Research Paper

Functional Consequences of Natural Substitutions in the GluR6 Kainate Receptor Subunit Ligand-Binding Site

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KEY WORDS

glutamate receptors, binding site, kinetics, dimer interface

ABBREVIATIONS

iGluR ionotropic glutamate receptors
GluR2 AMPA receptor subunit named GluR2
GluR6 Kainate receptor subunit referred to as GluR6
T/S/T The triple GluR6 point mutant A518T/A689S/N721T
GLU glutamate
KA kainate
AMPA α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
NMDA N-Methyl-D-aspartic acid
τDes desensitization time constant
τDeact deactivation time constant

NOTE

Supplementary Material can be found at: http://www.landesbioscience.com/supplement/KistlerCHAN1-6-sup.pdf

ABSTRACT

Differences in binding-site residues of GluR2 (AMPAR) and GluR6 (KAR) subunits have been identified that might account for their functional and pharmacological differences. Specifically, residues A518, A689 and N721 in GluR6 replace highly conserved threonine and serine residues found in other ionotropic glutamate receptor (iGluR) subunits. To define how these natural substitutions impact GluR6 function, we used patch clamp recording with ultrafast perfusion to characterize the effects of A518T, A689S and N721T on agonist potency, efficacy and response kinetics. We find these natural substitutions impact GluR6 function less than would be expected from reverse mutations in other iGluRs. There was little effect of individual or combined mutations on glutamate potency, deactivation or desensitization kinetics. Altered recovery kinetics were seen that were greater after combined mutations. Kainate potency and response kinetics were also unchanged in the mutants, whereas kainate efficacy was reduced in A518T and increased the T/S/T mutant relative glutamate. Notably, A518T and A689S mutation permitted AMPA to bind as a weak competitive antagonist, allowing AMPA to activate and fully desensitize the receptors. Alternative mutations altering side chain length at position 518 produced far greater changes in glutamate affinity and response kinetics than did the natural mutations. We conclude that these nonconserved residues in GluR6 define the size of the agonist-binding pocket, exerting a steric influence on the bound agonist and the extent of binding-domain closure that can influence agonist potency, deactivation, desensitization and recovery kinetics.

INTRODUCTION

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system. Three subtypes of ionotropic glutamate receptors (iGluRs) are named for their preferred agonists: AMPA, kainate (KA) and NMDA.1 Of these, AMPA receptors (AMPAR) are composed from GluR1-4 subunits, KA receptors (KAR) are composed from GluR5-7 and KA1-2, and NMDA receptors are composed from NR1, NR2A-D and NR3 subunits. All iGluRs have a common membrane topology but coassemble exclusively with other subunits of the same subfamily to form tetrameric channels.2,3 Membrane topology consists of an extracellular amino terminus, three transmembrane domains (M1, M3 and M4), a reentrant pore forming p-loop (M2), an extracellular M3-M4 loop, and an intracellular carboxyl terminus. The ligand-binding domain is composed of so-called S1 and S2 segments that are located extracellularly preceding M1 and between the M3-M4 transmembrane helices, respectively (Fig. 1A).4-6

The AMPAR, GluR2 S1S2 fusion protein and more recently the KAR, GluR5 and GluR6 S1S2 fusion proteins have been resolved by X-ray crystallography and shown to form homologous bi-lobed structures with the ligand-binding residues located in a solvent-accessible crevice formed by the S1 and S2 domains.6-8 Both GluR2 and GluR6 S1S2 proteins could be isolated as dimers. Most studies agree that AMPARs assemble and operate as dimers of subunits.9-11 KARs probably also assemble as dimers,12 whereas there is evidence to suggest they might operate as tetramers.10

