Targeting AMPA Receptor Gating Processes with Allosteric Modulators and Mutations

Nicholas A. Mitchell and Mark W. Fleck
Center for Neuropharmacology & Neuroscience, Albany Medical College, Albany, New York 12208

ABSTRACT Allosteric modulators and mutations that slow AMPAR desensitization have additional effects on deactivation and agonist potency. We investigated whether these are independent actions or the natural consequence of slowing desensitization. Effects of cyclothiazide (CTZ), trichlormethiazide (TCM), and CX614 were compared at wild-type GluR1 and “nondesensitizing” GluR1-L497Y mutant receptors by patch-clamp recording with ultrafast perfusion. CTZ, TCM, or L/Y mutation all essentially blocked GluR1 desensitization; however, the effects of L/Y mutation on deactivation and glutamate EC50 were three to five times greater than for modulators. CTZ and TCM further slowed desensitization of L/Y mutant receptors but paradoxically accelerated deactivation and increased agonist EC50. Results indicate that CTZ and TCM target deactivation and agonist potency independently of desensitization, most likely by modifying agonist dissociation ($k_{off}$). Conversely, CX614 slowed desensitization and deactivation without affecting EC50 in both wild-type and L/Y receptors. The S750Q or combined L497Y-S750Q mutations abolished all CTZ and TCM actions without disrupting CX614 activity. Notably, the S/Q mutation also restored L/Y deactivation and increased agonist EC50 by mutagenesis and possibly by allosteric modulators.

INTRODUCTION

AMPA-type glutamate receptors (AMPARs) are ligand-gated ion channels that represent the principal mediators of fast excitatory synaptic transmission in the mammalian CNS. The dysfunction of AMPARs has been linked to Alzheimer’s disease, depression, epilepsy, stroke, and neurodegenerative disease (1,2). Their implication in these various neuropathologies makes them attractive targets for the rational design and development of novel therapeutics.

AMPA-mediated synaptic currents are exceedingly brief (~1–2 ms), reflecting the transient elevation of glutamate in the synaptic cleft (3,4) and rapid inactivation of AMPARs by the combined processes of deactivation and desensitization (5–7). Deactivation is measured by the decay of current after the removal of agonist, which follows an exponential time course having a time constant ($t_{deact}$) of 0.5–2 ms. Desensitization is measured by the decay of current in the continuous presence of agonist and has a somewhat slower time constant ($t_{des}$) on the order of 1–10 ms (5,6,8). Both desensitization and deactivation are empirical measures of current decay from the open state, and so both measures necessarily involve the rate of channel closing ($\alpha$). Beyond that, it remains unclear to what extent the underlying rates of agonist dissociation ($k_{on}$) and isomerization to the desensitized conformation ($k_{des}$) are truly independent of one another. Nonetheless, because these rates collectively govern the magnitude and time course of synaptic transmission, an objective in the development of novel AMPAR modulators has been to target these processes selectively.

The molecular mechanisms of AMPAR gating and desensitization are the subject of intense investigation. Structural studies suggest that glutamate binds within an agonist-binding clamshell (9). The binding domains are linked to one another in a back-to-back dimer configuration (10,11) and held together in part by a salt-bridge hydrogen bond network at the dimer interface (12). AMPAR activation involves closure of the upper and lower lobes of the binding pocket around the agonist, which pulls against the rigid dimer interface to cause channel opening by rotational rearrangement of the transmembrane domains (13). Deactivation is thought to represent simply the reverse of this process, whereas desensitization is related to instability of the dimer interface contacts that are necessary to link the agonist-binding and transmembrane domains (11–14).

Allosteric modulators bind remotely but influence the agonist binding site or agonist-induced conformational rearrangements associated with channel gating (7,15–21). Interest in AMPAR allosteric modulators began in earnest with the discovery that cyclothiazide (CTZ) potentiates AMPAR currents in hippocampal neurons by slowing desensitization (21,22). CTZ binds within and stabilizes the glutamate receptor (GluR) dimer interface (11) and owes its preference for AMPAR-<i>flip</i> isomers (7,22,23) to a single serine residue (S750) in the <i>flip/flop</i> domain (7). In addition to slowing desensitization, CTZ also slows AMPAR deactivation (7) and increases the apparent affinity for activation by agonist (24). These secondary actions of CTZ might be an indirect secondary effect of the primary action of CTZ.
consequence of slowing desensitization or a direct effect of CTZ on agonist dissociation. The question is complicated to the extent that desensitization contributes to deactivation and limits agonist potency by preventing channel activation at low agonist concentrations (25,26).

To answer this question we used a combinatorial approach that employed a ‘‘nondesensitizing’’ AMPAR. Similar to CTZ, mutation of a leucine to a tyrosine at position 497 of GluR1 (27) (GluR2-L483Y (28)) greatly reduces AMPAR desensitization. The GluR1-L497Y receptor allowed us to discriminate between drug effects that were a consequence of blocking desensitization and those effects that were unique and separable from desensitization. The actions of a related benzothiadiazide, trichlormethiazide (TCM), and an unrelated Ampakine, 2H,3H,6aH-pyrrolidino[2’,1’-3’,2’]1,3-oxazinol6’, 5’-5,4]benzo[e]1,4dioxan-10-one (CX614), were also examined. GluR1-wt and GluR1-L497Y receptors were expressed in HEK 293 cells, and drug effects on measures of deactivation, desensitization, and agonist EC50 were determined using ultrafast solution exchange. An additional mutation, GluR1-S750Q (7), was used to confirm benzothiadiazide binding at the dimer interface and resulted in identification of S750 as a pivotal linkage that connects the L497Y mutation to the agonist-binding pocket. Results indicate that desensitization and deactivation can be selectively targeted, at least by site-directed mutagenesis, which suggests that future modulators may also be capable of such selectivity. Actions of modulators alone, and interactions between modulators and mutations on channel gating, are characterized in an effort to offer potential strategies for targeting specific AMPAR gating transitions.

