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A Point Mutation in the Glutamate Binding Site Blocks Desensitization of AMPA Receptors

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Summary

Desensitization of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors is thought to shape the synaptic response and act as a neuroprotective mechanism at central synapses, but the molecular mechanism underlying desensitization is poorly understood. We found that replacing the glutamate binding domain S1 of GluR3 (an AMPA receptor) with S1 of GluR6 (a kainate receptor) resulted in a fully active but completely nondesensitizing receptor. Smaller substitutions within S1 identified, besides two additional modulatory regions, a single exchange, L507Y, as is required and sufficient for the block of desensitization. This phenotype was specific for AMPA receptors and required an aromatic residue at this position. L507 lies between two residues (T504 and R509) that form part of the glutamate binding site. The physical proximity of these residues, which are involved in binding and gating, suggests they may form part of the link between these two events.

Introduction

L-glutamate, the major excitatory neurotransmitter in the brain, activates three distinct types of ionotropic receptors: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), and kainate receptors (reviewed by Watkins and Evans, 1981; Collingridge and Lester, 1989; Monaghan et al., 1989). AMPA and kainate receptors desensitize completely and rapidly in response to glutamate, with a time constant of ~5 ms (Mayer and Westbrook, 1987; Trussell and Fischbach, 1989; Jonas and Spruston, 1994; Trussell et al., 1994). This profound desensitization, together with slow recovery, is thought to play a role in determining synaptic amplitudes, particularly during high frequency of release; at synapses with multiple release sites; at elevated synaptic activity, when clearance of glutamate is slowed; and during brain damage that causes an increase in glutamate concentrations at the synapse (Trussell and Fischbach, 1989; Otis et al., 1996a, 1996b; reviewed by Jones and Westbrook, 1996). However, although substantial data are present for its role in synaptic function, desensitization at the molecular level is poorly understood.

There are four AMPA-selective subunits, GluR1–GluR4 (or GluRA–GluRD), that can form functional distinct channels in homo- or heterooligomeric assemblies. The kainate receptors assemble from two pools, GluR5–GluR7 and KA1–KA12 (reviewed by Seeburg, 1993; Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994). Topological studies divide the single subunit protein into several domains: (1) four hydrophobic domains, M1–M4, of which M1, M3, and M4 are thought to form transmembrane domains, while M2 forms a reentrant loop that lines the channel pore; (2) a short cytoplasmic C-terminal domain; and (3) two extracellular domains composed of the N terminus and the segment between M3 and M4 (reviewed by Hughes, 1995; Wo and Oswald, 1995).

The extracellular domains share homology to bacterial periplasmic amino acid binding proteins (PBPs; Nakanishi et al., 1990; O'Hara et al., 1993). The first ~400 amino acids are homologous to the lysine/isoleucine/valine-binding protein (LIVBP). The next ~150 amino acids preceding M1 and the segment between M3 and M4 are homologous to both the glutamine-binding protein (QBP) and the lysine/arginine/ornithine-binding protein (LAOBP). These two later segments, termed S1 and S2, were shown to determine agonist binding specificity of AMPA and kainate receptors (Stern-Bach et al., 1994). Site-directed mutagenesis studies further identified specific residues within these regions. Based on solved crystal structures of bacterial proteins, structural models for the glutamate binding site of the ionotropic receptors have been proposed (reviewed by Green et al., 1998; Paas, 1998). The function of the LIVBP-like domain is still unknown, although recently, it has been shown that it controls, together with a short segment at the C terminus of S1 (pre-M1), glycine-independent desensitization of the NMDA receptors (Krupp et al., 1998; Villarroel et al., 1998).

In AMPA receptors, desensitization is modulated by alternative splicing and RNA editing of segments in S2. The alternative spliced versions (known as "flip" and "flop") differ in their time course of desensitization and in their sensitivity to the desensitization blocker cyclothiazide (Sommer et al., 1990; Mosbacher et al., 1994; Partin et al., 1994), and some of the molecular determinants for these differences have been elucidated (Partin et al., 1995, 1996). The amino acid preceding the alternative spliced flip and flop modules is subject to RNA editing, and the edited channels possess faster recovery rates from desensitization (R/G site; Lomeli et al., 1994). Recently, it has also been demonstrated that residues at the N terminus of S2 modulate desensitization of GluR1 (Mano et al., 1996) and GluR6 receptors (Swanson et al., 1997). The role of S1 in the desensitization of AMPA receptors is unclear.

Although both AMPA and kainate receptors desensitize upon continuous application of glutamate, specific

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kinetic parameters vary considerably. These include, first, the extent of desensitization produced by various agonists. For example, kainate produces apparent nondesensitizing currents at AMPA receptors, while it elicits completely desensitizing currents at kainate receptors. Second, the time course for recovery from desensitization is ~50 times slower for the kainate receptors than for the AMPA receptors. Third, they have differential sensitivity to allosteric modulators. For example, Concanavalin A blocks desensitization at kainate receptors but is much less potent at AMPA receptors, while cyclothiazide blocks desensitization at AMPA receptors but has no effect on kainate receptors (reviewed by Bettler and Mulle, 1995). Based on these differences, structural elements specific for AMPA and kainate receptor desensitization may reveal underlying mechanisms of channel gating. We thus analyzed the kinetic properties of the GluR3–GluR6 chimeras that were previously used to investigate receptor pharmacology (Stern-Bach et al., 1994).

In the course of that study, we had identified certain chimeras that did not desensitize at all, while all other receptor channel properties remained intact. Following this unusual behavior, additional mutagenesis identified three regions in S1 of GluR3 that modified AMPA receptor desensitization, with a major role for L507, a residue contained in a structure controlling agonist binding.

Results

Replacing the Binding Domain S1 of GluR3 with the Corresponding S1 of GluR6 Results in a Fully Active but Completely Nondesensitizing Receptor

AMPA- and kainate-type glutamate receptor channels have characteristic desensitization and resensitization kinetics. These were measured in HEK293 cells transiently transfected with pcDNA3 vectors containing $GluR3_{flip}$ (an AMPA receptor) or GluR6 (a kainate receptor) cDNA. For all kinetic measurements, we used outside-out patch recordings in combination with a rapid solution exchange system (Clements and Westbrook, 1991; Colquhoun et al., 1992) in order to obtain solution exchanges faster than the rate of desensitization measured for these glutamate receptors. Patches were exposed to 0.5–2 s pulses of saturating glutamate concentrations (10 mM). As shown in Figures 1A and 1B, applying the agonist at a holding potential of −60 mV evoked a rapidly evolving and strongly desensitizing inward current for both GluR3 and GluR6 homomeric channels. The amount of desensitization, expressed as the ratio of peak to steady-state amplitude (P/S) was 46.2 ± 3.9 for $GluR3_{flip}$ (n = 13) and 236 ± 53 for GluR6 (n = 14). For the rate of desensitization (R_D, the inverse of the desensitization time constant), we measured 240 ± 15.4 and 225 ± 20 s^{-1}, respectively. Desensitization was blocked by cyclothiazide (100 μM) and Concanavalin A (1 μg/μl), when added to the agonist solution of $GluR3_{flip}$ or GluR6, respectively, resulting in P/S values close to one (data not shown). The rate of recovery from desensitization (R_R) was measured in paired pulse protocols and was ~50 times faster for $GluR3_{flip}$ than for GluR6 (29.9 ± 7.1 s^{-1} and 0.57 ± 0.06 s^{-1}, n = 10, respectively). The kinetic characteristics of $GluR3_{flip}$ and GluR6 are consistent with published values and are comparable to native channels (Trussell et al., 1988;

