Molecular Mechanism of AMPA Receptor Noncompetitive Antagonism

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Summary

AMPA-type glutamate receptors are specifically inhibited by the noncompetitive antagonists GYKI-53655 and CP-465,022, which act through sites and mechanisms that are not understood. Using receptor mutagenesis, we found that these antagonists bind at the interface between the S1 and S2 glutamate binding core and channel transmembrane domains, specifically interacting with S1-M1 and S2-M4 linkers, thereby disrupting the transduction of agonist binding into channel opening. We also found that the antagonists' affinity is higher for agonist-unbound receptors than for activated nondesensitized receptors, further depending on the level of S1 and S2 domain closure. These results provide evidence for substantial conformational changes in the S1-M1 and S2-M4 linkers following agonist binding and channel opening, offering a conceptual frame to account for noncompetitive antagonism of AMPA receptors.

Introduction

AMPA receptors (AMPARs) are glutamate-gated ion channels that mediate the majority of fast excitatory synaptic transmissions in the mammalian brain. They are part of a larger family of ionotropic glutamate receptors (iGluRs) that includes the closely related kainate receptors (KAR) and the NMDA receptors (NMDARs) (Dingledine et al., 1999). Due to the critical role that these receptors play at the synapse, there is great scientific interest in elucidating the molecular basis of receptor function. Dysregulation of glutamatergic synaptic transmission is also implicated in a variety of neurodegenerative and neuropsychiatric conditions (Choi, 1992; Franciosi, 2001). Thus, achieving an understanding of the way in which these receptors function may facilitate the development of new therapies to treat diseases of the central nervous system.

AMPARs assemble as tetramers in various combinations of four homologous subunits, termed GluR1-4 (or GluR-A to -D) (Dingledine et al., 1999). Like all iGluR subunits, the AMPAR subunits share a modular design consisting of an extracellular N-terminal oligomerization domain (NTD); an extracellular agonist-binding domain formed by two segments, S1 and S2; a channel-forming domain consisting of three transmembrane domains, M1, M3, and M4 and a reentrant loop M2; and an intracellular C-terminal trafficking and anchoring domain (CTD) (Figure 3; Madden, 2002; McFeeters and Oswald, 2004). Currently, structural data at atomic resolution are available only for the S1 and S2 domains, of which the first and most extensively characterized is that derived from the AMPAR GluR2 (Armstrong et al., 1998; Erreger et al., 2004; Mayer and Armstrong, 2004). Collectively, it has been shown that S1 and S2, which in the intact receptors are separated by the membrane regions M1 to M3, fold in a special manner, creating two globular domains (D1 and D2; Figure 4A, cartoon). Glutamate first docks in D1, which then promotes the rotation of D2 and closure of the binding cleft. Full agonists like glutamate and AMPA induce a large movement (~20º) resulting in full activation. Partial agonists like kainate induce an intermediate closure (~12º) and partial activation, and competitive antagonists like DNQX promote only a small extent of domain closure that is insufficient to trigger ion channel gating. Several studies have provided evidence that the agonist binding domains assemble as dimers, and the mechanism of desensitization has been further defined as a rearrangement of the dimer interface (Mayer and Armstrong, 2004; Sun et al., 2002). Therefore, the idea that agonist binding to S1 and S2 evokes significant conformational changes in the extracellular domains leading to channel opening is widely accepted. However, the mechanism by which these conformational changes are transduced to channel gating is still unclear. Gating is likely to involve the linker regions between the agonist-binding and channel-forming domains, namely the S1-M1, S2-M3, and S2-M4 linkers (Figure 4A; cartoon). These regions are postulated to contribute to an extended mass at the bottom of D2 (Abele et al., 1999) and are therefore likely to be coupled to the movement of D2 upon agonist binding. So far, experimental evidence for such conformational rearrangements is limited to the M3 linker (Erreger et al., 2004; Wollmuth and Sobolevsky, 2004).

There are a number of pharmacological agents that affect AMPAR function through interactions outside of the agonist-binding domain (Kew and Kemp, 2005). Thus, investigating the means by which binding of these ligands modulate channel gating may provide additional insight into mechanisms of receptor function. Toward this end, we have investigated the site of interaction with AMPAR of two selective noncompetitive AMPAR antagonists, GYKI-53655 (GYKI) and CP-465,022 (CP). GYKI belongs to a family of 2,3-benzodiazepines (Solyom and Tarnawa, 2002), and it is a more potent and selective analog of GYKI-52466, the first identified AMPAR noncompetitive antagonist (Donevan and Rogawski, 1993; Tarnawa et al., 1992). CP is a derivative of piraquilone and is ~100-fold more potent than GYKI on

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hippocampal neurons (Lazzaro et al., 2002; Menniti et al., 2000). Radioligand-binding assays suggested that the binding sites of these two compounds overlap with one another but that this site is distinct from the agonist-binding site (Lazzaro et al., 2002; Menniti et al., 2000). These antagonists are not open-channel blockers nor do they affect channel desensitization (Donevan and Rogawski, 1998; Lazzaro et al., 2002; Rammes et al., 1998), suggesting a mechanism of action not involving binding to the channel pore. However, there is an allosteric interaction between GYKI and the inhibitor of desensitization cyclothiazide (CTZ) (Donevan and Rogawski, 1998; Rammes et al., 1998), suggesting that the binding site for GYKI is affected by gating, although the molecular mechanism for this interaction is not known.

Here we show that GYKI and CP bind with different affinity to different gating states of the AMPAR. The highest affinity is for the closed state, most likely the resting rather than the desensitized state, and the lowest is for the open state, further depending on agonist efficacy to open the channel. Using AMPA/kainate chimeras, we show that GYKI and CP bind at the linker regions between S1 and S2 and the channel, specifically interacting with S1-M1 and S2-M4. Therefore, the change in antagonist binding affinity upon gating is indicative of substantial conformational changes in these linkers following agonist binding and channel opening. A model in which these noncompetitive inhibitors constrain the movements of these linkers provides the insight into the way agonist binding is transduced to channel gating through the linker regions. As such, our study provides a potential template for rational drug design.

Results

GYKI and CP are selective for inhibition of AMPAR compared to KAR (Lazzaro et al., 2002; Paternain et al., 1995). Consistent with these previous findings, GYKI and CP completely inhibited glutamate-induced currents of the AMPAR subunit GluR3 expressed in *Xenopus* oocytes at concentrations that had almost no effect at the GluR6 KAR (Figures 1A and 1B). As previously reported (Donevan and Rogawski, 1998; Palmer and Lodge, 1993), the GYKI inhibitory potency (measured as its IC_{50}) was markedly decreased in the presence of the desensitization blocker CTZ (~10-fold; Figure 1A). This shift was initially interpreted as an evidence of competition between GYKI and CTZ. However, later studies suggested that this interaction is rather allosteric. This was based on data from fast kinetic measurements, showing no change in the desensitization onset and recovery kinetics in the presence of GYKI and its inability to reverse the action of CTZ (Donevan and Rogawski, 1993, 1998; Rammes et al., 1998), the observation of a rather small shift in GYKI IC_{50} in the presence of kainate, and the fact that mutations that largely affected CTZ binding did not alter the sensitivity to GYKI (Partin and Mayer, 1996). Figure 1B shows that CTZ also reduces the potency of CP to inhibit glutamate-induced currents at a similar extent as seen for its effect on GYKI. In addition, a previous study using radioligand-binding assays found that CTZ does not interact directly with the CP binding site (Menniti et al., 2000).