MATERIALS AND METHODS

Materials

Ultrapure salts and chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture

Human Embryonic Kidney 293 fibroblasts (HEK293, CRL, ATCC, Manassas, VA) were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and 2 mM Glutamax (Life Technologies, Rockville, MD). Cells were incubated at 37°C in a 5% CO2 environment. Cells were plated into 25 cm2 Falcon flasks and passed twice weekly to fresh flasks without reaching confluence.

Transfections

Cells intended for transfection and subsequent recording were plated to poly-D-lysine-coated 35-mm NUNC dishes at a density of 80,000 cells/ml and transfected the following day using Lipofectamine 2000 reagents (Life Technologies). Cells were cotransfected with EGFP at a 9:1 ratio. cDNA plasmids containing GluR1, GluR1-L497Y, GluR1-S750Q, or GluR1-L497Y/S750Q were combined with Lipofectamine reagents for a final concentration of 1 μg/μl cDNA per 35-mm NUNC dish. Transfected cells were incubated for 36–72 h before use for electrophysiological recordings.

Patch-clamp recordings

Cells were continuously superfused with a standard extracellular solution containing (in mM): 150 NaCl, 3 KCl, 5 HEPES, 1 MgCl2, 1.8 CaCl2, 10 glucose, and 0.1 mg/ml phenol red, with an adjusted pH of 7.3. Transfected cells were identified by fluorescent expression of EGFP. Recording microelectrodes were fabricated from thin-walled borosilicate glass capillary tubes (TW150, World Precision Instruments, Sarasota, FL). Electrode open-tip resistance was typically 2–4 MΩ when filled with an intracellular solution comprised of (in mM): 135 CsCl, 10 CsF, 10 EGTA, 1 MgCl2, 0.5 CaCl2, pH 7.2, and 295 mOsm. Outside-out patch recordings were performed in voltage-clamp mode at a holding potential of −70 mV using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Current signals were filtered at 2–5 kHz with an eight-pole Bessel filter (Cygnum Technologies, Delaware Watergap, PA), digitized at 20 kHz, and stored on a Macintosh PowerPC-G3 computer using an ITC-16 interface (Instrutech, Port Washington, NY) under the control of the data acquisition and analysis program Synapse (Synergy Research, Silver Spring, MD). All recordings were performed at room temperature (20–22°C).

Ultrafast solution exchange

All recordings were conducted in the outside-out patch configuration. Ultrafast solution exchange was achieved by using an LSS-3100 piezotranslator (Burleigh Instruments, Fisher, NY). Control and agonist solutions were driven simultaneously at a rate of 0.25 ml/min through two parallel barrels of a θ-tube having a tip diameter ~200 μm. Allosteric modulators were pre-applied through the control barrel. Membrane patches were positioned in the control stream near the solution interface, and a piezotranslator was used to rapidly move the θ-tube ~100 μm such that the solution interface passed over the patch. The timing and rate of solution exchange were determined by open-tip junction currents at the conclusion of all recordings and were typically <200 μs (10–90% rise time); these were based on current deflections produced by a 5% change in NaCl concentration. Representative figure traces as well as those used for kinetic analyses were averages of 5–10 consecutive responses. Time constants for deactivation (τdact) and desensitization (τdes) were derived from one or two exponential fits as required using a least-squares fitting algorithm. Current decays were fit from 60% to 95% of peak to steady state. Glutamate was prepared as 1 M stock solution when filled with an intracellular solution containing (in mM): 150 NaCl, 3 KCl, 5 HEPES, 1 MgCl2, 1.8 CaCl2, pH 7.2, and 295 mOsm. Outside-out patch recordings were performed in voltage-clamp mode at a holding potential of −70 mV using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Current signals were filtered at 2–5 kHz with an eight-pole Bessel filter (Cygnum Technologies, Delaware Watergap, PA), digitized at 20 kHz, and stored on a Macintosh PowerPC-G3 computer using an ITC-16 interface (Instrutech, Port Washington, NY) under the control of the data acquisition and analysis program Synapse (Synergy Research, Silver Spring, MD). All recordings were performed at room temperature (20–22°C).

Statistical analyses

Mean EC50 and kinetic measures of deactivation and desensitization were compared using a one-factor ANOVA. Post-hoc analyses employed two-tailed Student’s t-tests with Bonferroni correction (where applicable). Paired t-tests were employed in some cases to compare pre- and postdrug measures from the same patches. Statistical significance levels are indicated in the figure legends. EC50 values were determined from best-fits of the logistic equation

$I = I_{\text{max}}/(1 + (EC50/\text{GLU}))^nH$

where I is the current at a given agonist concentration, $I_{\text{max}}$ is the maximal current at saturation, EC50 is the glutamate concentration giving half-maximal current, and nH is the Hill coefficient. A least-squares algorithm from KaleidaGraph software was used to generate curve fits (Synergy Software, Reading, PA). Best-fitting nH values were generally near unity (1.2–1.4). The number of independent observations from different patches is indicated (n) within figures.
Computational modeling of modulator and mutation effects