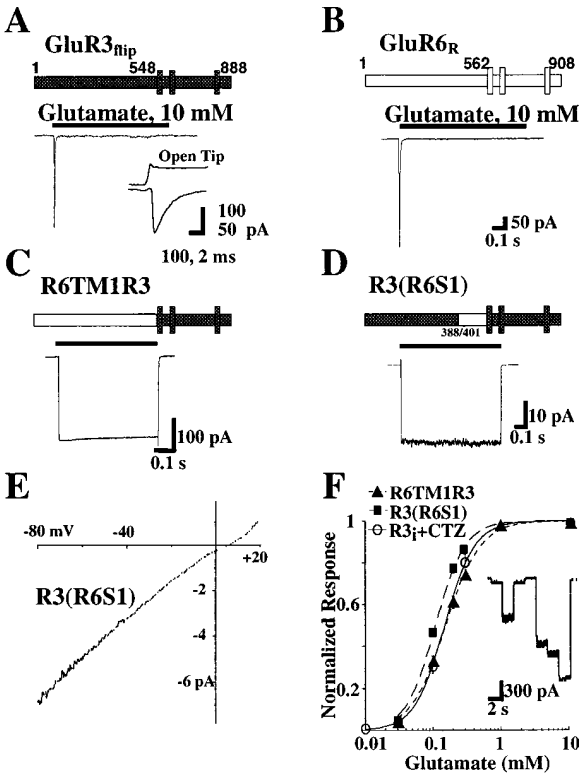

Figure 1. The Role of the N-Terminal Region in AMPA-Type Glutamate Receptor Desensitization

(A–D) Responses to rapid application of 10 mM glutamate from outside-out patches expressing homomeric receptors $GluR3_{flip}$ (A), $GluR6_R$ (B), $R6TM1R3_{flip}$ (C), and $R3(R6S1)_{flip}$ (D), measured at −60 mV. The subunit-type is illustrated above each trace. Black bars correspond to GluR3, white bars to GluR6. The three small vertical bars correspond to the transmembrane domains M1, M3, and M4, respectively. The numbers correspond to the first amino acid, to the beginning of M1, and to the last amino acid, respectively (A–C) and to those at the S1 junction (D). The amino acid numbering starts from the first methionine of the open reading frame. All responses are averaged from 2–50 episodes. Inset in (A) shows the same response on a 50-fold faster time scale. The solution exchange was estimated by the open tip current at the end of each experiment (as shown above, inset).

(E) Current–voltage relationship of $R3(R6S1)_{flip}$ in outside-out patch configuration. Voltage was ramped from −80 to +20 mV at 1 mV/ms. The trace represents an average of seven episodes in the presence of 10 mM glutamate after leak subtraction. Patch solutions contained no polyamines.

(F) Dose–response relationship for L-glutamate for $GluR3_{flip}$ (in presence of 100 μM cyclothiazide, white circles), $R6TM1R3_{flip}$ (black squares), and $R3(R6S1)_{flip}$ (black triangles), recorded in a whole-cell mode. Currents were normalized to the response at 10 mM. EC_{50} and hill slope values (n) were estimated by fitting the concentration–current relationship with the equation $Y = 1/(1 + [EC_{50}/(Glu)]n)$ and were 148 μM/1.95 for $GluR3_{flip}$, 155 μM/n = 1.66 for $R6TM1R3_{flip}$, and 107 μM/n = 2.02 for $R3(R6S1)_{flip}$, respectively. Data are from five to nine cells each (at −60 mV). Error bar represents standard error. Inset shows typical responses of a cell transfected with $R3(R6S1)_{flip}$ to a series of glutamate concentrations. The order of concentrations were control, 0.03, 0.1, 0.03, control, 0.2, 0.3, and 10 mM).

Sommer et al., 1990; Heckmann et al., 1996; Traynelis and Wahl, 1997).

To identify specific protein domains modulating receptor desensitization, responses to glutamate from chimeric GluR3–GluR6 receptors were analyzed (Stern-Bach et al., 1994). In contrast to both parent receptors, one N-terminal chimera, termed R6TM1R3 (Figure 1C), in which the entire extracellular N-terminal region of $GluR3_{flip}$ was substituted by the corresponding region of GluR6, showed complete removal of desensitization ($P/S = 1.02 \pm 0.01$, $n = 30$; Figure 1C).

Several studies have indicated that AMPA receptor desensitization is modulated by the "flip/flop" region located in S2 (Sommer et al., 1990; Mosbacher et al., 1994; Partin et al., 1994, 1995). Analysis of the flop version of chimera R6TM1R3 also showed complete removal of desensitization ($P/S = 1.07 \pm 0.04$, $n = 9$; data not shown), suggesting that the removal of desensitization does not require specific splice variants in the flip/flop cassette.

Based on the homology to bacterial proteins and functional studies, the N-terminal region can be separated in two, the LIVBP-like domain and the agonist binding domain S1. These two regions were examined separately by measuring the kinetic properties of chimera R3(R6S1), in which the GluR6 substitution was limited to S1, and chimera R6KBPR3, in which it was limited to the LIVBP-like region. Chimera R3(R6S1) exhibited a fully nondesensitizing response ($P/S = 1.01 \pm 0.01$, $n = 6$; Figure 1D), whereas chimera R6KBPR3 resulted in a receptor indistinguishable from GluR3 ($P/S = 56.2 \pm 22$, $R_D = 232 \pm 44\ s^{-1}$, $R_R = 19.3 \pm 5.2\ s^{-1}$, $n = 6$; data not shown). The LIVBP-like region was recently reported to affect glycine-independent NMDA receptor desensitization (Krupp et al., 1998; Villarroel et al., 1998). To further test its possible role in desensitization, we also checked the kinetic properties of the reverse chimera R3KBPR6 and of chimera NR1KBPR6, in which the LIVBP-like domain was taken from the NMDA receptor subunit NR1a (Stern-Bach et al., 1994). These two chimeras desensitized in a manner similar to GluR6 (R3KBPR6: $P/S = 94.3 \pm 23$, $R_D = 341 \pm 45\ s^{-1}$, $R_R = 0.24 \pm 0.07\ s^{-1}$, $n = 5$; and NR1KBPR6: $P/S = 83.1 \pm 33$, $R_D = 411 \pm 73\ s^{-1}$, $R_R = 0.31 \pm 0.09\ s^{-1}$, $n = 4$). Thus, abolishing desensitization in GluR3 by the chimeric exchange is exclusively a result of replacing the agonist binding domain S1.

We excluded for three reasons the possibility that the observed lack of desensitization for chimeras R6TM1R3 and R3(R6S1) could be due to some other form of kinetic change (for example, an increase of the desensitization rate to an extent that would impede detection in our experiments), and thus, the receptor is being observed in its desensitized state. First, desensitization of AMPA receptors can be blocked by cyclothiazide. Since both chimeras carry the "GluR3-flip" region important for cyclothiazide binding (Partin et al., 1995, 1996), any occluded desensitization should be revealed by an increase of the peak response in the presence of this drug. However, the addition of 100 μM cyclothiazide to the agonist solution (a concentration that increases peak responses of $GluR3_{flip}$ up to 3-fold, together with a complete block of desensitization; Partin et al., 1994) resulted in a 14% ± 4% and 12% ± 3% inhibition of the peak response of R6TM1R3 ($n = 13$) and R3(R6S1) ($n = 10$), respectively. This inhibition is similar to that observed for AMPA receptors saturated with cyclothiazide, after rapid removal of the drug from the external solution (Partin et al., 1993, 1994). Second, the steady-state amplitude of a desensitized AMPA receptor is in the range of 2.5% of the peak response. Assuming similar channel densities, the responses from the chimeric receptor should be quite small. However, patch responses were 156 ± 78 pA ($n = 30$), ~13-fold greater than the average peak responses to GluR3 and ~3-fold greater than responses of GluR3 when treated with cyclothiazide. Third, a desensitized receptor state should be reflective in its single channel behavior by either smaller conductance states, shorter mean open times, or longer shut times. On occasionally occurring patches that contained only a single chimeric channel, the channel opened to an apparent 23 pS state, with a very high open probability (88.3% ± 5% at 10 mM glutamate), similar to the conductance behavior observed with GluR3 single channels treated with cyclothiazide (Rosenmund et al., 1998).