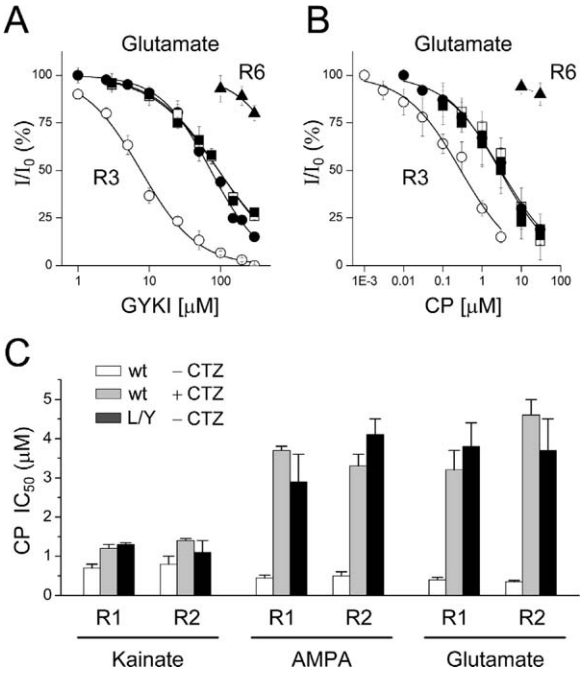

Figure 1. Effects of Receptor Desensitization and Agonist Efficacy on GYKI/CP

(A and B) Representative dose-inhibition measurements of GYKI (A) and CP (B) in the presence of 1 mM glutamate for GluR3 − CTZ (open circles), GluR3 + CTZ (solid circles), R3(L507Y) − CTZ (open squares), R3(L507Y) + CTZ (solid squares), and GluR6 + ConA (solid triangles). Responses (I) were normalized to glutamate-evoked currents without antagonist (I_0). Each point is a mean (±SD) of five to ten oocytes. GYKI IC_{50} values (μM): 7.6 ± 0.5 (−CTZ), 73 ± 3 (+CTZ) for GluR3, and 94 ± 4 (−CTZ), 94 ± 7 (+CTZ) for R3(L507Y). CP IC_{50} values (μM): 0.3 ± 0.1 (−CTZ), 3.0 ± 0.3 (+CTZ) for GluR3, and 3.2 ± 0.7 (−CTZ), 2.8 ± 0.3 (+CTZ) for R3(L507Y).

(C) CP IC_{50} values (±SD) in the presence of kainate (1 mM), AMPA (0.1 mM), or glutamate (1 mM) of wild-type (wt), GluR1, or GluR2 (unedited), without CTZ (white bars) or with CTZ (gray bars), and of R1(L497Y) and R2(L504Y) without CTZ (black bars), measured as in (B).

GYKI and CP Bind with Different Affinity to Different Gating States of the AMPAR

The above-described data further support an allosteric interaction between the CTZ and GYKI/CP binding sites and raise the hypothesis that the blocking of receptor desensitization, rather than CTZ binding itself, is responsible for the effect on GYKI/CP potency. To test this hypothesis, we measured GYKI and CP IC_{50}s at the nondesensitizing L-to-Y mutant of GluR3 (L507Y; Stern-Bach et al., 1998) in the absence and presence of CTZ. As seen in Figures 1A and 1B, the L-to-Y mutation occluded the effect of CTZ on the ability of both GYKI and CP to inhibit GluR3 receptors activated by glutamate. Similar observations were made for L-to-Y mutants of GluR1 and GluR2 receptors (Figure 1C). To further examine the effect of receptor desensitization on antagonist potency, we measured the ability of CP to inhibit the activity of the partially desensitizing agonist kainate in the absence or presence of CTZ. We found that CP inhibits kainate-induced currents with IC_{50} values slightly but significantly higher (~1.5-fold; $p < 0.05$) than those obtained for currents induced by the fully desensitizing agonists glutamate or AMPA

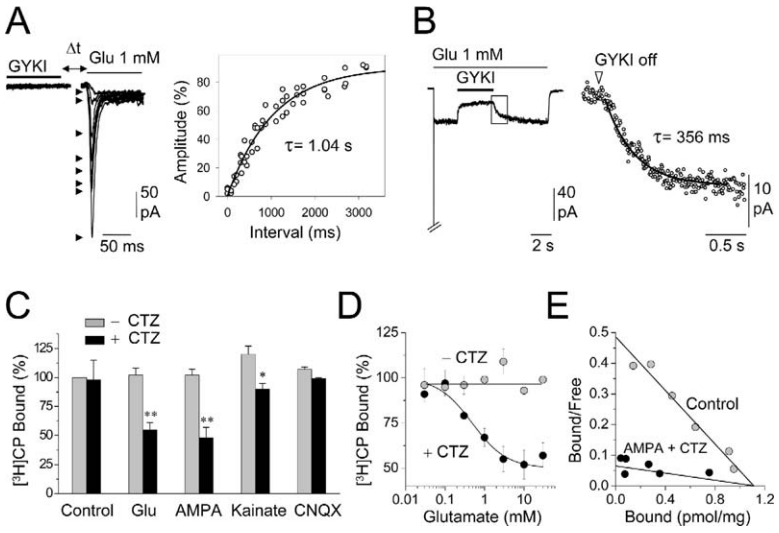

Figure 2. GYKI and CP Bind with Different Affinity to Different Gating Modes

(A and B) Measurements of GYKI unbinding rates from GluR1 receptors expressed in HEK293 cells. (A) To measure the unbinding rate of GYKI from the closed, nonactivated receptor channel, GYKI (30 μM) was applied to the cell for 10–20 s and then washed out. After several washout intervals (Δt), glutamate (1 mM) was applied, and the instantaneous evoked current was measured (arrowheads). Data points (14 cells) were normalized to the response in the absence of GYKI, pooled, and fitted to a single exponential with the indicated time constant (plot on the right). (B) To measure the unbinding rate of GYKI from activated receptor channels, GYKI was applied for 3.5 s once the receptors were activated and then washed off. The time course of current recovery denoted the unbinding rate of GYKI (panel on the right), which was well fitted by a single exponential of the indicated time constant. Traces on the right correspond to the recording fragment indicated by a box in (B).

(C–E) [^{3}H]CP-specific binding to rat-brain membrane. (C) Binding of [^{3}H]CP (3 nM) was measurend in the absence (control) or presence of glutamate (Glu; 10 mM), AMPA (0.1 mM), kainate (3 mM), or CNQX (0.1 mM), with (black bars) or without (gray bars) CTZ. Values were normalized to control without CTZ. Each bar is a mean (±SD) of three to four independent experiments done in triplicate (*$p < 0.05$, **$p < 0.01$). (D) [^{3}H]CP-specific binding (3 nM) to rat-brain membranes incubated with increasing glutamate concentrations, with (black circles) or without (gray circles) CTZ. Values were normalized to control (−CTZ/−glutamate). (E) Representative Scatchard plot analysis in the absence (control) or presence of AMPA (0.1 mM) and CTZ. Plots obtained in the presence of CTZ or AMPA alone were similar to control (data not shown). Mean Kd (±SEM) values were (nM): 3.7 ± 0.5, n = 9 (control), 3.3 ± 0.3, n = 3 (CTZ), 3.2 ± 0.5, n = 3 (AMPA), and 24 ± 7, n = 3 (AMPA + CTZ).

