currents were measured in the cell-attached configuration with an extracellular solution (in mM): of 142 KCl, 5.4 NaCl, 1.7 $MgCl_2$, 1.8 $CaCl_2$, 25 HEPES, pH 7.4 and the same solution in the pipette supplemented with 50 μM ACh. Currents were low-pass filtered at 5 kHz and acquired at 25 kHz using pClamp 6.0 (Axon Instruments). Single-channel transitions were detected with the use of a half-amplitude algorithm, and open-time histograms were binned and displayed using a square-root ordinate and a logarithmic time axis (Sigworth & Sine, 1987). The channel closing rate, α, was determined by fitting the open-time histograms to a single exponential distribution using a Levenberg-Marquardt algorithm. Only stretches of data without overlapping events were used in the analysis.

Results

We constructed a series of mutant γ subunits in which γL9′ was replaced by several different amino acids and examined receptor activation by ACh. Figure 1 shows currents elicited by an 800 msec application of various concentrations of ACh to voltage-clamped tsA cells transiently transfected with AChRs containing either wild-type γ or γL9′N subunits. In the case of each receptor, the amplitude and activation kinetics depend upon the agonist concentration. However, the concentration de-

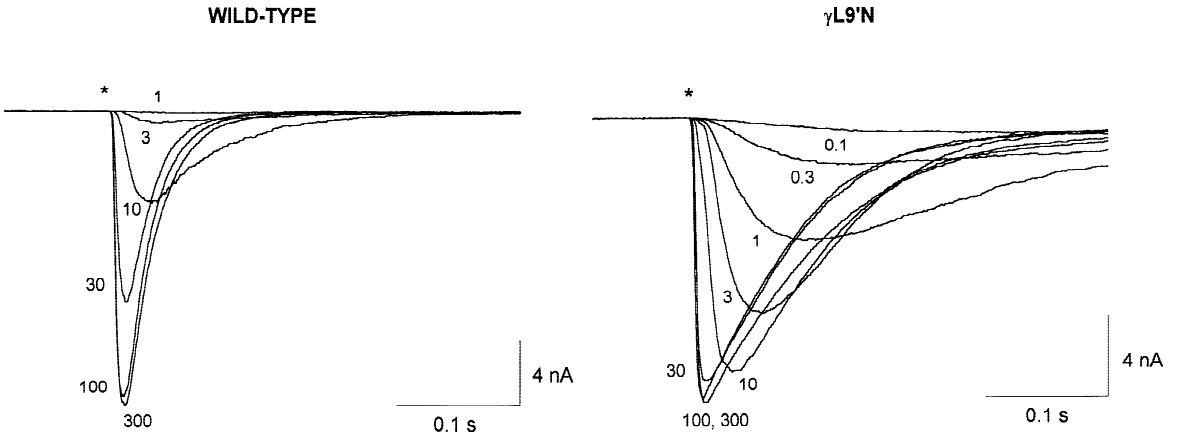

Fig. 1. Currents elicited from wild-type and γL9′N AChRs. Cells were transfected with either wild-type or γL9′N AChRs and currents elicited by an 800 msec application of the indicated concentration of ACh (in μM). Each family of traces was recorded from a single cell with a two-minute wash period between agonist application. Note the increased sensitivity to ACh and the reduced rate of desensitization of γL9′N receptors.

pendence of the current amplitudes recorded from cells expressing the two receptor types is quite different, with γL9′N receptors being activated by lower agonist concentrations and reaching maximal current levels at lower ACh concentrations than WT receptors.

The records shown in Fig. 1 suggest that the dose-response relationship of γL9′N receptors is different from that of wild-type receptors. To examine this more closely, dose-response relationships for wild-type receptors and receptors containing a series of γL9′ mutants (γL9′V, γL9′N, γL9′F, γL9′T, γL9′Y, and γL9′C) were determined. Figure 2 shows that each substitution results in a shift in the position of the dose-response curve towards lower concentrations relative to that of the wild-type receptor. The EC_{50} values for each receptor type depend upon which amino acid is at γL9′, with EC_{50} values ranging from 17.5 μM for wild-type receptors to 2.2 μM for γL9′Y receptors. As was the case for γL9′T (Filatov & White, 1995) or γL9′S (Labarca et al., 1995) receptors expressed in *Xenopus* oocytes, substitutions at position L9′ can markedly alter the equilibrium gating properties of AChRs expressed in transfected mammalian cell lines.