Finally, since S1 is exclusively located on the extracellular site and is part of the ligand binding domain, mutagenesis may influence agonist binding but not ion permeation. Consistent with that, we found no obvious differences in the current voltage properties between $GluR3_{flip}$ and the chimeras R6TM1R3 and R3(R6S1) (Figure 1E). Both chimeras responded to glutamate in a dose-dependent manner that was similar to that observed for $GluR3_{flip}$ (Figure 1F; see also Stern-Bach et al., 1994). As agonist potency strongly depends on receptor desensitization (Trussell and Fischbach, 1989; Patneau and Mayer, 1990; Patneau et al., 1993; Yamada and Tang, 1993; Partin et al., 1994), we removed desensitization of the native receptor $GluR3_{flip}$ by coapplying the desensitization blocker cyclothiazide. Based on the lack of receptor desensitization, these measurements were carried out in whole-cell recordings that allowed more accurate measurements of current amplitudes. Potency values obtained from patches showed identical values and were thus pooled. Interestingly, cyclothiazide reduced glutamate potency from 155 μM to 398 μM for R6TM1R3 ($n = 5$) and from 107 μM to 199 μM for R3(R6S1) ($n = 6$). A similar reduction in affinity was observed for [^{3}H]AMPA binding to rat brain membranes when treated with cyclothiazide (Kessler et al., 1996).

Taken together, these results show that abolishing desensitization in R6TM1R3 and R3(R6S1) does not result in gross alteration of other receptor channel functions. It also suggests that desensitization is an active gating process independent from the process of activation.

Three Distinct Regions in S1 Modify Desensitization Properties of GluR3 Receptors

The S1 region of GluR6 consists of 162 amino acids, of which 79 are different from GluR3. To identify the residue(s) responsible for regulating desensitization, we constructed 12 new "S1" chimeras, consisting of progressively smaller and complementary GluR6 substitutions (N1–N6 and C1–C6, Figure 2). All of the functional C-terminal chimeras altered the desensitization properties of the GluR3 "parent." Chimeras C6, C5, C3, and C2

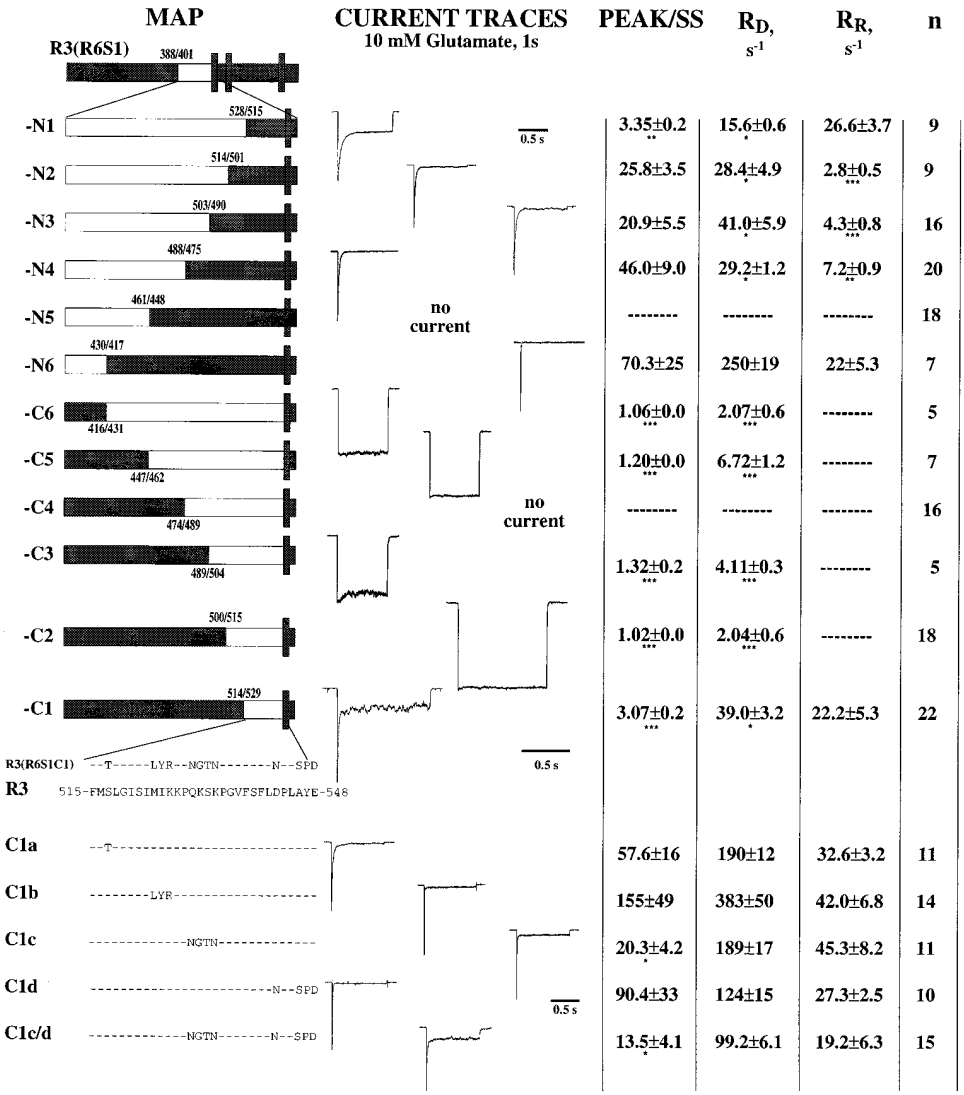

Figure 2. Desensitization Properties of GluR3–S1 Chimeras

(Left column) Map of chimeras and point mutations. Respective S1 regions are shown in black (GluR3) and white (GluR6). The junction residues, given by their number, are shown above each bar and correspond to the color code. GluR3–C1 residues 515–548 are shown in single letter code. Letters in C1 mutants indicate the GluR6 amino acid exchanges and their positions.

(Middle column) Typical current responses to a 1 s application of 10 mM glutamate. Vertical scale bars were omitted for display purposes. Peak response sizes ranged from 4–660 pA.

(Right column) P/S, R_D, and R_R values ± standard error from 5–22 measurements each. Stars under the values indicate significant differences compared with $GluR3_{flip}$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

did not desensitize, while C1 was partially desensitizing (Figure 2). The kinetics of C1 were significantly different from both $GluR3_{flip}$ ($p < 0.001$) and C2 ($p < 0.001$), suggesting that at least two sites within C2 modify desensitization.