(Figure 1C; compare white bars). The IC_{50} of CP for inhibition of kainate-induced currents was increased a further 2-fold in the presence of CTZ and in the L-to-Y mutant receptor (Figure 1C). These 2-fold increases in IC_{50}s contrast with the 10-fold increases in the presence of CTZ or the L-to-Y mutation when glutamate or AMPA are the receptor activators (Figure 1C). Comparable results were observed for GYKI. Taken together, these results imply that gating state strongly affects the interaction of GYKI and CP with the AMPAR. Furthermore, we directly measured the unbinding rate of GYKI from both unliganded and activated wild-type AMPARs, with the idea that a change in receptor affinity for GYKI upon gating would be accurately indicated by its dissociation rate constant. For these experiments, we expressed the GluR1 subunit in HEK293 cells, and the GYKI dissociation rate from unbound resting receptors was estimated with the following protocol. Cells under recording conditions were equilibrated with GYKI (30 μM) for 10–20 s and then washed out for defined times before applying a pulse of glutamate (1 mM) (Figure 2A). Instantaneous responses increased with washout intervals following a single exponential time course with a time constant of 1.04 s (single exponential adjusted to pooled data from 14 cells), which revealed the GYKI unbinding rate from resting receptors. To measure the dissociation rate from active GluR1 receptors, we applied a 3.5 s pulse of GYKI in the continuous presence of glutamate (Figure 2B). The current returned to the control value upon GYKI removal. Such a relaxation was better adjusted by a biexponential process, being the slowest one ($\tau \gg 1$ s) of minor amplitude. Therefore, we used the initial 1.5 s of the current relaxation to fit a single exponential, which on average presented a τ of 563 ± 179 ms (18 trials from 3 cells). These results indicate that active AMPARs present lower affinity for GYKI than resting nonactivated channels. We also evaluated direct binding of noncompetitive antagonists in radioligand-binding experiments using [^{3}H]CP-526,427 ([^{3}H]CP), a radioactive analog of CP. For these investigations we used purified rat-brain membranes (Menniti et al., 2000), as [^{3}H]CP-specific binding to recombinant receptors expressed either in oocytes or in HEK293 cells was too low for a reliable quantification (see Experimental Procedures). To enrich for receptors in the different gating states, washed membranes were incubated with (1) CTZ alone, enriching for the closed resting state; (2) agonist alone, enriching for the closed desensitized state; and (3) agonist + CTZ, enriching for the open state. While no significant change was obtained with CTZ alone or glutamate alone, in the presence of both CTZ and glutamate we observed a significant reduction in [^{3}H]CP binding (Figure 2C) that depended on the concentration of glutamate (Figure 2D). In the presence of CTZ, a similar decrease in [^{3}H]CP binding was observed for the other full-agonist AMPA (Figure 2C). However, a smaller change was obtained for the partial-agonist kainate, and no change was observed for the competitive antagonist CNQX (Figure 2C). Scatchard plot analysis demonstrated that the change in [^{3}H]CP binding was due to decreased binding affinity and not due to a decrease in the number of binding sites (Figure 2E). The reduction in binding affinity could however be underestimated in these experiments, since CTZ is less effective on AMPAR flop isoforms that are prevalently expressed in the brain (Dingledine et al., 1999).

Based on these results we conclude that GYKI and CP bind to the AMPAR with highest affinity to the closed (resting and desensitized) state and with lowest affinity to the open, nondesensitized state. Since GYKI has no

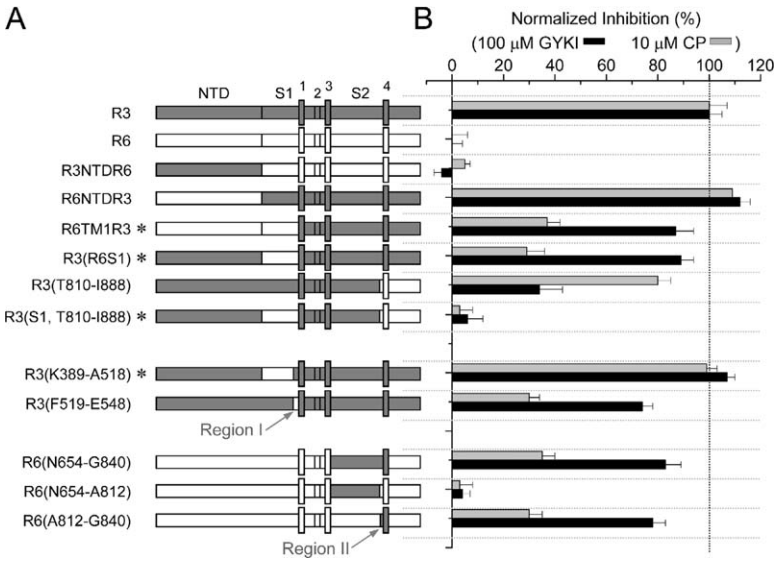

Figure 3. Subunit Sensitivity to GYKI and CP Is Specified by Two Regions Conferring Opposite Sensitivity to Each Antagonist

(A) Description of GluR3/GluR6 chimeras (GluR3, black bars; GluR6, white bars). Subunit topographical domains are indicated above the GluR6 bar (NTD, N-terminal ~400 amino-acid domain; S1 and S2, agonist binding domain; vertical bars, transmembrane regions M1, M3, and M4 and reentrant loop M2). Asterisks indicate nondesensitizing chimeras (Stern-Bach et al., 1998). The numbers in the names of the chimeras indicate the amino acids on the backbone subunit swapped between GluR3 and GluR6 [for example, R3(T810-I888) denotes that residues T810–I888 in GluR3 were replaced by the corresponding residues of GluR6].

(B) Inhibition of glutamate (1 mM) evoked currents by GYKI (100 μM; black bars) or CP (10 μM; gray bars). Currents were measured in the presence of CTZ and/or ConA, as appropriate, and inhibition values were normalized to those obtained for GluR6 (GYKI, 5% ± 2%; CP, 4% ± 2%) and GluR3 (GYKI, 59% ± 8%; CP, 92% ± 7%) as follows: $1 - (I/I_{0(mutant)} - I/I_{0(R3)})/(I/I_{0(R6)} - I/I_{0(R3)})$. Each bar is a mean (±SD) of 20 to 40 oocytes collected from at least two independent experiments. Note that GYKI concentrations higher than 100 μM could not be used due to the limited compound solubility.

effect on the onset of receptor desensitization (Figure 4C) or on its recovery from desensitization (Donevan and Rogawski, 1998), GYKI/CP most likely stabilize the resting state, thereby reducing the probability of channel opening. Furthermore, because the antagonist binding affinity inversely correlates with agonist efficacy, we also suggest that agonist binding to S1 and S2 and the subsequent "domain closure" leads to substantial conformational changes in the GYKI/CP binding site, thereby altering the binding affinity. This suggested that GYKI/CP bind to a site that is within the region of the AMPAR that transduces agonist binding to channel gating. To explore this possibility, we used a mutagenesis approach to identify the GYKI/CP binding site.