The current model of iGluR gating holds that ligand binding initiates closure of the inter-domain crevice, which pulls against the dimer interface connecting adjacent subunits to promote channel opening. In the continued presence of ligand, the receptors undergo another conformational change, entering a desensitized state wherein the dimer interface is disrupted and the ligand-binding site is uncoupled from the channel pore.13-16
is general agreement that greater binding-domain closure produces larger peak currents and faster, more complete desensitization. This has been demonstrated both in GluR2, comparing various 5-substituted willardine compounds\(^{17}\) and in GluR6 comparing other full and partial agonists.\(^{18}\) Once desensitized, receptors must reverse these conformational changes to recover from the desensitized state. The recovery process is much faster in AMPA than KARs and must involve reopening of the clamshell binding domain and dissociation of the agonist.\(^{19}\)

The S1S2 crystal structures have also provided confirmation of the specific residues involved in ligand binding, including similarities and differences that may account for some of the functional differences between iGluR subtypes. The primary ligand-binding residues in GluR6 are R523, T690 and E738, which are perfectly conserved among the AMPA and KAR subunits (Fig. 1).\(^{6-8,20}\) The GluR6 binding site also contains three natural substitutions of the secondary binding residues, A518, A689 and N721 that replace conserved threonine or serine residues found in other iGluRs. The functional consequences of these natural substitutions within the binding site are not known. In GluR3, T504A mutation (equivalent to GluR6-A518) was shown to reduce glutamate potency by 134-fold relative to wild-type.\(^{21}\) Similar observations were made in radio-ligand binding studies of the homologous chick-kainate-binding-protein.\(^{5}\) Likewise for NMDA receptors, NR2B-S664G mutation (equivalent to GluR6-A689) reduced glutamate potency by 100-fold relative to wild-type\(^{22}\) and NR2A-T671A (equivalent to GluR6-T690) reduced glutamate potency by 1000-fold.\(^{23}\)

It is striking, despite the natural substitutions of secondary ligand-binding residues, that AMPA and KARs have similar affinities and exhibit similar, rapidly desensitizing responses to their endogenous agonist, glutamate. Nonetheless, they exhibit significant functional and pharmacological differences.\(^{1,12,14,22}\) GluR6 possesses both sodium and chloride binding sites within the S1S2 domain that are required for function, while AMPA receptors are insensitive to sodium and chloride concentrations.\(^{10,24-26}\) Kainate acts as a partial agonist that produces rapidly desensitizing responses at KARs and partially or non-desensitizing responses at AMPARs. GluR6 does not respond to AMPA, whereas GluR5 and all of the AMPARs display fast desensitizing responses to AMPA. GluR6 also recovers very slowly from desensitization, over the course of several seconds, whereas AMPARs recover within tens of milliseconds. Considering the deleterious effects of binding-site mutations in other iGluRs, we hypothesized that the natural substitutions in the GluR6 binding site might contribute to these and other functional and pharmacological differences between AMPA and KARs.

The present study examines how these three natural substitutions at residues 518, 689 and 721 impact GluR6 function, and relates the findings to the model of iGluR gating initiated by binding domain closure. We postulated that mutating A518, A689 and N721 in GluR6 to the conserved threonine and serine residues would stabilize the glutamate bound conformation and thereby lower the EC\(_{50}\) for activation by glutamate. These mutations might also be expected to make GluR6 receptors more “AMPA like” with respect to kainate mediated desensitization and activation by AMPA. We further hypothesized these mutations should alter the extent of binding domain closure, thereby altering rates of desensitization and/or recovery. It has been previously demonstrated that mutation of N721 alone is enough to allow at least partial activation by AMPA.\(^{12,27}\) We hypothesized that mutation of A518 and A689 in addition to the N721T mutation would further enhance GluR6 receptor affinity for AMPA. To further test the binding domain closure model of activation and desensitization, we examined receptor kinetics and EC\(_{50}\) changes produced by smaller (A518G) and larger (A518V) aliphatic side-chain substitutions that open or occlude the binding pocket. Results suggest that the A518 and A689 substitutions in the GluR6
binding pocket interfere with AMPA binding but otherwise merely define the size of the agonist binding pocket and do little to alter glutamate- or kainate-evoked responses.