The 34 amino acid region replaced in C1 is proposed to include one of the hinge regions connecting the two agonist binding lobes (Stern-Bach et al., 1994; Sutcliffe et al., 1996; Swanson et al., 1997) and was recently found to be involved in glycine-independent NMDA receptor desensitization (pre-M1; Krupp et al., 1998; Villarroel et al., 1998). We further examined the role of the 12 residues in R3(R6S1C1) that are different from GluR3 by grouping them into four different chimeras (C1a-C1d; Figure 2). In comparison to the C1 replacement, the desensitization of all of the C1a-C1d chimeras was statistically different ($p < 0.05$), suggesting that multiple combinations of mutations are required to produce the C1 phenotype.

In addition to the replacements made at the C terminus of S1, those made at the N terminus also modified desensitization properties of $GluR3_{flip}$. Chimeras N2, N3, and N4 but not N6 exhibited significant reductions in both desensitization and resensitization rates (Figure 2). Thus, residues located in the region between R417 and Y474 may also be involved in desensitization. Further studies are required to evaluate their exact role.

In summary, three distinct regions in S1 modify desensitization properties of GluR3 receptors. The first is situated between R417 and Y474 (a cross of N4 and N6

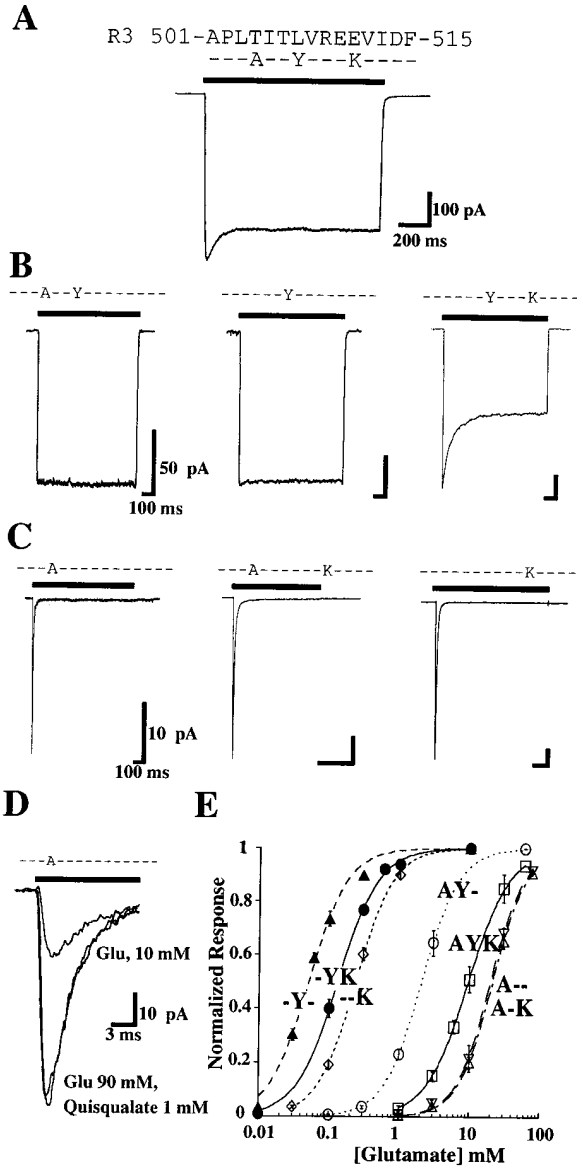

Figure 3. Mutations of T504A, L507Y, and E511K on $GluR3_{flip}$ Differentially Control Desensitization and Agonist Binding

GluR6 residues that replace GluR3 residues are indicated by a one letter code above each trace.

(A) A response from a patch containing receptors with the triple point mutations T504A, L507Y, and E511K. Specific values obtained from 13 measurements were P/S = 1.51 ± 0.07 and R_D = 50.3 ± 8 s^{-1}.

(B) Representative responses from receptors containing the L507Y mutation alone (middle, P/S = 1.01 ± 0.01, n = 12) or in combination with T504A (left, P/S = 1.09 ± 0.04, n = 8) or E511K (right, P/S = 2.1 ± 0.14; R_D = 8.6 ± 0.45 s^{-1}; n = 21).

(C) Representative responses from receptors mutated in T504A (left, P/S = 26.0 ± 5.1; R_D = 90.3 ± 7 s^{-1}; n = 11), E511K (right, P/S = 43.3 ± 8; R_D = 90.3 ± 7 s^{-1}; n = 11), or combined (middle, P/S = 30.7 ± 7.2; R_D = 186 ± 43 s^{-1}; n = 9). All receptors were activated by 10 mM glutamate, except for those containing the T504A mutation, where a concentration 90 mM has been used.

(D) Superimposed responses from a patch containing R3(T504A) to 1 mM quisqualate and 10 mM and 90 mM glutamate as indicated. L-quisqualate- (1 mM) induced desensitization was similar to desensitization evoked by glutamate (P/S = 24.0 ± 7.1, R_D = 360 ± 68 s^{-1}, n = 8).

(E) Dose–Response relationships to glutamate of the mutants shown in (A) through (C) (indicated by letter code on each trace) were measured as described in Figure 1F; desensitizing receptors were measured in the presence of 100 μM cyclothiazide. EC_{50} and hill slope values (n) were L507Y (-Y-) = 48 μM/n = 1.66, L507Y + E511K (-YK) = 131 μM/n = 1.52, E511K (—K) = 236 μM/n = 1.64, T504A + L507Y (AY-) = 2.09 mM/n = 1.64, T504A + L507Y + E511K (AYK) = 9.6 mM/n = 1.48, T504A (A—) = 19.9 mM/n = 1.81, and T504A + E511K (A-K) = 21.2 mM/n = 1.81.

exchanges), the second between A501 and D514 (a cross of C2 and C1 exchanges), and the third between F515 and E548 (C1).

A Single Exchange in Proximity to Residues that Bind Glutamate Removes Desensitization of GluR3 Receptors

The region substituted in the C2 chimera, excluding C1 (i.e., A501–D514), contains only three amino acids that differ between GluR3 and GluR6. These are T504A, L507Y, and E511K (Figure 3). A simultaneous exchange of all three of these amino acids resulted in a barely desensitizing receptor (Figure 3A). The replacement of single amino acid residues within these positions reveals that L507Y accounted entirely for the removal of desensitization (Figure 3B, middle). Its effect was slightly reduced when combined with E511K (Figure 3B, right) but not with T504A (Figure 3B, left). In addition to glutamate, quisqualate (1 mM; P/S = 1.03 ± 0.06, n = 34) or AMPA (1 mM; P/S = 1.05 ± 0.03, n = 24) also elucidated nondesensitizing responses, with an identical efficacy of opening as glutamate (glutamate/quisqualate = 1.02 ± 0.02 and glutamate/AMPA = 0.97 ± 0.03, n = 7, respectively). Desensitization was also abolished by the L507Y mutation when introduced into the flop version of GluR3 (P/S = 1.01 ± 0.04, n = 12; data not shown). In contrast to the L507Y exchange, T504A, E511K, or their combined exchange had no effect on the desensitization rate (Figure 3C) nor on the resensitization properties of $GluR3_{flip}$ (data not shown). Moreover, desensitization of these three later mutants was completely blocked by cyclothiazide (100 μM). In contrast, cyclothiazide reduced peak response of L507Y by 9.6% ± 2.7% and reduced the affinity for glutamate from 48 to 262 μM (n = 6), similar to what was observed for the nondesensitizing chimeras R6TM1R3 and R3(R6S1).