Receptor Sensitivity to GYKI and CP Is Conferred by Residues in the S1-M1 and S2-M4 Linker Regions

To identify the GYKI/CP interacting site(s), we took advantage of the iGluR subtype selectivity of these compounds and measured the ability of each antagonist to inhibit the activity of various GluR3/GluR6 chimeras expressed in *Xenopus* oocytes. Because several of these chimeras differ highly in their desensitization kinetics and agonist efficacy profiles (Stern-Bach et al., 1994, 1998)—parameters found to also affect the GYKI/CP inhibition potency—all measurements were done on glutamate-induced currents in the presence of desensitization blockers (CTZ and/or ConA as appropriate; see Experimental Procedures). Collectively, the analysis of the set of chimeras shown in Figure 3 narrowed down the effects of GYKI and CP to two short segments, one consisting of 30 residues preceding M1 [region I, exemplified by chimera R3(F519-E548)], and the other consisting of M4 and 7 preceding residues [region II, exemplified by chimera R6(A812-G840)]. Exchanges containing region I affected mainly the inhibitory potency of CP [compare R6TM1R3 and R3(R6S1) to wild-type GluR3], while exchanges containing region II were more effective on GYKI [compare R3(T810-I888) to wild-type GluR3, and R6(N654-G840) and R6(A812-G840) to wild-type GluR6]. However, full conversion of sensitivity was conferred only through the combination of both regions I and II, as inferred from chimera R3(S1, T810-I888).

Subsequent site-directed mutagenesis inside regions I and II identified specific residues in each region reproducing the effects seen for the larger exchanges (Tables S1 and S2, respectively; see the Supplemental Data available online). As summarized in Figure 4A, the residues affecting sensitivity to GYKI (marked by $) are (for the most part) different from those affecting sensitivity to CP (marked by Δ); however, they are in close proximity to one another. In the intact receptor, these residues are part of the peptides linking the C termini of S1 and S2 to the respective trasmembrane domains M1 and M4 and are here referred to as S1-M1 and S2-M4 linkers (Figure 4A). Unfortunately, these linker sequences (as well as the S2-M3 linker) are omitted from the crystallized S1 and S2 construct (Armstrong et al., 1998).

Figure 4B further summarizes the impact of the specific amino-acid substitutions on the GYKI/CP sensitivity of GluR3 and GluR6, respectively, and reinforces at the level of point mutations that while GluR3/GluR6 substitutions in S1-M1 were more potent on sensitivity to CP and those in S2-M4 mainly affected sensitivity to GYKI, full conversion of receptor sensitivity to either antagonist depended on the reciprocal exchanges in both S1-M1 and S2-M4 linkers. We further noted that while the analysis shown in Figure 4B is in the presence of desensitization blockers, when GluR3 mutants were tested without CTZ, the antagonists' potency increased 5- to 8-fold, as observed for wild-type GluR3 (Table S3). Therefore, gating likely causes the same conformational changes in each of the mutated linkers as in the wild-type subunits leading to changes in antagonist efficacy.

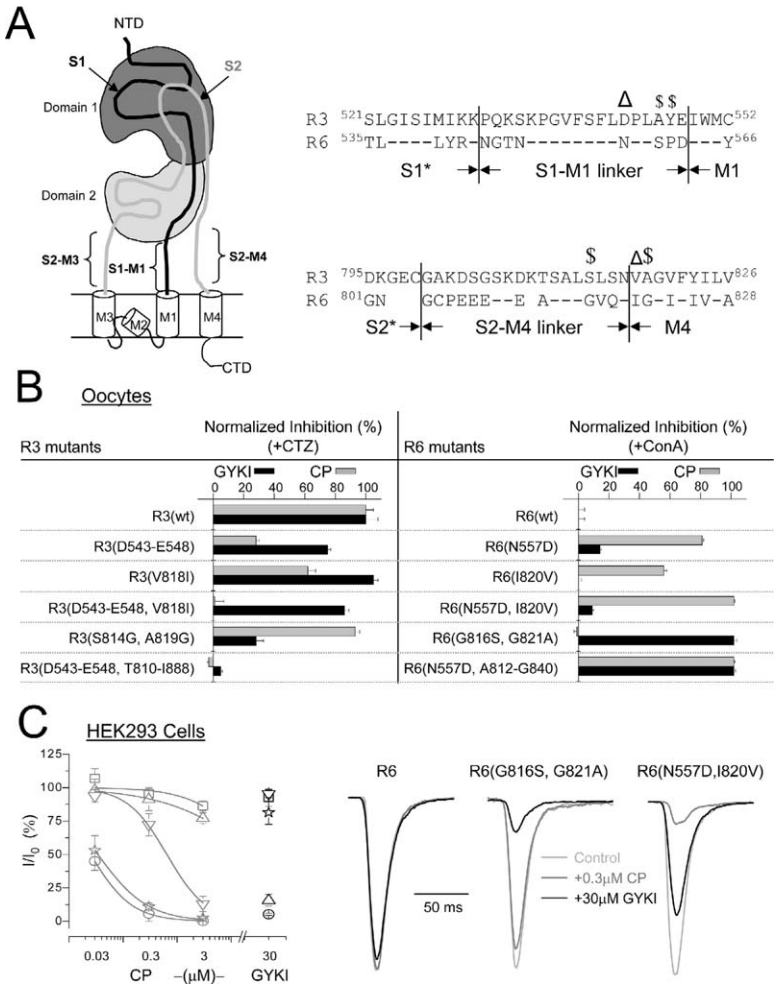

Figure 4. Conversion of GYKI/CP Sensitivity between GluR3 and GluR6 by Single Amino-Acid Substitutions

(A) (Left) Schematic model of the organization of S1 (black line) and S2 (gray line) forming domains 1 and 2 (dark and light gray, respectively) of the agonist-binding core. The three linkers connecting S1 and S2 to the transmembrane domains M1, M3, and M4 are marked as S1-M1, S2-M3, and S2-M4, respectively. GluR3/GluR6 sequence alignment within parts of regions I and II (Figure 3) are shown on the right; amino-acid numbering is shown as superscript. The boundaries marked by S1* and S2* are according to the crystallized S1 and S2 construct of GluR2 (Armstrong et al., 1998), thus omitting several amino acids N-terminal to M1 and M4, marked as S1-M1 and S2-M4 linkers, respectively. The signs $ and Δ indicate residues affecting sensitivity to GYKI and CP, respectively.

(B) Inhibition of glutamate (1 mM) evoked currents from oocytes expressing specific mutations in S1-M1 and S2-M4 as indicated. The mutant name denotes the amino acid substituted between GluR3 and GluR6. An exchange of more than one residue within a specific region is indicated by the respective amino-acid numbering on the backbone subunit [for example, R3(D543-E548) denotes the substitutions D543N, A546S, Y547P, and E548D made on GluR3]. The experiments were done in the presence of desensitization blockers, as indicated, and the mutants' inhibition values were normalized as described in Figure 3. Each bar is a mean (±SD) of 20 to 40 oocytes collected from at least two independent experiments.