**MATERIALS AND METHODS**

**Site-directed mutagenesis.** Point mutations were introduced in the rat GluR6 sequence using the QuickChange method with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) and custom primers from Sigma Genosys (Woodlands, TX). Mutagenesis primers were 29–31 bases in length and contained 1–3 base substitutions that added or eliminated diagnostic restriction sites. Mutant cDNAs were amplified in transformed DH5α E. Coli and purified with Qiafilter maxiprep kits (Qiagen, Los Angeles, CA). Mutant cDNAs were selected by restriction enzyme digest and confirmed by automated DNA sequencing (Center for Comparative Functional Genomics, University at Albany, NY). Mutations were based on sequence alignments and comparisons of GluR2 and GluR6 and crystal structures, which are nearly identical despite the natural substitutions (Fig. 1). Structures are not available to confirm how mutations altered the binding site conformation or binding domain closure, however functional studies did not imply any deleterious or unusual effects on agonist binding or gating properties.

**Cell cultures and transfections.** Human Embryonic Kidney 293 (HEK293) fibroblasts were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and 2 mM Glutamax (Invitrogen) and incubated at 37°C in a 5% CO2 environment. Cells were maintained in 25 cm² flasks and passed twice weekly into fresh flasks. Extra cells were plated on poly-D-lysine coated 35 mm NUNC dishes at a density of 60–80,000 cell/ml. Cells were transfected using Lipofectamine 2000 (Invitrogen) with GluR6 and EGFP cDNAs at a 9:1 ratio and used 18–48 hours later.

**Patch-clamp recording.** Standard extracellular solution consisted of (in mM): 150 NaCl, 3KCl, 5 HEPES, 1 MgCl₂, 1.8 CaCl₂, 10 glucose, and 0.1 mg/ml phenol red, pH 7.3. Recording electrodes were made of thin walled borosilicate glass tubes (TW150-F, World Precision Instruments, Sarasota, FL). Electrodes had resistances of 2–4 MΩ when filled with intracellular solution consisting of (in mM): 135 CsCl, 10 CaF, 5 HEPES, 5 EGTA, 1 MgCl₂, 0.5 CaCl₂, pH 7.2, 295 mOsm. Outside-out patch recordings were performed in voltage clamp mode at -70 mV using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Current signals were filtered at 2 kHz with an eight-pole bessel filter (Cygnus Technology, Watergap, PA) and digitized at 5-20 kHz. Data were acquired and stored on a Macintosh PowerPC-G3 computer using an ITC-16 interface (Instrutech Corporation, Long Island, NY) controlled by the data acquisition/analysis program Synapase (Synergy Research, Silver Spring, MD).

**Solution exchange.** Ultrafast solution exchange rates between 200–500 μs were achieved using a piezo-based system (Burleigh Instruments, Fishers, NY). Control and agonist solutions were driven through two barrels of a theta tube simultaneously at a rate of 0.3 ml/min. Outside-out patches were positioned in the control stream near the interface between control and agonist solutions. The piezo system was used to move the theta tube ~70 μm, rapidly bringing the patch in and out of agonist solution. Response decay time constants (τ) were derived using a least squares algorithm in synapase; tdesensitization was measured following 1 ms agonist pulses, tdesensitization was measured during 25–50 ms agonist pulses. Recovery time constants (recovery) were derived from twin pulse experiments in which 10 ms agonist pulses were delivered at interpulse intervals in 100 or 250 ms increments, as required, with 10–30 sec between trials. Percent recovery was determined from the amplitude ratio of the test pulse to the conditioning pulse of individual trials; data were excluded that showed more than 15% rundown over trials.