Interestingly, we found that all mutants containing the T504A exchange evoked a weak response to 10 mM glutamate—usually a saturating concentration ($GluR3_{flip}$ receptors; Figure 1F). Responses evoked by quisqualate (1 mM) applied to the same patch were usually about 3-fold greater than the response evoked by 10 mM glutamate (see Figure 3D). The difference between glutamate and quisqualate was observed on both desensitizing and nondesensitizing receptors. Since T504 is proposed to directly interact with glutamate (Stern-Bach et al., 1994; Paas et al., 1996; Laube et al., 1997) and resides near R509, a residue shown by mutagenesis to be critical for agonist binding (Uchino et al., 1992), we tested whether a change in glutamate efficacy or affinity had occurred. Dose–response analysis revealed that all mutants containing the T504A substitution exhibited a less than 50-fold increase in the EC_{50} for glutamate (Figure 3E). The responses at saturating concentrations equaled

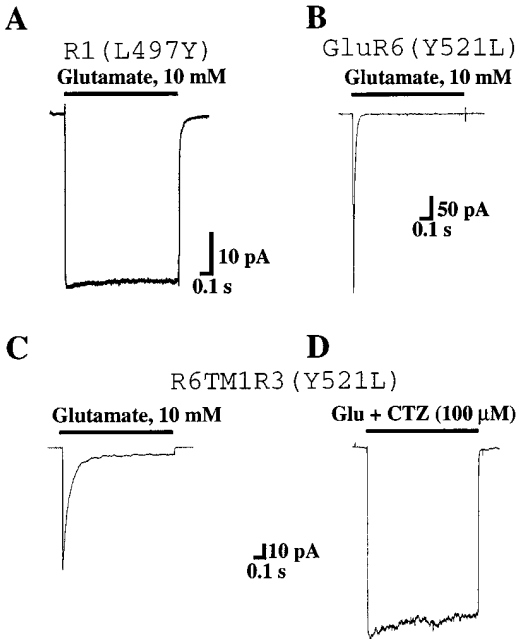

Figure 4. Specificity of L507 for AMPA Receptor Desensitization

(A–D) A typical response to application of 10 mM glutamate, at −60 mV, obtained from a patch containing the mutants GluR1(L497Y)$_{flip}$ (A), GluR6(Y521L) (B), and R6TM1R3(Y521L) (C). In (D), glutamate evoked current in the presence of 100 μM cyclothiazide from the same patch as in (C).

the response amplitude of quisqualate (Figure 3D), indicating that the efficacy of channel opening was not affected by the mutation T504A.

The effects on glutamate potency of both positions T504A and L507Y were independent of each other, as the introduction of the T504A mutation led to a parallel reduction of potency (Y > AY = 44-fold; YK > AYK = 73-fold; K > AK = 90-fold; Figure 3E), suggesting that the mechanisms of the affinity shift were independent. In summary, the mutations in the region T504–E511 reveal an intriguing convergence of agonist binding and receptor desensitization.

Specificity of Position L507 to AMPA Receptor Desensitization

The AMPA receptor subunits GluR1–GluR4 share high sequence homology in the S1 region (>85%), suggesting that a leucine-to-tyrosine exchange on other AMPA receptor subunits as well as in native AMPA receptors would lead to the same phenotype. We tested this by mutating position L497Y on GluR1$_{flip}$, the subunit that shares the least homology with GluR3$_{flip}$. This mutant also resulted in a complete block of desensitization (Figure 4A).

To further test the specificity of site L507, we performed a reverse mutation on the kainate receptor GluR6. Mutant R6(Y521L) was almost identical in its kinetics when compared with the wild-type GluR6 receptor (Figure 4B). This result implies that Y521 is not involved in kainate receptor desensitization, although it was possible that a change to something other than leucine might have an effect. However, mutations of Y521 to glycine (n = 7), valine (n = 4), and glutamate (n = 4) resulted in desensitization properties indistinguishable from GluR6 wild-type (data not shown). Therefore, this particular site (R3–507/R6–521) appears to be specific for AMPA but not kainate receptor desensitization.

The N1 replacement (Figure 2), which includes the L507Y mutation, resulted in a partially desensitizing receptor, similar to that found in the double mutation L507Y + E511K, compared with L507Y alone (Figure 3B, right versus middle). Thus, the control of desensitization by position 507 either may be modulated by other residues, or position 507 is necessary but not specific for the control of desensitization. To test this, we first measured the effect of a reversed Y-to-L mutation on the nondesensitizing R6TM1R3 chimera (see Figure 1C). The resulting R6TM1R3(Y521L) receptor gained back almost complete desensitization but with a 16-fold slower rate (R_D = 15.4 ± 1.1 s^{-1}; P/S = 11.5 ± 2.1, n = 8; Figure 4C). Desensitization was blocked by cyclothiazide (Figure 4D), and resensitization was not different compared with GluR3$_{flip}$ (R_R = 14.2 ± 4.2 s^{-1}, n = 3), suggesting that the kinetic characteristics of mutant R6TM1R3(Y521L) resemble those of GluR3.

Next, we further examined the role of position E511. Recent molecular modeling of the glutamate binding domain predicts that the region T506–V512 is α-helical (see Figure 7). Both L507 and E511 are situated on the surface of lobe 1, with about the same orientation. The interaction observed between these two sites could thus be explained by either specific interactions between positions 511 and 507 or by the entire α helix nonspecifically controlling desensitization. We therefore tested whether a tyrosine residue at position 511 will also result in a nondesensitizing receptor. However, mutant R3(E511Y) exhibits desensitization properties characteristic of the wild-type receptor (P/S = 43.9 ± 13; R_D = 383 ± 50 s^{-1}; R_R = 27.9 ± 8.0 s^{-1}, n = 4; data not shown). Taken together, we can conclude that L507 is specifically required for AMPA-type receptor desensitization to occur but with an additional modulatory effect of surrounding residues on this position.

Removal of Desensitization Requires the Exchange of L507 to an Aromatic Residue

To understand the nature of the removal of desensitization by the L507Y mutation, we introduced residues other than tyrosine in this position (Figure 5). Of the 11 mutations tested, desensitization was blocked by three changes, to phenylalanine (F; P/S = 1.08 ± 0.11, n = 7; Figures 5A and 5D), tryptophan (W; P/S = 1.01 ± 0.03, n = 5; Figure 5D), and histidine (H; P/S = 2.03 ± 0.4, n = 6; Figure 5D), all aromatic. The partial desensitization observed for mutation L507H may be due to the slightly smaller size of the imidazole ring rather than its protonation state, since we observed similar behavior at different pH values (data not shown). Exchanges to the aliphatic alcohol side chains serine (S; Figure 5B) and threonine (T; Figure 5C) resulted in fully desensitizing receptors, with a significant faster desensitization rate, R_D, for the L507T. A similar increase was also observed by the mutation to asparagine (N; Figure 5D). Finally,

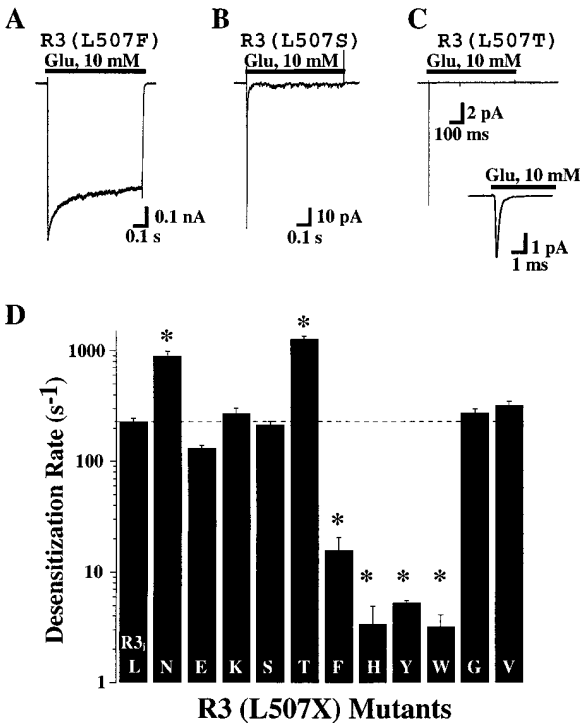

Figure 5. Aromatic Residues in Position 507 Remove Desensitization

(A–C) A typical response to application of 10 mM glutamate, at −60mV, obtained from a patch containing the $GluR3_{flip}$ mutants L507F (A), L507S (B), and L507T (C). Inset in (C) shows the same trace on a faster time scale. The time constant of desensitization is 0.72 ms.