Computational models were constructed, and simulations were tested, using AxoGraph software (Axon Instruments). Several kinetic schemes have been used to model AMPAR behavior (7,25,29), and the underlying assumptions of modeling AMPAR behavior have been well described (29,30). We adopted the 4-site scheme of Robert and Howe (29), which was simplified by excluding secondary desensitized states. Briefly, the model assumes that 1), AMPARs are tetrameric channels (2,31,32) that possess four agonist binding sites (9,10) and exhibit three intermediate conductance levels (31,33), 2), agonist binding initiates either channel activation or desensitization, and 3), the decay during prolonged agonist pulses represents receptor entry into desensitized states. The parameters of the models were optimized to simultaneously reproduce the values of activation ($r_{act}$), deactivation ($r_{deact}$), desensitization ($r_{des}$), and agonist potency (EC50) that were observed experimentally for wild-type, modulator-bound, and mutant receptors; the latter required iterative changes to one or more rate constants: $k_0/k_{off}$, $B_{act}$, or $k_{act}/k_{deact}$. Initially, rate constants were modified individually, and the effects of these changes on channel behavior were recorded. Subsequent modifications to multiple rate constants were then combined in an iterative manner to arrive at an optimal solution (see Table 2). Simpler two-site kinetic schemes required similar solutions except that the changes were quantitatively different.

RESULTS

CTZ and L497Y mutation block desensitization and slow deactivation

Many effects of CTZ and the L497Y mutation on GluR function are documented, but it is difficult to compare these various results from different preparations, methods, and laboratories (7,22,23,27,34,35). Specifically, we sought to compare (quantitatively) the effects of CTZ and L497Y mutation on desensitization, deactivation, and agonist potency (EC50) in GluR1 AMPARs. Our initial experiments therefore replicated findings from other sources not only to confirm the findings but also to make them quantitatively comparable for subsequent studies (Fig. 1). Consistent with previous reports, homomeric wild-type GluR1 flip channels in outside-out membrane patches deactivated in <1 ms (Fig. 1 C) and desensitized to <1% of peak within 2–3 ms (Fig. 1 A). Preapplication of 100 μM CTZ or insertion of a leucine-to-tyrosine (L/Y) mutation at position 497 of the mature protein prevented desensitization during 50-ms pulses of 10 mM glutamate. Prolonged exposure to glutamate for up to 2 s typically produced <25% decay to steady state in GluR1 + CTZ and L/Y mutant receptors (Fig. 1 B, Table 1). Although desensitization is slowed extensively by CTZ and the L497Y mutation, as indicated in Fig. 1, receptors did still desensitize (see Table 1 for values). Differences in the time course of deactivation were obvious from the relaxation kinetics after glutamate removal; decay of currents for GluR1 + CTZ appeared much faster than GluR1-L497Y. To characterize deactivation kinetics more precisely, and for comparison to wild-type GluR1, brief 1-ms exposure and rapid removal of 10 mM glutamate were used to minimize wild-type desensitization and to focus on the faster processes of channel closing ($\alpha$) and agonist dissociation ($k_{off}$). These effects are depicted in Fig. 1 C and summarized in Table 1. Deactivation time constants ($t_{deact}$) were significantly slower than wild type in both cases ($P < 0.001$ unpaired Student’s t-test), but more so for the L/Y mutant than for GluR1 + CTZ. Preincubation of GluR1 receptors with 100 μM CTZ slowed receptor deactivation by 2.4-fold, whereas the L497Y mutation slowed GluR1 deactivation by more than 13-fold compared to wild type. These values are similar to those reported elsewhere (7,34). Activation rates were evaluated by comparing the current onset time constants ($t_{act}$) of receptors at glutamate concentrations ranging from 0.3 to 3 mM. As expected, activation rates were concentration dependent; the relation between concentration and activation was not different between groups, indicating that rates of agonist binding ($k_{on}$) and channel opening ($B_{act}$) were not modified by these manipulations.
**TABLE 1** Mutation and drug effects on functional measures

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ CTZ</th>
<th>+ TCM</th>
<th>+ CX614</th>
</tr>
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<tr>
<td><strong>GluR1-wt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_{\text{des}} )</td>
<td>2.2 ± 0.1</td>
<td>ND</td>
<td>14 ± 1*</td>
<td>7.1 ± 0.3*</td>
</tr>
<tr>
<td>( \tau_{\text{deact}} )</td>
<td>0.9 ± 0.1</td>
<td>2.2 ± 0.3</td>
<td>4.7 ± 0.6</td>
<td>6.4 ± 1.3*</td>
</tr>
<tr>
<td>EC50</td>
<td>779 ± 72</td>
<td>83 ± 13</td>
<td>178 ± 13</td>
<td>657 ± 98</td>
</tr>
<tr>
<td>( I_{\text{ss}} ) (%)</td>
<td>&lt; 1</td>
<td>&gt; 95</td>
<td>86 ± 1</td>
<td>39 ± 4</td>
</tr>
<tr>
<td><strong>GluR1-L497Y</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_{\text{des}} )</td>
<td>12 ± 1</td>
<td>4.4 ± 0.6</td>
<td>5.4 ± 0.6</td>
<td>22 ± 1*</td>
</tr>
<tr>
<td>( \tau_{\text{deact}} )</td>
<td>20 ± 4</td>
<td>121 ± 16</td>
<td>68 ± 18</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>EC50</td>
<td>&gt; 95</td>
<td>&gt; 95</td>
<td>&gt; 95</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>( I_{\text{ss}} ) (%)</td>
<td>&lt; 1</td>
<td></td>
<td>2 ± 1</td>
<td>16 ± 1</td>
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<tr>
<td><strong>GluR1-S750Q</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_{\text{des}} )</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>4.2 ± 0.8*</td>
</tr>
<tr>
<td>( \tau_{\text{deact}} )</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>2.3 ± 0.2*</td>
</tr>
<tr>
<td>EC50</td>
<td>885 ± 98</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>( I_{\text{ss}} ) (%)</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>2 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td><strong>GluR1-L497Y, S750Q</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_{\text{des}} )</td>
<td>16 ± 1*</td>
<td>16 ± 1*</td>
<td>NA</td>
<td>22 ± 3*</td>
</tr>
<tr>
<td>( \tau_{\text{deact}} )</td>
<td>1.3 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>NA</td>
<td>2.2 ± 0.2*</td>
</tr>
<tr>
<td>EC50</td>
<td>1015 ± 169</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>( I_{\text{ss}} ) (%)</td>
<td>73 ± 3</td>
<td>73 ± 3</td>
<td>69 ± 1</td>
<td></td>
</tr>
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</table>