(C) Inhibition of glutamate (3 mM) evoked currents from HEK293 cells expressing GluR1 (circles), GluR6 (squares), R6(G816S,G821A) (up triangles), R6(N557D) (inverted triangles), and R6(N557D,I820V) (stars). Three concentration of CP (0.03, 0.3, and 3 μM) and one of GYKI (30 μM) were tested. Cells were first exposed for 500 ms to 3 mM glutamate (I_0), washed for 10 s, and than exposed for 1 s to the antagonist alone, followed by a 500 ms application in the presence of glutamate (I). Each point is a mean (±SD) of three to five cells. Shown on the right are representative superimposed normalized traces of GluR6, R6(G816S,G821A), and R6(N557D,I820V) without antagonist (control; light gray), plus 0.3 μM CP (gray), or 30 μM GYKI (black).

Corresponding mutations in the AMPAR GluR1 subunit produced similar effects on GYKI and CP sensitivity (data not shown). For all subunit backgrounds, the mutations affecting GYKI/CP sensitivity had no significant effect on functional expression or glutamate EC_{50} as compared to the wild-type subunits (data not shown). Finally, measurements in HEK293 cells of peak current responses of the GluR6 mutants, without blocking receptor desensitization, confirmed the results observed in oocytes and also confirmed the observation made on AMPAR that GYKI and CP do not alter onset of receptor desensitization (Figure 4C; see Donevan and Rogawski, 1998).

The simplest interpretation of the above results is that GYKI and CP physically bind to the S1-M1/S2-M4 linkers, either to the particular identified residues or in their vicinity. Interestingly, however, driven by the finding that in NMDAR negative allosteric modulators such as infenprodil and zinc bind to the NTD (Herin and Aizenman, 2004), a computer-modeling study suggested how GYKI may bind to the NTD of GluR1 (De Luca et al., 2003). Therefore, it is possible that GYKI/CP inhibition may result from allosteric interactions between the NTD and the S1-M1/S2-M4 linkers. However, we found that a GluR1 mutant lacking the NTD, which was fully active (e.g., Pasternack et al., 2002), retained the inhibition by GYKI and CP with IC_{50} values identical for inhibition of the full-length receptor (data not shown), therefore excluding the involvement of the NTD in GYKI/CP binding.

In summary, the results show that residues responsible for sensitivity to GYKI and CP, although not identical, reside in two small regions of the AMPAR that link the agonist-binding domain to the membrane-spanning domains that form the AMPAR channel pore. This suggests that the GYKI/CP binding site is located in regions hypothesized to transduce agonist binding into channel gating. Thus, the mechanism of inhibition of AMPARs for these compounds is likely to be disruption of this transduction process.

CP Interacts with S1-M1 and S2-M4 Linkers Derived from Adjacent Subunits

Based on the finding that GYKI/CP sensitivity depends on both S1-M1 and S2-M4 linkers, a question remains whether their antagonistic action proceeds via an intra- or intersubunit binding mode. To distinguish between the two possibilities, we tested coexpression of CP-sensitive and CP-insensitive subunits carrying additional mutations that allowed preferential activation of only one subunit type in the heteromeric assembled receptors. Our experimental approach was based on the assumption that agonist binding to each subunit in a given receptor additively contributes to channel gating in a subunit-independent manner. Evidence for this assumption comes from single-channel analysis on recombinant and native channels (Rosenmund et al., 1998; Smith and Howe, 2000) demonstrating that AMPARs open into three graded and defined conductance states—small, medium, and large (~6, 12, and 18 pS for GluR2)—corresponding to two, three, and four agonist-bound subunits per receptor (binding to only one subunit is apparently undetectable or ineffective). According to this assumption, in heteromeric receptors containing both antagonist-sensitive and -insensitive subunits, currents mediated by agonist binding to the antagonist-sensitive subunits should be fully inhibited. On the other hand, currents mediated by agonist binding to the antagonist-insensitive subunits should not be inhibited if the antagonist exclusively binds within the neighboring subunits. However, if the antagonist binds between S1-M1 and S2-M4 linkers of neighboring subunits, an intermediate inhibition value would be expected in both experimental paradigms (as observed for subunits mutated in only one of these linkers; Figure 4B). Because CP is more potent than GYKI, the following experiments were done with subunits engineered to have or lack sensitivity to CP.

For the first scenario, we used the CP-insensitive mutant of GluR3, R3(D543-E548, V818I), in which we additionally mutated the conserved arginine in the binding pocket (R509) to alanine, therefore impairing glutamate binding [marked as $R3_Q(CP^-/Glu^-)$; Figure 5A]. For the CP-sensitive subunit, we used the edited form of GluR2 containing an arginine (R) at the Q/R-edited site in M2 (Seeburg, 1996) [marked as $R2_R(CP^+/Glu^+)$; Figure 5A]. Based on previous studies, the R form generates low-conducting receptors (Hume et al., 1991; Mansour et al., 2001; Swanson et al., 1997) that are largely retained in the endoplasmic reticulum (Greger et al., 2002); however, when assembled with unedited subunits containing glutamine at this position (Q form), the channels are targeted to the membrane and are highly conducting. As expected, no currents could be recorded from either one of these subunits when expressed alone, while protein expression was significantly high (Figure 5A). In contrast, when they were coexpressed at a 1:1 ratio, we obtained large glutamate-evoked currents, and these were partially inhibited by CP (57% ± 9%; n = 7). At these conditions, glutamate-evoked currents are mediated only by the heteromeric channels in which the $R2_R(CP^+/Glu^+)$ subunits contribute the active glutamate-binding sites. Therefore, the observation that the activation of these channels by glutamate binding only to the CP-sensitive subunits was not fully inhibited by CP suggests that CP most probably binds between neighboring subunits. As a control, we used the R509A mutation in the GluR3 wild-type background, thus having an operational CP binding site, but not for glutamate [marked as $R3_Q(CP^+/Glu^-)$; Figure 5A], and when this subunit was expressed with $R2_R(CP^+/Glu^+)$ the resulting glutamate-evoked currents where fully inhibited by CP (97% ± 2%, n = 7; Figure 5A).