One of the effects of substitutions at L9′ in both muscle-type and homomeric α7 AChRs expressed in *Xenopus* oocytes was the marked slowing or elimination of desensitization (Revah et al., 1991; Filatov & White, 1995; Labarca et al., 1995). The current traces in Fig. 1 also show a slight (approximately twofold) slowing of the rate of desensitization for the γL9′N receptors, although the effect is not as pronounced as in the case of receptors expressed in oocytes. Figure 3 shows the concentration dependence of the time constant for inactivation as a function of the ACh concentration normalized for the EC_{50} for WT and γL9′N receptors. The time constant for desensitization of γL9′N receptors is approximately 2-fold higher (i.e., desensitization is slower) than that for WT receptors across the entire concentration range. Similar results were obtained for the other γL9′X receptors (*data not shown*). Thus, the effect of the L9′ mutations on the desensitization of receptors expressed in mammalian cells is less than the effects observed in receptors expressed in *Xenopus* oocytes.

Previous work on muscle AChRs has shown the substitution of either a threonine (Filatov & White, 1995) or serine (Labarca et al., 1995) at position 9′ resulted in an increase in the channel mean open time for receptors expressed in *Xenopus* oocytes. We interpreted these data to mean that the mutations stabilized the OPEN state of the channel. We have carried out measurements of the channel mean open time for wild-type and γL9′X mutant receptors. Figure 4 shows cell-attached single-channel recordings from cells transfected with either wild-type and γL9′N receptors. It can be seen that γL9′N receptors spend more time in the OPEN state than do wild-type receptors (mean open times of 23.0 msec for γL9′N *vs.* 4.5 msec for wild-type). The Table gives the mean open times of all seven receptors examined in this study. Most mutant receptors examined had longer mean open times than wild-type receptors.

Discussion

Work from a number of laboratories working on ligand-gated ion channels has demonstrated that mutations at L9′ have a significant effect on channel gating, resulting in a shift in the agonist dose-response curve (Revah et al., 1991; Yakel et al., 1993; Filatov & White, 1995; Labarca et al., 1995; Chang et al., 1996; Tierney et al., 1996; Chang & Weiss, 1998). In the case of the muscle-type AChR, substitutions of threonine or serine at L9′ result in a shift in the dose-response curve towards lower concentrations (Filatov & White, 1995; Labarca et al.,

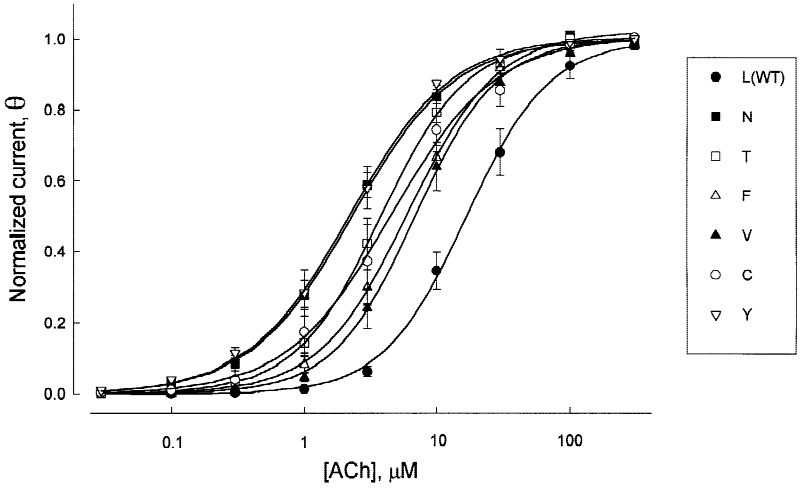

Fig. 2. Dose-response curves for AChRs with various substitutions at γL9′. Dose-response curves were determined for AChRs with wild-type (●), γL9′V (▲), γL9′F (△), γL9′T (□), γL9′C (○), γL9′Y (▽), or γL9′N (■) subunits as described in Materials and Methods. Each point represents the mean ± SEM of 3–5 determinations. The solid curves are drawn according to Eq. 1 with EC_{50} values of 17.5 μM (wild-type), 7.1 μM (γL9′V), 5.8 μM (γL9′F), 4.0 μM (γL9′T), 4.9 μM (γL9′C), 2.2 μM (γL9′Y), and 2.4 μM (γL9′N). Note that the position of the dose-response relationship depends upon the substitution at γL9′.