Data from 10 mM glutamate-evoked currents with drug concentrations as follows: cyclothiazide (CTZ, 100 \( \mu \)M), trichloromethiazide (TCM, 500 \( \mu \)M), CX614 (100 \( \mu \)M). \( \tau \)-values are given in ms and EC50 values are \( \mu \)M. Steady-state current amplitudes \( (I_{\text{ss}}) \) during 50-ms glutamate pulses are given as percentage of the corresponding peak current. ND denotes responses having < 5% decay during 50 ms glutamate pulses. In some cases, slow \( \tau_{\text{des}} \) values and steady-state ratios were estimated from 2-s agonist pulses; values were 13 ± 1 ms and 86 ± 4% for WT + CTZ and 73 ± 11 ms and 74 ± 1% for L497Y. No desensitization was seen for L497Y + CTZ within 2 s \( (I_{\text{ss}} \text{ < 1%}) \). NA, not analyzed. *\( \tau_{\text{des}} \) values are given for the desensitizing fraction of the response; \( I_{\text{ss}} \) values for the same groups reflect the magnitude of the steady-state component after 50-ms glutamate pulses. Biexponential; \( \tau \)-values are given for the weighted average of fast and slow components. For GluR1 + CX614 deactivation was 2.9 ± 0.1 ms (70%) and 16.7 ± 0.5 ms (30%). In other cases the slow component was <10%.

**CTZ and L497Y mutation enhance agonist potency**

Immediately after the application of glutamate, receptors begin to desensitize at a rate of \( \sim 450 \text{ s}^{-1} \) \( (\tau_{\text{des}} \sim 2.2 \text{ ms}) \). This limits their contribution to the peak response and theoretically should do so most profoundly at lower agonist concentrations where agonist-binding rates \( (k_{\text{on}}) \) are equivalent to or slower than the rate of desensitization. Thus, blocking entry into the desensitized states should facilitate receptor activation and increase the apparent affinity for glutamate. To confirm this relation, we compared the effects of CTZ and the L/Y mutation on the glutamate concentration-response relation. The curves were derived using a 10 mM maximum concentration of glutamate and sequential application of six lower agonist concentrations before returning to the maximum concentration to assess rundown; data from patches that exhibited >20% rundown of the maximal currents were excluded. Peak response data were normalized to the maximal concentration, plotted as a function of glutamate concentration, and fit by a logistic equation (see Methods). Best-fitting EC50 values are given in Table 1. Notably, even though both conditions achieved near-complete block of desensitization, CTZ produced only a 9.4-fold increase in agonist potency, whereas the L497Y mutation provided nearly a 40-fold increase in agonist potency relative to wild-type controls (Figs. 1 D and 2 F and Table 1). This more pronounced effect of the L497Y mutation on agonist potency was paralleled by slower deactivation rates (Figs. 1 C and 2 E). Although both CTZ and the L/Y mutation produced equivalent block of desensitization, their additional effects on deactivation and EC50 were evidently very different.

**Effects of CTZ on L497Y mutant AMPARs**

The quantitatively different effects of CTZ and the L/Y mutation on deactivation and agonist EC50 could be explained in one of two ways. 1) Both CTZ and the L/Y mutation have independent actions on desensitization, deactivation, and agonist binding. Their varied effects are best explained by a difference in efficacy for deactivation and agonist affinity. 2) Slowing deactivation and lowering the EC50 is a consequence of blocking desensitization for both GluR1 + CTZ and L/Y receptors, but only CTZ has an additional negative allosteric effect that increases receptor deactivation and decreases the apparent affinity for agonist.