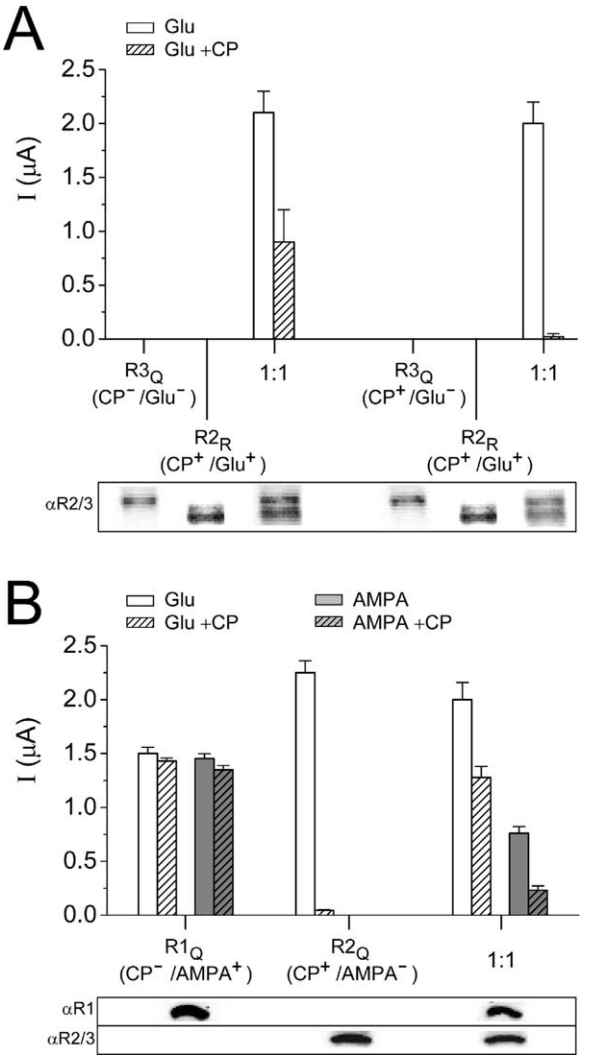

Figure 5. Coexpression of CP-Sensitive and CP-Insensitive Subunits Suggests an Intersubunit Binding Mode

(A) Expressions (alone and at 1:1 ratio) of R3(D543-E548, R509A), the CP-insensitive GluR3 subunit lacking a functional glutamate binding site marked as $R3_Q(CP^-/Glu^-)$, and edited GluR2, the CP-sensitive GluR2 R-form subunit marked as $R2_R(CP^+/Glu^+)$.

(B) Expressions (alone and at 1:1 ratio) of R1(D533N, I806V), the CP-insensitive GluR1 subunit marked as $R1_Q(CP^-/AMPA^+)$, and unedited Q-form R2(L650T), the CP-sensitive GluR2 subunit lacking functional AMPA binding-site marked as $R2_Q(CP^+/AMPA^-)$. All subunits are included in addition to the L-to-Y nondesensitizing mutation. Bars represent current amplitudes (±SD) mediated by the respective agonist (glutamate, 1 mM; AMPA, 0.1 mM) in the absence or presence of CP (30 μM) as indicated (n = 7–10 oocytes each). Western blot analysis of subunit protein expression is shown on the bottom. Experiments were repeated twice with similar results.

In the second assay, we coexpressed the CP-insensitive mutant of GluR1, R1(D533N, I806V) [marked as $R1_Q(CP^-/AMPA^+)$; Figure 5B], with the unedited Q form of GluR2 having the mutation L650T, which impairs binding of the agonist AMPA but not of glutamate (Armstrong et al., 2003) but otherwise carries an intact binding site for CP [marked as $R2_Q(CP^+/AMPA^-)$; Figure 5B]. As expected, when expressed alone, $R1_Q(CP^-/AMPA^+)$ exhibited similar current amplitudes when activated by glutamate or AMPA, and these currents were hardly affected by CP (5% ± 2% and 7% ± 3%, respectively; n = 10). Conversely, glutamate but not AMPA activated the $R2_Q(CP^+/AMPA^-)$ receptors, and this activation was fully inhibited by CP (98% ± 1%; n = 10). When these subunits were coexpressed at a 1:1 ratio, we observed large glutamate-evoked currents and intermediate AMPA-evoked currents, and both were inhibited by CP (36% ± 8% and 70% ± 6, respectively; n = 10). Under these conditions, glutamate-evoked currents must be mediated by all resulting receptor combinations, while AMPA-evoked currents must be exclusively mediated by receptors containing the CP-insensitive $R1_Q(CP^-/AMPA^+)$ subunits, thereby exhibiting the intermediate amplitude of AMPA/glutamate-evoked currents (38% ± 3%) as compared to the homomeric expressions of $R1_Q(CP^-/AMPA^+)$ (97% ± 3%) and $R2_Q(CP^+/AMPA^-)$ (0.6% ± 0.5%), respectively. According to the working hypothesis of subunit-independent gating, if binding of CP is exclusively within the subunit monomer, AMPA-evoked currents should not be inhibited by the antagonist, while glutamate-evoked currents should be partially prevented. The fact that AMPA-evoked currents were significantly inhibited by CP thus supports the intersubunit-binding model.

Discussion

In the present study, we have mapped the binding site for two noncompetitive antagonists, GYKI and CP, on the AMPAR. Although these molecules are representatives of two structurally distinct classes of compounds, radioligand-binding studies indicated that the binding site for these two drugs essentially overlaps (Menniti et al., 2000). Both GYKI and CP are highly selective for inhibition of AMPAR as compared to the closely related KAR, as shown previously (Lazzaro et al., 2002; Paternain et al., 1995) and as demonstrated here (Figure 1). Using a domain-swapping strategy between the AMPAR GluR3 and the KAR GluR6 (Figure 3) followed by site-directed mutagenesis (Tables S1 and S2), we demonstrate that the residues critical for conferring GYKI/CP sensitivity are located in small regions of the AMPAR that unambiguously lie close to the putative junction at the plasma membrane of the linkers S1 and S2 with the transmembrane-spanning regions of M1 and M4, respectively. At the amino-acid level, the residues critical for GYKI and CP binding are distinct but in close proximity to one another (Figure 4), consisting of the overlapping binding sites on one hand and the distinct structures of GYKI and CP on the other. Substitutions in S1-M1 were more effective on CP, while those in S2-M4 affected GYKI binding to a greater extent. Significantly, however, substitutions on both the S1-M1 and S2-M4 linkers were required to fully convert the sensitivity between GluR3 and GluR6 and vice versa (Figure 4). Thus, it appears that the GYKI/CP binding site spans these two linker regions.

The question remained as to whether the GYKI/CP binding site spanned S1-M1 and S2-M4 within a single subunit or across adjacent subunits. To investigate these two possibilities, we performed coexpression and activity studies in oocytes with AMPAR subunits in which either the agonist binding or CP binding was abolished by appropriate point mutations. This approach assumed that each subunit gates independently (Rosenmund et al., 1998; Smith and Howe, 2000). We observed under several conditions that the degree of inhibition of channel activity by CP could only be accounted for by an interaction of the molecule with linkers derived from adjacent subunits (Figure 5). Thus, the GYKI/CP binding site resides in the multimeric complex of the fully assembled AMPAR.

Manipulations that reduce AMPAR desensitization (i.e., increase open state) significantly decreased the potency of GYKI and CP for inhibition of AMPAR activity (Figure1). These manipulations included both pharmacological (addition of CTZ; Patneau et al., 1993) and structural (L-to-Y mutation; Stern-Bach et al., 1998), in both cases resulting in block of receptor desensitization. Radioligand-binding studies in rat-brain membranes revealed that the binding affinity of CP is significantly reduced (>10-fold) by the presence of agonist (AMPA or glutamate) plus CTZ, i.e., conditions that enrich for the presence of receptors in the open, nondesensitized state (Figure 2). In addition, electrophysiological measurements in wild-type AMPARs (i.e., with intact desensitization) revealed that the dissociation constant of GYKI from the open state is 2-fold larger than that from the closed state (Figure 2). These data indicate that GYKI and CP bind better to a closed state of the AMPAR channel. The apparent loss of this binding site in the open, nondesensitized receptors is parsimoniously accounted for by a conformational change in the S1-M1 and S2-M4 linkers that distort the binding site such that GYKI and CP affinities are reduced. In addition, the tight correlation between GYKI/CP binding and agonist efficacy to open the channel (Figure 2) further provides a strong link between gating state and rearrangements at the linker interface.