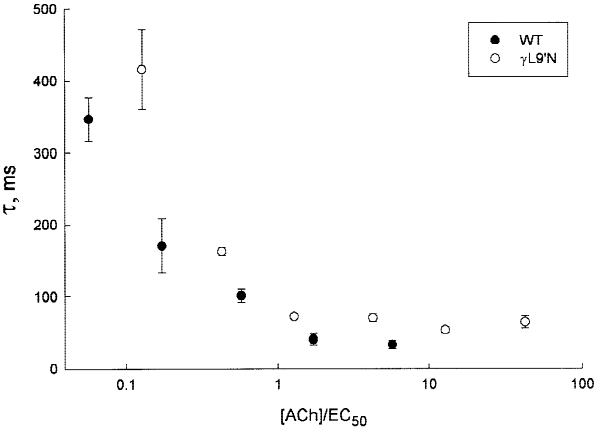

Fig. 3. L9′ mutations do not markedly affect the kinetics of receptor desensitization. The rate of desensitization was estimated by fitting the decay of the ACh-evoked currents to a single-exponential time course for various ACh concentrations (normalized to the EC_{50}) for WT (●) and γL9′N (○) receptors. Note that across the entire concentration range, the γL9′N receptors desensitize approximately twice as slowly as do WT receptors.

1995). Furthermore, the extent of the shift in the dose-response curves depends only on the number of subunits mutated, rather than the particular subunits mutated, suggesting that each mutation had an independent effect on channel gating. Finally, single-channel analysis demonstrated that the underlying mechanism was an increase in the channel mean-open time. Based on these results, we hypothesized that L9′ moved into a polar environment when the channel went from the CLOSED to OPEN conformation, and having a polar residue such as threonine or serine at position 9′ created an interaction which stabilized the OPEN state relative to the wild-type (Filatov & White, 1995). As more subunits were mutated, additional stabilizing interactions would be created, leading to an even greater stabilization of the OPEN state of the channel.

If this model is correct, then the strength of this interaction should depend upon the polarity of the residue at position 9′, and the position of the dose-response curve and the channel mean open time should vary as the polarity of the residue at position 9′ is altered. In this study, we systematically varied the polarity of the residue substituted at position γL9′ (as defined using the Eisenberg hydrophobicity scale (Eisenberg et al., 1984)) and measured the gating properties at both the macroscopic and single-channel levels of the receptors. Figure 5 shows the EC_{50} values and the channel closing rate constant, α, for each mutant receptor plotted against the Eisenberg hydrophobicity scale value for the substituted residue. Both sets of parameters decrease as the hydrophobicity decreases, suggesting a strong relationship between the polarity of the residue and the energetics of the gating process. In addition, the fact that both parameters show essentially the same sensitivity to the substituent hydrophobicity (as shown by the parallel nature of the lines for both parameters) suggests that, as expected, these two measures of channel gating are affected by the same physical processes. The EC_{50} and α values do not correlate with other properties of the amino acid side chains, such as volume or accessible surface area (*not shown*). However, since there is some scatter in the data, it is possible that other properties of the side chains may have some second-order effects.

One significant difference between our results using receptors expressed in transfected cells and results obtained by us and others in previous studies using AChRs expresesd in *Xenopus* oocytes is the relatively minor effect that the γL9′X mutations have on receptor desensitization in transfected cells. The reason for this is unclear; however, the time course of AChR desensitization in *Xenopus* oocytes is much slower than that of AChRs expressed in mammalian cells, suggesting that the desensitization process is perturbed in oocytes. Therefore, any effects on desensitization observed with receptors