To determine which of these explanations was valid, we examined the effects of CTZ on L/Y mutant receptors. L/Y mutant receptors showed little desensitization during 50-ms agonist pulses in the absence or presence of CTZ. Longer agonist pulses (2 s) allowed L/Y receptors to desensitize to \( \sim 75\% \) of their peak currents (Fig. 2 B, see Table 1 for values). This residual desensitization was blocked by pre-application of 100 \( \mu \)M CTZ (Fig. 2 B) and was accompanied by an unexpected threefold faster deactivation of L497Y mutant receptors (Fig. 2 B, C–E). CTZ also produced a rightward shift in the glutamate concentration-response curve (Fig. 2, D–F); EC50 values were sixfold higher after preincubation with CTZ. All CTZ effects were reversed by prolonged washout of the drug. In summary, CTZ further slowed GluR1-L497Y receptor desensitization but paradoxically accelerated receptor deactivation and decreased agonist potency. This result indicates that CTZ and the L/Y mutation effects are additive with respect to desensitization but competitive with respect to deactivation and EC50. Such competition, albeit only for the latter effects, implies convergence at a common site or process that could result from CTZ binding in proximity to the L/Y residue (11) or at another unknown site. We therefore questioned whether the non-competitive and competitive effects might be mediated by CTZ binding to multiple sites.
Confirmation of a single CTZ binding site in L497Y receptors

To determine if these separate and opposing actions of CTZ required multiple binding sites, we introduced the S750Q mutation to both GluR1-wt and GluR1-L497Y constructs to prevent CTZ from binding to its known binding site at the dimer interface. Previous studies have shown that CTZ binding critically involves the SNQ residue at position 750 (in GluR1), with serine (S) being the most active and glutamine (Q) being inactive (7,11). The GluR1-S750Q single mutant served as a control for these experiments. The GluR1-S750Q mutant exhibited desensitization and deactivation kinetics that were slightly faster than those of wild-type GluR1 (Fig. 3, A–C). GluR1-S750Q receptor rates of deactivation and desensitization were consistent with those previously reported (7). We also determined that the GluR1-S750Q receptor EC50 for glutamate was essentially unchanged from GluR1-wt (see Table 1). Addition of the S750Q mutation to the L/Y mutant resulted in several unexpected and significant changes in receptor phenotype, the most important of which was elimination of both the positive and negative allosteric effects of CTZ (Fig. 3, B–D). As expected, all CTZ effects on GluR1-wt and L497Y mutant channels are made possible by the S750 residue and therefore a single binding site.

FIGURE 2 Inverse actions of CTZ at L497Y mutant receptors. (A) Current traces showing desensitization kinetics of GluR1-L497Y in the absence and presence of 100 μM CTZ during 50-ms pulses of 10 mM glutamate. CTZ appeared to have little effect on L497Y currents during 50-ms pulses. (B) GluR1-L497Y mutant receptor currents were nondesensitizing during 50-ms pulses, but longer pulses (2 s) disclosed a partial and slowly desensitizing response to 10 mM glutamate. The residual L497Y receptor desensitization was alleviated after preincubation with 100 μM CTZ. (C) Deactivation kinetics of GluR1-L497Y (1-ms pulse, 10 mM glutamate) in the absence and presence of CTZ. Deactivation was about three times faster after preincubation with 100 μM CTZ. (D) Concentration-response curves for the GluR1-L497Y mutant in the absence and presence of CTZ. Data are fit by the logistic equation (see Methods); error bars represent SEM. (E, F) Summary bar plots comparing deactivation time constants (E) and agonist potency (F) for GluR1-wt and the GluR1-L497Y mutant receptors in the absence and presence of CTZ. Data are fit by the logistic equation (see Methods); error bars represent SEM. Numbers of observations are given in parentheses. ***P < 0.001 versus R1-wt controls; 111P < 0.001 comparing GluR1 + CTZ versus GluR1-L497Y; 11P < 0.001 comparing GluR1-L497Y in the absence and presence of CTZ. Unpaired t-tests were used with Bonferroni correction for multiple comparisons.

Double mutant GluR1-L497Y/S750Q

The GluR1-L497Y/S750Q double mutant may represent the keystone to understanding how CTZ and L/Y mutation effects are both related to the S750 residue. Comparable to the GluR2-L483Y/S754D mutant described by Sun et al. (11), the GluR1-L497Y/S750Q mutant desensitized partially during 50-ms glutamate pulses. The extent of desensitization was far from complete, and its onset was sevenfold slower than GluR1-wt but still fourfold faster than the L/Y parent (see Table 1). Equilibrium (steady-state) currents during 50-ms glutamate pulses were 73 ± 3% of peak (Fig. 3 B). These measures were notably similar to the equilibrium currents recorded for WT + CTZ and L497Y mutant receptors during the 2-s agonist pulses (Fig. 1 B). Closer examination of the GluR1-L497Y/S750Q double mutant indicated that deactivation of this receptor was 10-fold faster than that in the L/Y parent, and the EC50 for glutamate was ~40-fold higher. Both of these values are remarkably comparable to GluR1-wt and the GluR1-S750Q mutant (see Table 1 and Figs. 1 C, 2 C, and 3 C for comparison). These observations indicate that effects of the L/Y mutation on deactivation and agonist potency are not a consequence of slow desensitization but occur independently. Moreover, this mutation demonstrates that the S750 residue is required not only for all the effects of CTZ but also for the effects of the L497Y mutation on deactivation and agonist potency (Fig. 1, C and D, and 3 D). Faster deactivation and reduced agonist potency of the CTZ bound L/Y receptor implies that CTZ disrupts a direct or indirect interaction between Y497 and S750 to promote faster channel closing or agonist dissociation.