The effects of gating on GYKI/CP binding to S1-M1 and S2-M4 linkers thus suggest movement of these linkers during channel gating. We propose that this movement is part of the mechanism transducing agonist binding into channel opening, and restriction of this movement underlies the molecular mechanism of inhibition by these compounds (see Figure 6 for illustrative model). The effects of gating on GYKI/CP binding may be due to separation of the linkers one from another and/or conformational changes within the linkers. Models of AMPAR gating suggest that agonist binding leads to outward lateral rotation of the individual subunits in the dimeric assembly (Figure 6, arrow 1; Sun et al., 2002). Such a rotation is thus likely to increase the distance between S1-M1 and S2-M4 linkers from adjacent subunits, making binding of GYKI/CP to two binding sites on adjacent subunits less likely. Conformational changes within the linker domains are supported by the observation that reduction of binding to one of the

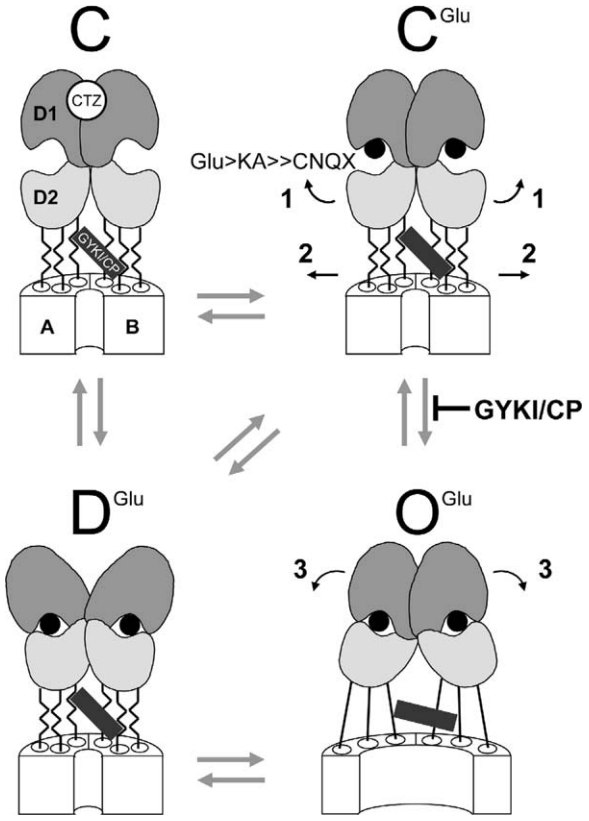

Figure 6. Schematic Model for GYKI/CP Inhibition of AMPAR

Shown are two subunits (A and B) forming one out of the two dimers assembling an active channel (the NTD is omitted). Four equilibrium states of AMPAR receptor/channel modes are drawn: C, agonist-unbound/closed-resting; C^{Glu}, agonist-bound/closed-resting; O^{Glu}, agonist-bound/open-conducting; D^{Glu}, agonist-bound/closed-desensitized. Agonist (black circles) docking in D1 induces rotation of D2 (arrow 1), closing the binding cleft. The movement is greater for full agonists such as glutamate and AMPA, intermediate for partial agonist like kainate, and almost none for competitive antagonists like CNQX. This movement is assumed to produce channel opening (arrow 2). In the next step (arrow 3), breakdown of the D1 interface promotes closing of the channel, entering into the desensitized state. This step is inhibited by CTZ (white circle) binding in the D1 dimer interface and by the L-to-Y mutation. Based on present results, we suggest that the dimer interface extends to the linker regions. GYKI/CP (black rod) bind to the linkers originated from adjacent subunits. GYKI/CP binding affinity is high for agonist-unbound (C) or desensitized (D^{Glu}) states but low to activated channels (O^{Glu}). GYKI/CP binding to the S1-M1 and S2-M4 linker regions stabilize a configuration of these linkers such that agonist binding fails to induce a sufficient conformational change to pull open the channel. Thus, the interplay between rearrangements at the D1 dimer interface and those at the linker regions explains the allosteric interaction observed between CTZ and GYKI/CP.

linkers does not abolish the gating effects on GYKI/CP binding (Table S3). Thus, a model providing the simplest explanation for the present findings with GYKI and CP is that these compounds, by binding to the S1-M1 and S2-M4 linker regions, stabilize a configuration of these linkers such that agonist binding fails to induce a sufficient conformational change to pull open the channel (Figure 6).

Overall, our findings confirm previous models but reveal new aspects of AMPAR physiology. Studies on the Lurcher mutation (Klein and Howe, 2004; Kohda et al., 2000) and utilizing cysteine-substituted mutagenesis (Sobolevsky et al., 2003, 2004; Yelshansky et al., 2004) have established that the S2-M3 linker plays an important role in transduction of agonist binding to channel gating in the AMPAR. Residues significant for GYKI/CP action on this segment could not be revealed by the chimeric screening approach because segment S2-M3 is identical in GluR3 and GluR6 subunits. Yet, in addition to S2-M3, our results with GYKI and CP disclose a crucial role of the other two linkers in gating. Of special significance is the involvement of S2-M4, which has been systematically omitted from models of channel gating (Wollmuth and Sobolevsky, 2004), mostly due to lack of homology of M4 to the potassium channel (Wo and Oswald, 1995) and its absence in GluR0, a prokaryotic glutamate-gated channel (Chen et al., 1999). Therefore, a plausible interpretation of our data is that all three linker regions are involved in mediating the transduction of agonist binding to channel gating of the AMPAR. Yet, it is possible that the state dependence may arise solely from the movement of M3 and that GYKI/CP binding to S1-M1 and S2-M4 allosterically interferes with this movement. However, previous studies on NMDAR have implicated the S1-M1 linker in desensitization (Krupp et al., 1998; Villarroel et al., 1998), and cysteine-substituted mutagenesis showed a state-dependent accessibility of residues in S1-M1 and S2-M4 linkers to cysteine-modifying reagents (Sobolevsky et al., 2002), further supporting their direct involvement in channel gating.

A notable observation from the site-directed mutagenesis was the ability to confer to GluR6 complete sensitivity to either GYKI or CP through single-amino-acid substitutions on the S1-M1 and on S2-M4 segments (Figure 4). The homology of the S1-M1 and S2-M4 linkers between GluR3 and GluR6 is quite low. However, the fact that complete sensitivity to GYKI/CP can be conferred through these amino-acid substitutions suggests a high degree of topological homology. Furthermore, since the GYKI/CP binding site occurs between adjacent subunits, the topological homology in this region is conserved across that multimeric receptor complex. This finding adds support to the prevailing hypothesis that there is a high degree of conservation in the gating mechanism across the different subtypes of glutamate receptors (Erreger et al., 2004).