(D) R_D for various substitutions at 507, indicated by a one letter code for the respective amino acids. Significant deviations from the native receptor R3(L507), left, are indicated with an asterisk.

exchanges to the basic/positively charged lysine (K), acidic/negatively charged glutamate (E), or to the relatively small side chains valine (V) and glycine (G) had no apparent effect on desensitization when compared with $GluR3_{flip}$ (Figure 5D).

Kainate Elicits Fast Desensitizing Currents at AMPA Receptors

A structural tie between agonist binding and desensitization could be the basis for the observation that AMPA receptor desensitization depends on the agonist used. Kainate applied to AMPA receptors induces rapid, much weaker desensitizing responses, with considerably lower agonist efficacy than AMPA or glutamate (Patneau and Mayer, 1991; Patneau et al., 1993). If kainate binding and activation induce conformational changes other than those induced by glutamate (particularly the one associated with desensitization), the degree of glutamate-induced desensitization expressed by a receptor should not influence its kainate response. Responses evoked by saturating kainate concentrations (5–10 mM) from the GluR3–S1 chimeras (see Figures 1 and 2) were all essentially nondesensitizing. The efficacy of kainate was maximal for nondesensitizing receptors and was positively correlated (r = 0.91) to the degree of inhibition of

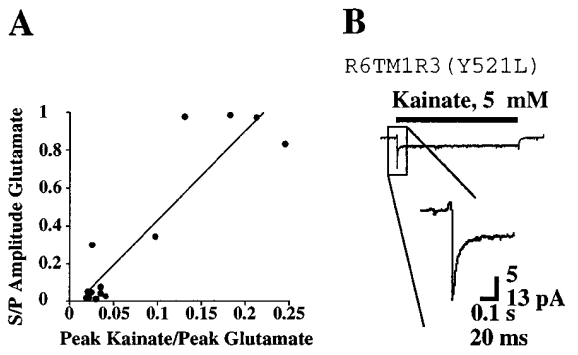

Figure 6. Kainate Elicits Fast Desensitizing Responses at AMPA Receptors

(A) Plot correlates the efficacy of kainate currents (expressed as the ratio of peak kainate current to peak glutamate current) to the P/S of glutamate currents from all receptors examined in Figures 1 and 2 (represented as black circles). Correlation coefficient was 0.91.

(B) A typical response to application of 5 mM kainate, at −60 mV, obtained from the 16-fold slower desensitizing chimera R6TM1R3 (Y521L). Data are from the same patch as shown in Figures 4C–4D. Insert exemplifies kainate current desensitization.

glutamate-induced desensitization (Figure 6A). The fully desensitizing receptors exhibited a peak glutamate/kainate response ratio (G/K) of 53.2 ± 5.3 (n = 55), while for all nondesensitizing mutants, G/K was 6.21 ± 0.7 (n = 66). The lack of apparent receptor desensitization, in contrast to native AMPA receptors (Patneau et al., 1993), may result from desensitization kinetics that are considerably faster than those of activation, thus being either not measurable or overlooked. To test this idea, we took advantage of the 16-fold slower desensitizing receptor R6TM1R3(Y521L). Desensitizing responses to kainate were now apparent (P/S = 2.71 ± 0.2; R_D = 155 ± 28 s^{-1}; n = 6; Figure 6B). Similar results were obtained from chimeras N1–N3 (n = 21; Figure 2 and data not shown), indicating the validity of this aforementioned hypothesis. As kainate responses evoked on AMPA receptors appear rapidly desensitizing and respond comparably as well as glutamate to the removal of desensitization by the S1 chimeras, the conformations the receptor undergoes upon agonist binding occurs regardless of which agonist is used. Differences in respect to the agonist may be the speed of the desensitization process, however.

Discussion

The process of glutamate receptor desensitization is thought to be coupled to binding of the agonist. However, little is known about the structures and mechanisms underlying the gating process. By following the abnormal nondesensitizing behavior of a chimeric GluR3–GluR6 receptor, we were able to detect a single mutation, L507Y, that abolished GluR3 receptor desensitization. L507 resides in between two residues that are involved in glutamate binding, T504 and R509. Moreover, an identical mutation made on GluR1, another AMPA receptor subunit, also resulted in a nondesensitizing receptor. In contrast, the corresponding reverse

mutation, Y521L, made on the kainate receptor GluR6, had only a marginal effect on receptor desensitization.

Two conclusions can be drawn based on these results. First, our data directly link the conformational change induced by the binding of the agonist to both activation and desensitization, since we observed three intertwined residues on one secondary structure element that independently control agonist binding and desensitization. Following binding, activation and desensitization then proceed in two independent and separable conformational changes. Second, the relatively clean separation of desensitization without or only mildly affecting binding or activation suggests that activation, but not desensitization, is a conserved process within the glutamate receptor family. Since site L507 was found to be required for AMPA but not kainate receptor desensitization, AMPA and kainate receptors probably have unique structural motifs that shape their desensitization properties.

Technical Limitations of Mapping Structures to Measurements of Desensitization

Two main problems arise in studying the molecular events underlying AMPA receptor channel desensitization. A mutational approach generates uncertainty over how mutations to single residues or segments affect the structure of the receptor in general. However, we believe this is a minor problem in our study. Chimeric exchanges between AMPA and kainate receptors represent conservative mutations, as both receptors share high sequence homology. We observed no drastic changes whether only the residue L507 or the entire N-terminal part of the protein has been exchanged with GluR6 residues. In addition, the apparent nondesensitizing behavior did not alter other receptor channel properties. On the other hand, a major limitation of the "chimeric" approach is that structures controlling desensitization that are common to both receptors will not be detected. For example, the modest effect (compared with L507Y) observed by the replacements made N- and C- terminal to L507 (i.e., N2, N3, N4, and C1; Figure 2) may be due to highly conservative changes, and thus specific residues in these positions may have a more important role in desensitization than we observed.

Another problem arises from the methods we used to detect desensitization. An accurate measurement requires that channel activation occur much faster than the rate of desensitization. Although a fast flow system with solution exchange times of 300 μs and high agonist concentrations were used, one cannot exclude that the rate of desensitization exceeds activation. However, in all cases where single channels from nondesensitizing receptors could be observed ($n = 34$), the same large conductance sizes (23 pS) and high open probability ($\sim$90%) at high agonist concentrations were seen. Therefore, gating modes carrying larger currents, as has been observed for the steady-state response of the nondesensitizing receptors, are unlikely.