Effects of other allosteric modulators on L497Y mutant receptors

The appearance of negative allosteric effects of CTZ (i.e., faster deactivation and reduced agonist potency) in addition
FIGURE 3 Negative allosteric actions of CTZ require the S750 residue. (A) Wild-type GluR1 and GluR1-S750Q mutant responses to 50-ms application of 10 mM glutamate are overlaid to compare desensitization kinetics. The GluR1-S750Q mutant was unaltered after treatment with 100 μM CTZ. (B) The GluR1-L497Y mutant and GluR1-L497Y/S750Q double mutant responses to 50-ms application of 10 mM glutamate are overlaid to compare desensitization kinetics. Note that the GluR1-L497Y/S750Q double mutant exhibits some desensitization (average ≊ 27%), which is absent or slower in the L/Y parent. GluR1-L497Y/S750Q mutant kinetics were unaltered after treatment with 100 μM CTZ. (C) Deactivation kinetics of the GluR1-S750Q mutant receptor are similar in the absence and presence of 100 μM CTZ. Traces are pre- and postdrug responses to 1-ms applications of 10 mM glutamate. The GluR1-wt trace is provided for reference. (D) Deactivation kinetics of the GluR1-L497Y/S750Q double mutant are unaltered by 100 μM CTZ. The overlay is provided for direct comparison of pre- and postdrug kinetics and the GluR1-wt response. (E) Summary bar plot comparing the desensitization time constants of GluR1 wild-type and mutant receptors. Error bars represent SEM. Numbers of observations are given in parentheses. The GluR1-L497Y/S750Q double mutant receptor displayed a partial block of desensitization (73 ± 3% nondesensitizing); τdes of the remaining decay from peak to steady state was 16.4 ± 0.4 ms. ND indicates the majority of current was nondesensitizing during 50-ms agonist pulses. (F) Summary bar plot comparing deactivation time constants for GluR1-S750Q and GluR1-L497Y/S750Q double mutant receptors in the absence and presence of CTZ. Error bars represent SEM. Numbers of observations are given in parentheses. Pre- and postdrug measures were not significantly different for either receptor by paired t-tests. A significant difference was disclosed only between GluR1-S750Q and GluR1-L497Y/S750Q (**P < 0.01, unpaired t-test with Bonferroni correction).
Modeling of modulator and mutation effects on AMPA channel function

To further inform conclusions about the effects of modulators and mutations on AMPAR gating, we simulated their actions by computational modeling. We began with a simplified version of the Robert and Howe model (29), as shown in Fig. 5, which includes four binding sites linked to three subconductance states displayed by AMPARs (31,34). The assumptions of the model have been described (29), and the kinetic rate constants that were derived for GluR1-wt (Table 2) produced an accurate simulation of the GluR1-wt phenotype ($\tau_{\text{des}}$ 2.1 ms, $\tau_{\text{deact}}$ 0.8 ms, and 597 $\mu$M EC50) (Fig. 5, B and C).

We first determined the minimum changes necessary to reduce the rate and extent of desensitization to simulate GluR1-CTZ and GluR1-L497Y. A “nondesensitizing” phenotype was replicated in several ways, including slowing entry into the desensitized states ($k_{\text{des}}$), accelerating exit from the desensitized states ($k_{\text{res}}$), or simply eliminating the desensitized states. Changing $k_{\text{des}}$ and $k_{\text{res}}$ had complementary effects except that $k_{\text{res}}$ primarily impacts the magnitude of the steady-state current, whereas $k_{\text{des}}$ governs the rate of decay.
Optimized reaction rates are given in Table 2. (B) Kinetic scheme of the four-site model used for simulations. The form of the model and starting rate constants were adapted from Robert and Howe (29), where R represents receptors in the closed, activatable state, D represents the desensitized receptor state, O represents the open-channel state, and subscripted numbers indicate the number of agonist molecules bound in the various states. O2, O3, and O4 conductance levels were 8, 15, and 23 pS, respectively. Agonist binding (k+1) and unbinding (k−1) reactions are indicated by right and left arrows. Channel-opening (κ), -closing (σ), desensitization (δ), and resensitization (γ) reactions are indicated by up or down arrows. Optimized reaction rates are given in Table 2. (B) Simulated current traces comparing the kinetics of deactivation (1 ms agonist pulses, left) and desensitization (50 ms agonist pulses, right) of the models after optimization of model parameters to match experimental results. (C) Simulated concentration-response curves from the optimized models. Peak currents were measured from 50-ms simulations, normalized, and plotted relative to their individual maxima. Data are fit with the logistic equation (see Methods). Best-fitting EC50 values are given in the figure.

from peak to steady state and has greater impact on the peak current amplitudes. Changing these rates together gave the best approximation of CTZ effects (40-fold slower kdes, 4-fold faster kres), whereas slowing k<sub>des</sub> alone by 150-fold was sufficient to model the L/Y mutant currents. These modifications gave the best approximation of CTZ and L/Y mutant currents having an initial peak and slow decay to a steady-state level at ~80% of peak.