**Data analysis and statistics.** Desensitization and deactivation kinetics for mutants were compared with a single factor ANOVA. Post hoc comparisons were made with a Bonferroni multiple comparison test. Dose-response data were normalized to the maximal response obtained with 10 mM glutamate and expressed as a percentage. The data were plotted in Kaleideograph and fit with the logistic equation:

\[
I_{\text{glu}} = \frac{I_{\text{max}}}{1 + (\text{EC}_{50}/[\text{glu}])^{nH}}
\]

Eqn. 1

where \( I_{\text{max}} \) is the maximum current, \([\text{glu}]\) is the concentration of glutamate, \( nH \) is the Hill coefficient, and \( \text{EC}_{50} \) is the concentration of glutamate that produces a half-maximal response. Log \( \text{EC}_{50} \) values were compared by nonlinear regression analysis in Graph Pad Prism with a Bonferroni correction for multiple comparisons. For recovery kinetics, test pulse amplitudes at various ISI were normalized to the conditioning pulses and expressed as a percent recovery. In order to plot the average recovery for each population of receptors, averages and SEMs for each time point were computed and fit with the equation:

\[
I_t = I_{\text{max}} - I_0(\text{e}^{-t/\tau})
\]

Eqn. 2

where \( I_t \) represents recovery at a given time interval, \( I_{\text{max}} - I_0 \) is the plateau recovery current, \( t \) is time, and \( \tau \) is the recovery time constant determined by least-squares fit to the data. We note that our recovery fits are approximations that assume single exponential kinetics whereas others have demonstrated that GluR6 recovery is a multistep process, where recovery at very short interpulse intervals is best fit with a sigmoidal curve. For statistical analysis, \( \tau \) recovery values were calculated for individual patches and one-way ANOVA was performed to assess the main effects of mutation on the recovery time constant. Post hoc comparisons were done with a Bonferroni multiple comparison test. AMPA inhibition curves were plotted in Kaleideograph and with the equation:

\[
I = I_{\text{max}}/1+([\text{AMPA}]/(\text{IC}_{50})^{nH})
\]

Eqn. 3

where \( I_{\text{max}} \) represents the maximum response elicited with a fixed concentration of agonist, \( I \) represents the response after preincubation with AMPA, \([\text{AMPA}]\) is the antagonist concentration, and \( \text{IC}_{50} \) represents the concentration of antagonist that inhibits half the response, and \( nH \) is the Hill coefficient. IC\(_{50}\) values were statistically compared by nonlinear regression analysis in Graph Pad Prism and corrected for multiple comparisons with a Bonferroni correction. For AMPA, Ki values were estimated with the equation:

\[
K_i = \text{IC}_{50}/(1+[\text{agonist}]/\text{EC}_{50})
\]

Eqn. 4

where \( \text{IC}_{50} \) represents the dose of agonist required to inhibit functional responses 50%, and \( \text{EC}_{50} \) represents the dose of agonist that elicits 50% of a functional response.
Generation of GluR2 and GluR6 glutamate bound structures. Images were generated using the DeepView/SwissPDBviewer v3.7 alpha (http://www.expasy.org/spdbv). Structures were obtained from the RCBS Protein Data Bank at http://www.pdb.org.

Amino acid numbering. We use the numbering of full-length GluR6 beginning with the initiation methionine and including the 31 amino acid signal peptide. Residues in other iGluRs may be numbered according to the full-length or mature protein as given in the specific work cited.

RESULTS

Effects of A518T, A689S, and N721T mutations on glutamate potency and kinetics. The A518 residue in GluR6 was replaced by the conserved threonine found in other AMPA and kainate receptors using site-directed mutagenesis. Recombinant GluR6 receptors were expressed in HEK293 cells and examined by patch clamp recording. Currents were elicited from outside-out patches by ultrafast application and removal of glutamate (agonist). We determined the agonist potency and the kinetics of deactivation, desensitization and recovery for wild-type GluR6 and the A518T mutant. Figure 2 shows the glutamate dose response curve for A518T was left-shifted by less than a half order of magnitude. The EC50 was 606 ± 60 μM compared to 1040 ± 60 μM for GluR6-WT. Deactivation and desensitization kinetics were tested by 1 ms and 25 ms pulses, respectively, of 10 mM glutamate (Fig. 3A, C and D). The τ deactivation for A518T was 1.88 ± 0.11 ms (n = 6) compared to 1.56 ± 0.16 ms (n = 6) for GluR6-WT. The τ desensitization for A518T was 3.43 ± 0.34 ms (n = 10) versus 3.35 ± 0.23 ms (n = 11) for GluR6-WT. Recovery kinetics were also examined by fast application of 10 mM glutamate using a twin pulse protocol (Fig. 3B and E). The τ recovery for A518T was 2.16 ± 0.21 s (n = 8) versus 2.35 ± 0.30 s (n = 5) for GluR6-WT. Deactivation, desensitization and recovery kinetics were not significantly different between wild-type and the A518T mutant.