The problem of detection is more apparent for the interpretation of kainate-induced currents. It was previously shown that kainate can evoke very rapidly desensitizing responses at AMPA receptors (Patneau et al., 1993). Our results further support this observation. In general, responses to kainate from chimeras that were nondesensitizing had a higher efficacy compared with responses evoked by desensitizing receptors, suggesting that kainate efficacy is correlated with the degree of receptor desensitization. The maximal efficacy of kainate we observed was in the range of 25%, however. Furthermore, using the 16-fold slower but fully desensitizing chimera R6TM1R3(Y521L), kainate-induced desensitization became more apparent (Figure 6B). Thus, it is likely that the differences in desensitization rates observed for kainate versus glutamate are based on the ability of kainate-activated AMPA receptors to make the desensitization process more rapid, and thus, to make it hard to detect. This may suggest that the first step required for the desensitized state occurs as fast as binding and possibly faster than activation.

Structural Elements Unique for AMPA Receptor Desensitization

Both GluR6 and GluR3 receptors desensitize rapidly and almost completely in response to glutamate. The conservative mutational analysis we have employed screens for structural motifs that are unique for both receptors. This was shown most dramatically with the identification of position L507. Contrary to the corresponding position 521 in GluR6 receptors, L507 was required and sufficient to control AMPA receptor desensitization. The specific phenotypes observed with the L507 mutations and the rescue of AMPA-like desensitization with chimera R6TM1R3(Y521L) indicate a major role of this residue in the process of AMPA but not kainate receptor desensitization. In support of this, L507 is conserved for all AMPA receptors, while in kainate receptors, GluR5/6 contains a tyrosine in the corresponding position 521 and GluR7 contains a histidine. This hypothesis that AMPA and kainate receptors exhibit unique structural elements awaits proof, however. Data obtained using a complementary approach to kainate receptor desensitization may provide an answer.

Another region, R417–Y474 (as defined by the chimeras N2–N4 versus N6; Figure 2), seems to contain structures unique for the extremely slow recovery from desensitization of kainate receptors, as the introduction of this segment in AMPA receptors significantly slowed resensitization rates (Figure 2). The underlying mechanism requires more detailed examination. Moreover, the exchange of this region also significantly reduced the rate of desensitization. As with the region surrounding L507, it contains residues that line the agonist binding pocket (e.g., T423–S427 and D471–Y474), further supporting the close molecular tie between agonist binding and desensitization. Additional mutagenesis is required to identify the specific sites involved.

Previous mutational studies indicated other regions that are involved in AMPA/kainate receptor desensitization. Mutations of residue S650 at the N terminus of S2 in GluR1 (Mano et al., 1996) and the corresponding site A689 of GluR6 (Swanson et al., 1997) have been shown to change desensitization properties. However, a similar mutation (S680A) made on GluR3 or on the nondesensitizing R3(L507Y) had no effect on their kinetic properties

(P/S = 49.9 ± 17, n = 5 and P/S = 1.05 ± 0.02, n = 5, respectively). Another study on the mechanism of cyclothiazide, an allosteric modulator that blocks AMPA but not kainate receptor desensitization, suggests some common form of desensitization. A serine residue in position S780 (S750 in $GluR1_{flip}$), part of the flip/flop module (see Figure 7), is required for the block of desensitization by cyclothiazide (Partin et al., 1995). It was further shown that the introduction of serine in the corresponding position of GluR6 enabled modulation by cyclothiazide.

NMDA receptors also desensitize upon application of glutamate, although on a different time scale and involving multiple mechanisms. Using homologous exchanges between the desensitizing NR2A subunit and the nondesensitizing NR2C subunit, it has been recently found that glycine-independent desensitization of NMDA receptors is controlled by two distinct domains, one adjacent and preceding the binding domain S1 (the LIVBP-like region, pre-S1) and another immediately preceding M1 (pre-M1; Krupp et al., 1998; Villarroel et al., 1998). A role for the pre-S1 region in AMPA receptor desensitization was not apparent in our study. The complementary exchanges of this region between GluR3 and GluR6, or even with the NMDA receptor subunit NR1, did not alter the desensitization properties of the parent receptor. As discussed above, the limitation of our mutagenesis approach does not rule out the participation of residues N-terminal to S1 in AMPA receptor desensitization. It is interesting to note that two other regions in NR2A, termed N6 and module 5 (Villarroel et al., 1998), were also found to modulate NMDA receptor desensitization. Segment N6 contains the site corresponding to L507 in GluR3, and module 5 is located at the N terminus of S2.

The modulation we observed in the C1 region of GluR3 (pre-M1 of NMDA receptors) is complex and involves multiple substitutions (Figure 2). Significant changes in the P/S ratio rather than the desensitization rate were observed. However, in contrast to the NMDA receptors, the replacement of small subsets of residues was not sufficient in modulating desensitization. Together with the ~10-fold difference in desensitization rates, proposing a common mechanism for pre-M1 and C1-like desensitization would be premature. Resolution of the three-dimensional structure awaits to clarify the situation.

The Role of L507 in AMPA Receptor Desensitization

Based on homology to the bacterial PBPs and subsequent experimental data, current structural models suggest that the glutamate binding domain is formed by two lobes that bind the agonist molecule between them (Stern-Bach et al., 1994; Paas et al., 1996; Sutcliffe et al., 1996; Swanson et al., 1997). S1 and the C-terminal half of S2 form the larger lobe 1, while the N-terminal half of S2 forms the smaller lobe 2 (see Figure 7). According to the proposed "Venus flytrap" model (Mano et al., 1996), the agonist binds first to lobe 1, establishing an "open-bound" configuration, and then interacts with lobe 2, forming the "closed-bound" configuration. The open-bound form has been proposed to induce channel activation, while additional interaction with lobe 2 causes channel desensitization. However, L507 is part of a short α helix within lobe 1 (Figure 7), positioned on the surface and close and opposite to the residues involved in agonist binding (T504 and T509), arguing strongly that agonist binding initiates the desensitization process within lobe 1.

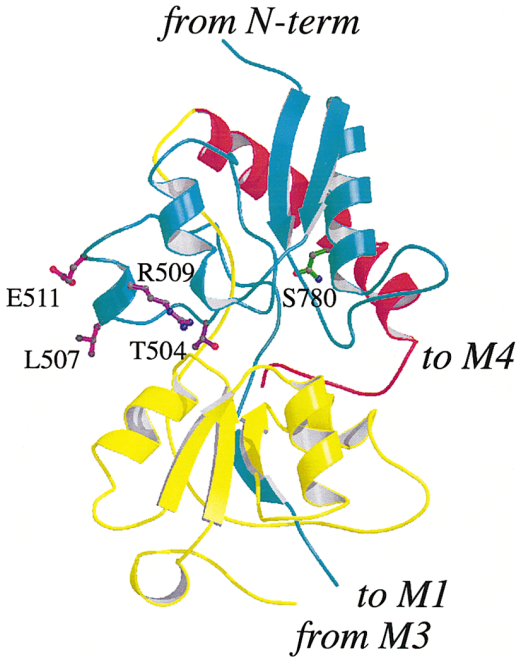

Figure 7. A Model for the GluR3 S1–S2 Binding Domains Based on the Resolved "Closed" Structure of LAOBP

This image was generated as described in Stern-Bach et al. (1994). S1 and S2 are colored blue and gold, respectively, except for the flip/flop region in S2, which is colored red. The side chains of T504, L507, R509, E511, and N780 are marked. The orientation of the structure with respect to the membrane is unknown. The orientation of L507 is similar when modeled according to QBP (T. Green, personal communication).

The movement of L507 during binding (perhaps together with structures within R417–Y474) may therefore be the first in the cascade of conformational changes leading to desensitization. The subsequent changes may be within the subunit monomer and/or involve neighboring subunits forming the oligomeric receptor channel complex. Such interactions could be confined to lobe 1, between lobes 1 and 2, or between lobe 1 and another unknown structure. Currently, most elements found to affect desensitization are contained within lobe 1. The N-terminal region of S1 (R417–Y474) modulated de- and resensitization rates, and the C1 segment traversing toward lobe 2 and the membrane affected the extent of desensitization. The flip/flop module, connecting lobe 1 to the membrane, regulates recovery, desensitization rates, and modulation by the desensitization blocker cyclothiazide.