Regardless of how the "nondesensitizing" phenotype was modeled, reducing or eliminating desensitization from the model by itself predicted very little change in either τ<sub>deact</sub> (0.8 to 1.0 ms) or EC50 (570 to 416 μM). In addition to alterations in k<sub>deact</sub>/k<sub>res</sub>, more realistic models of CTZ and L/Y mutation effects on these measures required additional changes to agonist dissociation rates (k<sub>a</sub>). They could not be reproduced by slowing channel-closing rates (α), which slowed deactivation but did not lower the agonist EC50. CTZ effects on GluR1 were best simulated having 3.6-fold slower k<sub>off</sub> than wild type (τ<sub>deact</sub> 2.3 ms, EC50 125 μM), and L/Y mutation effects required a 20-fold slower k<sub>off</sub> (τ<sub>deact</sub> 13.9 ms, EC50 25 μM). All other modulator and mutation effects, except for CX614, were readily simulated by altering these same rates (Fig. 5, B and C, and Table 1). The additive effects of CTZ on L/Y mutant desensitization were reproduced in the L/Y model having fourfold faster k<sub>res</sub> as in the CTZ model, whereas the acceleration of deactivation and reduction of agonist potency by CTZ was readily simulated by increasing k<sub>off</sub> in the L/Y model by threefold (τ<sub>deact</sub> 3.84 ms, EC50 71 μM). The best-fitting model describing the behavior of the L/Y-S750Q double mutant receptor required 20-fold slower k<sub>des</sub>, sixfold faster k<sub>res</sub>, and 30% faster k<sub>off</sub> as compared to GluR1-wt. Notably, the small change in k<sub>off</sub> made here was also sufficient to produce the slight increase in EC50 and faster τ<sub>deact</sub> and τ<sub>des</sub> seen for the GluR1-S750Q mutant (Table 1).

In contrast to these, the preferential effects of CX614 on deactivation with lesser effect on agonist EC50 were reproduced by slowing channel closing rates threefold in the wild-type model. This change alone slowed τ<sub>des</sub> and τ<sub>deact</sub> 2.6-fold because both are measured as decay of current from the open states and are therefore limited by channel closing. Truly accurate simulations also required fivefold faster k<sub>res</sub> to reproduce the larger equilibrium currents associated with CX614 without further slowing of desensitization (28) (Table 1).

Our experimental results can therefore be reasonably well described by modifications to existing models of GluR function. Within the context of these models, the multiple
Biophysical Journal 92(7) 2392–2402

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effects of modulators and even the single-point mutations cannot be simulated by changes to desensitization alone ($k_{\text{des}}/k_{\text{res}}$) but require additional changes to agonist dissociation ($k_{\text{off}}$) or channel closing ($\alpha$).

DISCUSSION

AMPA gating

Emerging interest in the development of AMPAR allosteric modulators as potential therapeutics makes it increasingly important to understand both the effects of lead compounds and the nature of the gating processes they modulate. AMPAR gating is an allosteric process by which glutamate binding promotes conformational transitions from closed to open and desensitized channel states. Although the molecular details of channel opening and desensitization are unclear, it has been proposed that closure of the ligand-binding domain around ligand pulls against a rigid dimer interface to cause opening of the channel pore. Desensitization is thought to result from disintegration of the dimer interface, which uncouples ligand binding from channel opening. Functional and atomic-level structural analyses support this model of AMPAR gating (11–14,28,29,40,41). Mutations that promote stability of the dimer interface slow desensitization, as in the case of L497Y, whereas mutations that destabilize the dimer interface accelerate desensitization (12,14,40). CTZ binds to specific residues within the dimer interface to promote dimer stability and slow desensitization (7,11).

Modulator and mutation effects on channel gating

The structural model does not explain why the effects of modulators and mutations that stabilize the dimer interface are not limited to desensitization. Rather, such manipulations tend to produce a constellation of effects, simultaneously altering deactivation, desensitization, and agonist potency. These secondary effects on deactivation and agonist potency might result in part or entirely from the slowing of desensitization itself, to the extent that desensitization limits channel activation at low agonist concentrations and provides a route of inactivation from the ligand-bound and possibly open states. Otherwise, they might reflect secondary actions of drugs and mutation on channel-closing ($\alpha$) or agonist dissociation ($k_{\text{off}}$) rates that are entirely independent of effects on desensitization. If the former were true, we hypothesized that block desensitization by any means should have quantitatively similar effects on deactivation and agonist EC50. Moreover, these effects of modulators should be occluded in cases where desensitization is already blocked by mutation.

To test these predictions, we compared the effects of CTZ and TCM on GluR1-wt and GluR1-L497Y mutant receptors. Consistent with previous reports, CTZ, TCM, and L/Y mutation profoundly slowed desensitization of GluR1 during prolonged exposure to agonist (7,27,34,36). Yet, despite the near absence of desensitization during test pulses, these treatments had quantitatively very different effects on deactivation and glutamate potency (see Table 1). Furthermore, although CTZ and TCM further slowed desensitization in the L/Y mutant, they paradoxically accelerated deactivation and reduced agonist potency, opposite their normal effects on GluR1-wt. Results therefore indicate that the effects on deactivation and agonist potency are not a consequence of slower desensitization. Predictions of computational models support this conclusion and further suggest that CTZ, TCM, and L/Y mutation alter desensitization ($k_{\text{des}}/k_{\text{res}}$) and agonist binding ($k_{\text{off}}$) independently. The effects on $k_{\text{des}}/k_{\text{res}}$ most likely involve greater dimer stability, whereas the modulation of $k_{\text{off}}$ appears to involve interactions with the S750 residue specifically. Greatest support for this conclusion comes from the L497Y-S750Q double mutant where S/Q mutation restores L/Y mutant deactivation and EC50 to wild-type levels.

Aniracetam and CX614 are unique from CTZ and TCM. These modulators bind between the lower lobes of the ligand-binding domain within the dimer interface, where they inhibit the relaxation from the closed- to open-cleft configuration (28). These and related benzamide modulators generally slow receptor deactivation with relatively less effect on desensitization or agonist potency. Their actions persist in the L497Y and S750Q mutants and are best modeled by assuming faster recovery from desensitization and slower channel closing (7,28,37,38,42).