In summary, our results define sites of interaction of two noncompetitive antagonists, GYKI and CP, with the AMPAR, providing a conceptual mechanism for noncompetitive antagonism of receptor activity. It comes from the fact that domain movements leading to channel opening are likely restricted upon antagonist binding. Indeed, AMPA receptors N-terminal seem to undergo a substantial movement upon agonist binding, leading to pulling apart the linker segments forming the gating pathway. Interestingly, the GluR6 residue G821 in S2-M4, found to play a significant role in conferring sensitivity to GYKI when mutated to the corresponding alanine of GluR3 (Table S2), enables enhancement of GluR6 currents by local anesthetics such as halothane (Minami et al., 1998). This raises the intriguing possibility that compounds that affect conformational changes in the linkers could modulate up or down the coupling

efficiency between the agonist-binding and channel domains, thus acting as positive or negative allosteric modulators. Once atomic structural resolution is available for this region, the identification and definition of these sites would allow rational drug design of molecules limiting or enhancing AMPAR or KAR activity in an agonist-independent manner. Such compounds may provide the much-needed pharmacological probes to explore new therapeutic uses of glutamate-receptor-subtype-selective antagonists.

Experimental Procedures

Molecular Biology and In Vitro cRNA Transcription

All AMPAR constructs used in this study contained the flip isoform. The majority of the chimeras presented in Figure 3 have been described previously (Ayalon and Stern-Bach, 2001; Stern-Bach et al., 1994, 1998). The other chimeras were constructed by a similar approach. Point mutations were constructed by the QuikChange method (Stratagene, La Jolla, CA). The templates GluR3(Q, flip) GluR1(Q, flip), and GluR6(VCQ) were originally obtained from S.F. Heinemann (Salk Institute, La Jolla, CA). Mutants were first inserted into the pGEMHE vector (a gift from E. Liman, University of Southern California, Los Angeles, CA) for expression in oocytes and subsequently moved to pCDNA3 (Invitrogen, San Diego, CA) for expression in HEK293 cells. The regions amplified by PCR were verified by double-strand DNA sequencing. In all cases, numbering of amino acids starts from the first methionine of the ORF. GluR2(L650T) (L493Y/ Q-form/ flip) in pGEMHE was obtained from E. Gouaux (Columbia University, New York, NY). For expression in oocytes, plasmids were linearized with NheI, and capped cRNA was transcribed in vitro using T7 RNA polymerase (mMessage mMachine; Ambion, Austin, TX). The quality of transcripts was assessed by agarose gel electrophoresis and ethidium bromide staining and quantified by analytical UV spectroscopy (ND-1000; NanoDrop Technologies, Inc. Rockland, DE).

Heterologous Expression and Electrophysiological Recordings

Stage V-VI *Xenopus laevis* oocytes were prepared as described previously (Stern-Bach et al., 1994). Oocytes were injected up to 24 hr after preparation with 5–10 ng of cRNA in 50 nl/oocyte and were assayed 1–3 days later. Two-electrode voltage-clamp recordings were carried out at room temperature using GeneClamp500 connected to DIGIDATA1200 and pCLAMP8 (Axon Instruments, Foster City, CA). Pulled-glass capillaries (Sutter Instruments, Novato, CA) were filled with 3 M KCl and had a resistance of 0.5–1 MΩ. Oocytes were continuously perfused with recording solution containing 10 mM HEPES (pH 7.4), 90 mM NaCl, 1 mM KCl, and 1.8 mM $MgCl_2$ or $CaCl_2$. Where indicated, to avoid interference from receptor desensitization, oocytes were treated with cyclothiazide (0.1 mM, mixed in the agonist solution; RBI, Natick, MA) or with concanavaline A (ConA; 1 mg/ml, 5–10 min before recording; Sigma, St. Louis, MO) to block AMPAR or KAR desensitization, respectively. Currents were usually recorded at −70 mV, unless otherwise stated. Glutamate and kainate were obtained from Sigma (St. Louis, MO), AMPA and CNQX were obtained from Tocris (Bristol, UK), GYKI-53655 (LY300168) was a gift from Eli Lilly and Company (Lilly Corporate Center, Indianapolis, IL), and CP-465,022 was synthesized in-house (Pfizer, Groton, CT). Dose-inhibition curves were usually constructed by applying one to three concentrations per oocyte of antagonist mixed in agonist solution, and currents (I) were normalized to the steady-state current obtained with agonist alone (I_0). Data were analyzed using ORIGIN 6.0 software (OriginLab, Northampton, MA). Receptor expression and electrophysiological measurements in HEK293 cells were done as previously described (Lazzaro et al., 2002). Currents were measured under the whole-cell configuration of the patch-clamp technique, and solutions were applied with a fast-perfusion system as previously (e.g., Paternain et al., 2003). While the IC_{50}s for GYKI inhibition of AMPARs were similar in oocytes and HEK293 cells, these values differed for CP by ~5-fold in oocytes and HEK cells. Values in HEK293 cells were similar to those observed for CP inhibition of native AMPA receptors (Lazzaro et al., 2002).

Rat Forebrain Membrane Preparation and [3H]CP-526,427 Binding

Membrane preparation and binding assays were done as previously described (Menniti et al., 2000). Forebrains of adult male Sprague-Dawley or SABRA rats were homogenized in 0.32 M sucrose at 4ºC. The crude nuclear pellet was removed by centrifugation at 1000 × g for 10 min, and the supernatant was centrifuged at 17,000 × g for 25 min. The resulting pellet was resuspended in 5 mM Tris acetate (pH 7.4) at 4ºC for 10 min to lyse cellular particles and was again centrifuged at 17,000 × g. The resulting pellet was washed twice in Tris acetate, resuspended at 10 mg of protein/ml and stored at −70ºC until use. Immediately before binding assays, membranes were thawed, homogenized, and diluted to 0.5 mg of protein/ml with 50 mM Tris-HCl (pH 7.4). For competition assays, the various compounds were added to the membranes and incubated for 10 min at 30ºC in a shaking water bath prior to the addition of 3 nM [3H]CP-526,427 (specific activity, 24.36 Ci/mmol). After incubation for an additional 20 min, samples were filtered onto Whatman GFB glass-fiber filters, washed twice with ice-cold Tris-HCl buffer, and the radioactivity trapped on the filter was quantified by liquid scintillation counting. Nonspecific binding was determined in parallel incubations containing 50 μM unlabeled CP-465,022. Specific binding was defined as total binding minus nonspecific binding. Scatchard analyses were performed similarly by incubating different concentrations of [3H]CP-526,427 with membranes. Binding assays performed on intact oocytes or on membranes prepared from either oocytes or HEK293 cells (Stern-Bach et al., 1994) expressing various combination of AMPAR subunits resulted in only ~5% specific binding using radioligand concentrations of 3–100 nM; thus preventing reliable measurements on recombinant subunits. This high background most probably resulted from combination of the high lipophilicity of CP (~50% background on rat-brain membranes; Menniti et al., 2000) and relative low receptor expression as compared to the native material.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/48/2/279/DC1/.

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