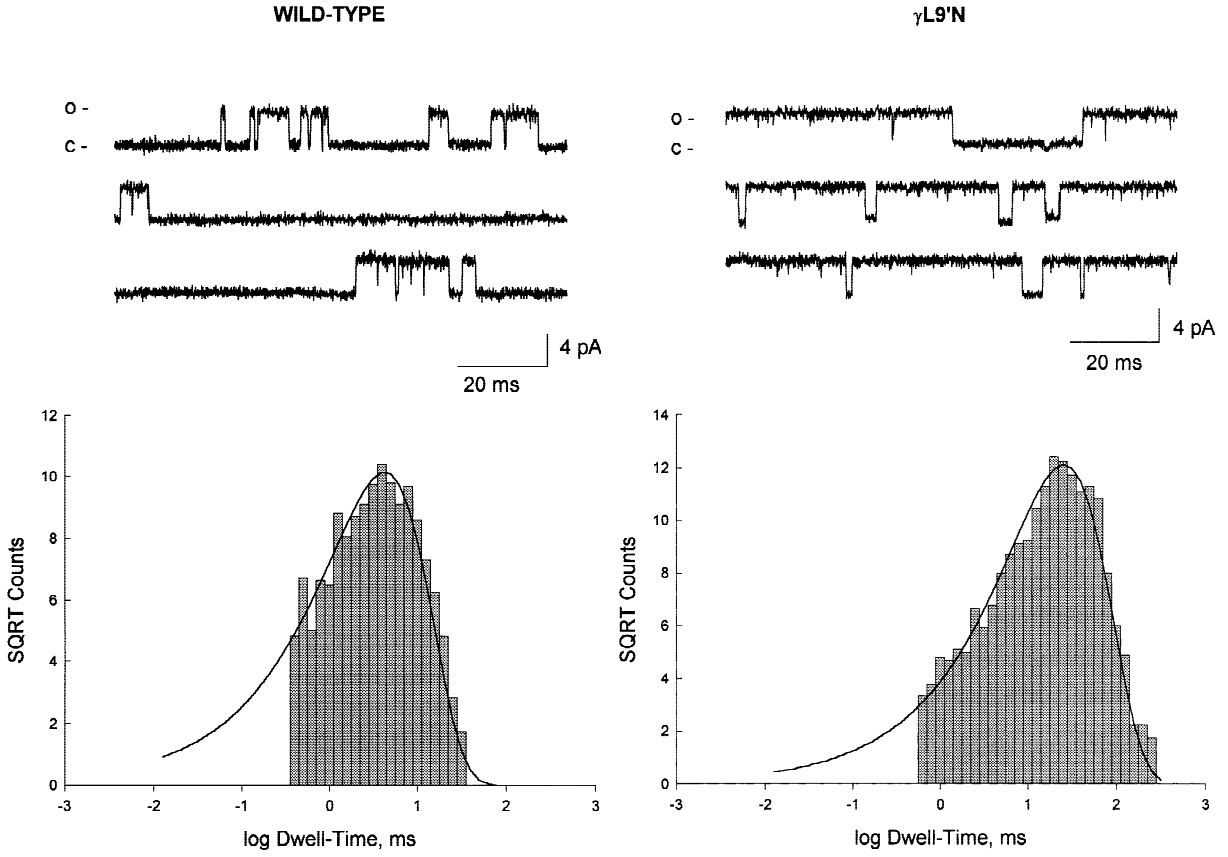

Fig. 4. Substitution at γL9′ alters the single-channel mean open time. Single-channel currents were recorded in the cell-attached mode from cells expressing either wild-type or γL9′N AChRs. The mean open times (along with the error estimate returned by the fitting routine) for wild-type and γL9′N receptors were 4.23 ± 0.04 and 25.54 ± 0.03 msec, respectively.

Table. Single-channel kinetics

Receptor	Mean open time, msec	α, $msec^{-1}$
Wild-type	4.5 ± 0.6 (5)	0.22 ± 0.03
γL9′V	3.8 ± 1.1 (5)	0.26 ± 0.08
γL9′F	1.7 ± 0.4 (6)	0.61 ± 0.15
γL9′T	6.5 ± 1.2 (9)	0.15 ± 0.03
γL9′N	23.0 ± 1.6 (8)	0.04 ± 0.01
γL9′Y	4.7 ± 1.1 (3)	0.25 ± 0.07
γL9′C	5.2 ± 0.2 (3)	0.18 ± 0.01

Single-channel currents were measured in the cell-attached configuration in the presence of 50 μM ACh. The mean open times were determined for each patch from the fits of the open times to a single exponential distribution as described in Materials and Methods. Each value represents the mean ± SEM of measurements from the number of patches given in parentheses.

expressed in oocytes may not be observed in receptors expressed in mammalian cells.

Investigations on other ligand-gated ion channels have also supported the notion that the polarity of the residue at position 9′ affects the gating process. Mutating L9′ to polar residues in the α7 neuronal AChR (Revah et al., 1991) and the $5HT_3R$ (Yakel et al., 1993) resulted in a reduction in the EC_{50} values, although the relationship between polarity and changes in EC_{50} values was not examined in detail. Studies using unnatural amino acid substitution in the muscle-type AChR (Kearney et al., 1996) also suggest that polarity of the amino acid at position 9′ plays a role in receptor gating. In that study, small changes in residue polarity due to insertion of a $–CH_2–$ group or replacement of an –O– group by $–CH_2–$ resulted in small changes in the EC_{50}, with a decrease in EC_{50} as the polarity of the residue increased. Although estimates of the polarity of many of these unnatural amino acids are not available, for the most part the changes in polarity were quite minor, as were the changes in the gating parameters. Our data, on the other hand, encompass a much wider range of polarities, and provide a more comprehensive test of our original hypothesis.