Alternative interpretations of results

There are several possible explanations for the conflicting actions of these modulators on deactivation and agonist potency in the L/Y mutant. One interpretation is that modulator-receptor interactions supersed interactions made by the mutant tyrosine residue so that L/Y mutant and wild-type receptors behave similarly when bound to modulators. Indeed, $\tau_{\text{des}}$ and EC50 values of WT + CTZ (or TCM) and L/Y + CTZ (or TCM) are more similar than those of L/Y receptors in the absence and presence of modulators (Table 1). However, several observations contradict this explanation. First, both modulators further slowed desensitization of L/Y mutant receptors. The additive nature of effects suggests that both the modulator and L/Y interactions across the dimer interface remain intact. Second, $\tau_{\text{des}}$ and EC50 values are significantly different between WT + CTZ (TCM) and L/Y + CTZ (TCM), in some cases by two- to threefold. Unfortunately, the L/Y + CTZ crystal structure has not been solved, which might further refute this explanation. More limited insight may be gained by comparison of related structures, including GluR2 + AMPA (PDB: 1FTM), GluR2 + GLU + CTZ (PDB: 1LBC), and GluR2-L483Y + AMPA (PDB: 1LB8) (10,11). Superimposition of these structures indicates that the L/Y residue is 8 to 8.7 Å from the nearest
point of CTZ, suggesting there is unlikely to be any direct interaction between the L/Y residue and modulators. Examination of all residues within 4 Å of CTZ, the L/Y residue, or the bound agonist in the superimposed structures reveals only minuscule differences in the position or orientation of these residues that cannot account for the profound effects of CTZ or TCM on L/Y deactivation and agonist potency. We could find no indication that CTZ supersedes any direct interactions made by the L/Y residue.

A more plausible explanation for the seemingly paradoxical actions of modulators on L/Y mutant receptors is that CTZ and TCM have two opposing actions. The first is to block desensitization, which by itself only modestly slows deactivation and increases agonist potency. The second is to destabilize agonist binding, which accelerates deactivation and decreases agonist potency. Quantitative differences among CTZ, TCM, and L/Y mutation effects and their paradoxical interactions can thus be explained by how effectively these manipulations engage the two processes. Some previous studies have alluded to a second inhibitory action of CTZ that occurs independently of desensitization. Patneau et al. (43) noted a modest inhibition of kainate-evoked currents in native GluRs from hippocampal neurons. Others have reported reduced affinities for radioligands binding to native or recombinant AMPARs treated with CTZ (44,45) and an approximately twofold increase in the agonist EC50 at some nondesensitizing GluR chimeras (27). These data are most consistent with effects on agonist dissociation (koff). All of the effects on desensitization, deactivation, and EC50 can be attributed to a single CTZ binding site because they are abolished by the S750Q mutation (7,11). Moreover, the profound slowing of desensitization in the L/Y-S750Q double mutant without effects on τdeact or EC50 argue that the S750 residue is directly involved in modulation of koff in addition to its being required for modulator binding.

The role of S750 in modulation

S750Q mutation in wild-type GluR1 has only a modest impact on receptor function (7,11,23). Such a profound effect on L/Y mutant receptor function is therefore quite remarkable. Sun et al. (11) reported a similar, slowly desensitizing phenotype for the GluR2-L483/S754D double mutant. These mutations target the same sites we examined in GluR1 except that the S/D mutation alone accelerates desensitization more than S/Q (7,11); τdeact and EC50 values were not available for comparison. The authors concluded that these two residues act independently to regulate the transitions into and out of the desensitized states. Our results support their conclusion with respect to the rate and extent of desensitization, which we agree is most readily explained by the effects of these mutations on dimer stability. On the other hand, these residues clearly interact with respect to deactivation and agonist potency. In fact, our data suggest that the S750 residue is essential for modulation of koff by L/Y mutation. Unfortunately, because this residue is required for CTZ binding, its downstream involvement in benzothiadiazide modulation cannot be ascertained. Nonetheless, the L/Y-S750Q double mutant clearly demonstrates that desensitization can be modified independently of deactivation, at least by mutation targeting the dimer interface.

Future design of more selective modulators

Insights from comparison of these modulators and mutations may offer strategies to more selectively target desensitization or other functional measures. CTZ and TCM differ by an R3 substitution, which differentiates their size, hydrophobicity, and charge. It may be significant that that the R3 moiety occupies a hydrophobic pocket near the L/Y residue (11). Although CTZ and TCM have substantially different affinities for AMPARs, both produce similar block of desensitization. More importantly, their modulatory effects on τdeact and EC50 are significantly different, suggesting that other R3 substitutions may provide a means to selectively target these parameters. Numerous benzothiadiazides have been described, including additional R3 substitutions, some of which slow AMPAR desensitization (21). Aside from CTZ and the present characterization of TCM, none of these compounds has been thoroughly examined for effects on AMPAR deactivation or agonist potency. Additional characterization of this family of compounds could be useful in the generation of more functionally selective modulators that would have both experimental utility in dissecting GluR function and therapeutic value in correcting GluR dysfunction in disease.

The authors are grateful to Drs. Mark L. Mayer, Peter Seeburg, and Yael Stern-Bach for providing cDNA constructs and to Dr. Gary Rogers and Cortex Pharmaceuticals for CX614. We thank Dr. Hermes H. Yeh for critically reading a previous version of this manuscript.

This work was supported by NIH NS040347.

REFERENCES