As L507 is proposed to be on the surface of lobe 1 pointing away from the center, the current structural models cannot predict the segment(s) that interacts with L507. The observation that the reverse mutation Y-to-L on the nondesensitizing chimera R6TM1R3 restored desensitization almost completely suggests that L507 has

to interact with residues C-terminal to it, assuming a yet unproven intrasubunit interaction. Candidate amino acids may be those found at the N terminus of S2 forming lobe 2 or in the flip/flop region contained in lobe 1. However, none of these structures seems to interact directly, based on predicted long distances to L507 (see Figure 7).

Two other possibilities exist. First, one could speculate that L507 (alone or in concert with other residues) interacts directly with the conduction pathway, as the orientation of the lobes relative to the membrane is not yet defined. An analogy may be found in the α7 nicotinic receptor, where a leucine ring in the transmembrane domain is thought to control agonist-dependent desensitization (Labarca et al., 1995). Alternatively, L507 may interact with L507 (or another residue(s) located on neighboring subunits, as they may have close contact at this position. An aromatic bulky residue at this position may destabilize the oligomeric interaction and thus hinder conformational processes underlying desensitization. This is supported by the orientation of residue S780 (S750 in $GluR1_{flip}$), found to be critical for cyclothiazide binding. S780 is facing the same direction as L507 (Figure 7), and it was proposed that cyclothiazide might bind between subunits to accomplish its action on desensitization (Partin et al., 1995).

Desensitization, Open Probability, and Agonist Affinity

Our present results, together with our recent discovery that single channel conductance is controlled by the number of activated subunits (Rosenmund et al., 1998), indirectly suggest that desensitization controls the efficacy of channel opening and dissociation kinetics. As full conductance is reached by the concerted activation of four subunits, activation kinetics (that are agonist concentration–dependent) compete with the ongoing desensitization kinetics, with the result that a higher concentration of agonists are needed to reach the maximum current. In experiments done with single channels of the nondesensitizing receptor R6TM1R3 (Rosenmund et al., 1998), following removal of the agonist, we observed latencies for the switch from the large, fully occupied conductance state to smaller conductances that were longer than predicted (C. R., unpublished data). We interpret these slowed "dissociation kinetics" as trappings of the agonist, owing to the high open probability of the large conductance state (assuming that dissociation is not possible in the open channel state). This may explain the observed apparent increase in agonist affinity either by mutation of site 507 or by coapplication of cyclothiazide as well as the slowed current deactivation upon removal of desensitization (Patneau et al., 1993; Yamada and Tang 1993; Partin et al., 1994). In the α7 nicotinic receptors, mutations that reduce desensitization also result in an apparent increased affinity for agonists (Revah et al, 1991; Bertrand et al., 1992). Since an 80 pS state was observed in addition to the original 45 pS, the apparent increase in affinity has been explained by making the desensitized state permeable by these mutations (Bertrand et al., 1992). However, since we did not observe any additional conductance state(s) by our mutations (Rosenmund et al., 1998 and unpublished data), we favor a model in which the mutations at site L507 prevent entry into the desensitized state.

Taking into account the $\sim$3-fold reduction in glutamate affinity by cyclothiazide, as observed for the nondesensitizing mutants and in binding studies (Kessler et al., 1996), the actual EC_{50} value for wild-type $GluR3_{flip}$ (compared with the measured 148 μM) could be very similar to that obtained for the point mutant R3(L507Y) (48 μM). In this respect, the effect of removal of desensitization by the drug at the glutamatergic synapse may be underestimated. Besides a reported action of cyclothiazide on release (Diamond and Jahr, 1995), an increased efficacy of opening based on the removal of desensitization could be offset by a reduction of receptor affinity, particularly in synapses that show a low degree of postsynaptic receptor saturation.

Concluding Remarks

Following abnormal behavior of chimeric AMPA/kainate receptors, we were able to identify critical residues for AMPA receptor desensitization. Our results also provide a strong molecular basis for the hypothesis that receptor desensitization is directly linked to agonist binding. Applying a similar mutagenesis approach on GluR6 may further extend our knowledge of the mechanisms of glutamate receptor desensitization.

Glutamate receptor stripped of desensitization may serve as a biosensor for glutamate concentrations (sniffer patch) in screening for neuroprotective and nootropic drugs, and it provides a powerful tool for investigating receptor channel properties masked so far by the fast onset of receptor desensitization. For example, by studying the single channel properties of the nondesensitizing chimera R6TM1R3, we were able to detect three conductance states that depend on the number of agonists bound, a finding that led us to propose that glutamate receptors are tetramers (Rosenmund et al., 1998).

Experimental Procedures

Construction of Chimeras and Mutants

Chimeras N1–N6 and C1–C6 were made as previously described (Stern-Bach et al., 1994). Point mutations were synthesized by the PCR-based method described by the QuickChange mutagenesis (Stratagene). All mutants were first subcloned by an appropriate digest in $GluR3_{flip}$-pGEMHE and subsequently moved into pCDNA3 (InVitrogen) for expression in mammalian cells. All mutations were verified by double strand DNA sequencing. The original flop module of chimeras R6TM1R3 and R3(R6S1) (Stern-Bach et al., 1994) has been replaced with the corresponding flip module by using a SalI/XbaI digest of $GluR3_{flip}$. Amino acid numbering starts from the first methionine of the open reading frame.

Electrophysiology

Outside-out patches were obtained from the human embryonic cell line HEK293 (ATCC), expressing homomeric channels composed of rat $GluR3_{flip}$, $GluR6_R$, or chimeras 12–96 hr after transfection by the $Ca_2(PO_4)_3$-method (Chen and Okayama, 1988). Transfected cells were detected as described (Margolskee et al., 1993). All kinetic measurements were obtained from outside-out patches to maximize solution exchange rates. After excision of the patch, the patch was moved into a stream of a rapid perfusion system (Clements and Westbrook, 1991; Colquhoun et al., 1992). Solution exchange (20%–80% to peak) was judged by open tip control by diluting the control solution with 2% water ranging from 0.3–0.6 ms. Experiments were

performed at room temperature (20°C–25°C). Repeated agonist application was done at 0.2–0.02 Hz. Recovery from desensitization was measured by paired pulse application of the agonist. Pipettes were filled with a solution containing 150 mM CsF or CsCl, 20 mM HEPES, 10 mM NaCl, and 10 mM EGTA, adjusted to 305 mOsm (pH 7.3). Holding potential was usually −60 mV. Currents were amplified with an Axopatch amplifier 200 B (Axon Instruments), filtered at 1–10 kHz, and digitized at 2–20 kHz with the pClamp 6.0 (Axon Instruments) acquisition system. The extracellular medium contained 170 mM NaCl, 10 mM HEPES, 2–4 mM $CaCl_2$, and 2–4 mM $MgCl_2$, adjusted to 330 mOsm (pH 7.25). Agonist solutions were made by mixing external medium with isotonic (330 mOsm [pH 7.3]) agonist stock solutions by replacing NaCl with the agonist. Analysis was performed with Axograph 3.5 software, and exponentials were fitted by the squared error method. Multiple measurements from one patch were averaged, and the results were treated as one experiment. The significance of results was determined by analysis of variance followed by Dunns posthoc comparison and are indicated when $p < 0.05$.

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