However, not all ligand-gated ion channels show this type of sensitivity to the polarity of the residue at L9′. Chang and Weiss (1998) examined the gating behavior of monomeric ρ1 GABA receptors with a number of substitutions at position 9′. Replacement of L9′ by hydrophilic (Y, S, T) or small (G, A, V) residues produced spontaneously opening receptors (suggesting a de-

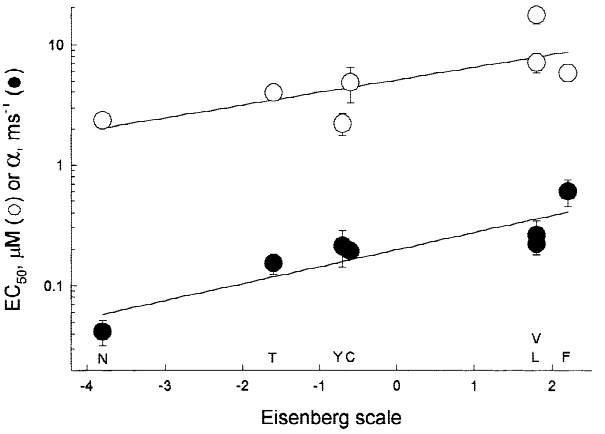

Fig. 5. The midpoints of the macroscopic dose-response curves and channel closing rates vary with residue polarity at position 9′. EC_{50} values (○) and channel closing rate constants (α; ●) for receptors various amino acid substitutions at γL9′ were determined and plotted against the Eisenberg hydrophobicity value for each residue. The solid lines represent fits of the data to either log(EC_{50}) = m * (Eisenberg value) + b or log(α) = m * (Eisenberg value) + *b*. The slopes of the fitted lines were 0.11 ± 0.04 (EC_{50} data) and 0.14 ± 0.03 (α data). The single-letter amino acid code for each residue examined in this study are placed on the *x*-axis at the appropriate Eisenberg hydrophobicity value.

stabilization of the CLOSED state), while replacement by F and V had no effect on the EC_{50}. In this case, while it is clear that L9′ plays a role in the gating of GABA ρ1 receptors, its role may be a bit more complex than in other receptors.

Our results support the notion that L9′ moves into a polar environment when the channel is in the OPEN state; however, they do not provide information about what constitutes this environment. Examination of the sequences of the M1 and M3 domains of the γ subunit identify several polar residues (γC252, γS256, γS257, γT316, γN322, γS323) in the center of these regions that might serve as a polar "partner" for L9′. Mutation of these residues to a nonpolar residue might create the opposite effect on the L9′ mutations; namely, nonpolar residues at L9′ might stabilize the open state, while polar residues at L9′ might destabilize the open state. However, we have been unable to detect any significant change in the mean open time of γS256A, γS257A, γN322A, and γS323A mutant receptors, suggesting that these particular residues do not function as the polar partner for L9′. An alternate, perhaps more obvious, candidate for the polar environment is the lumen of the channel itself, which is, after all, a polar, aqueous environment (Lewis & Stevens, 1983). Scanning cysteine accessibility mutagenesis analysis of the α subunit of the mouse muscle AChR shows that L9′ is more susceptible to the labeling reagent methanethiosulfonate ethylammonium (MTSEA) when the channel is in the OPEN conformation than in the CLOSED conformation (Akabas et al., 1994). This increased susceptibility suggests that L9′ is more exposed to the aqueous lumen of the channel in the OPEN state of the channel, and thus the lumen itself could provide the polar environment that L9′ moves into upon channel opening.

In summary, our data provide evidence in support of the notion that during channel opening, L9′ moves into a polar environment, and the interactions that L9′ makes with this environment are one factor that determines the stability of the OPEN state of the AChR channel. Given the highly conserved nature of L9′ in the members of the ligand-gated ion channel family, interactions between L9′ and its local environment plays an important role in determining the equilibrium between the OPEN and CLOSED states of the liganded receptor for all members of this gene family.

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