The A689 residue in GluR6 is replaced by a conserved serine in AMPA and NMDA receptors. Consistent with a previous report of GluR6 mutations, the A689S mutation by itself did not alter deactivation or desensitization kinetics when tested with 10 mM glutamate (Fig. 3A, C and D).2 The τ deactivation for A689S was 1.79 ± 0.19 ms (n = 8) and τ desensitization was 3.18 ± 0.13 ms (n = 24). Moreover, the EC50 for activation was 937 ± 140 μM, which was also not different from wild-type (Fig. 2). Recovery kinetics were 42% faster than GluR6-WT when tested with 10 mM glutamate (p < 0.05). The τ recovery for A689S was 1.35 ± 0.05 s (n = 11) compared to 2.16 ± 0.21 s (n = 8) for GluR6-WT (Fig. 3B and E).

The N721 residue in GluR6 is replaced by a conserved threonine in AMPA receptors. Mutation of N721 to a threonine had little impact on glutamate affinity, but robust effects on glutamate mediated kinetics. The EC50 for activation was 1300 ± 62 μM, not significantly different from wild-type (Fig. 2E). The τ deactivation for N721T was 1.33 ± 0.12 ms (n = 4), which was also not significantly different from GluR6WT. Interestingly, The τ desensitization was 1.63 ± 0.10 ms (n = 9), which was significantly faster than GluR6WT. The τ recovery for N721T was 6.29 ± 0.31 s (n = 4) compared to 2.16 ± 0.21 s (n = 8) for GluR6-WT, which was significantly slower. (Fig. 3B and E).

We further tested how restoring both the conserved threonine (A518T) and serine (A689S) residues simultaneously would impact the apparent affinity and kinetic properties of GluR6 receptors. The EC50 of activation by glutamate for the double mutant A518T/A689S was significantly lower, approximately half that of wild-type, 506 ± 50 μM (p < 0.05) (Fig. 2D). Likewise, τ deactivation was 2.32 ± 0.17 ms (n = 5) and τ recovery was 4.27 ± 0.28 s (n = 11), both approximately 2-fold slower than GluR6-WT (Fig. 3A–C and E) (p < 0.05). However, as with the individual mutations, τ desensitization remained unchanged in the A518T/A689S double mutant at 3.17 ± 0.13 ms (n = 25) when tested at 10 mM glutamate (Fig. 3A and D).

Similarly, the triple mutant A518T/A689S/N721T also displayed an increase in glutamate affinity with an EC50 of 513 ± 100 μM, which was significantly lower than GluR6WT (Fig. 2F). Unlike the double mutant, the τ deactivation was not significantly slower than that of GluR6-WT, with a value of 1.21 ± 0.06 ms (n = 5). Similar to the single mutant N721T, the triple mutant had significantly faster desensitization kinetics, 1.50 ± 0.04 ms (n = 5), and significantly slower recovery kinetics, 15.08 ± 0.70 s (n = 4) (Fig. 3).

KA actions in A518T, A689S and N721T. Kainate behaves as a partial agonist at GluR6, and changes in the relative affinities or efficacies of kainate and glutamate are reflected in the normalized kainate dose response curves (Fig. 2A–F). Kainate-evoked currents were recorded from outside-out patches at various concentrations and plotted relative to the maximal responses elicited by 10 mM glutamate in the same patches. These are plotted alongside glutamate and AMPA concentration response curves in Figure 2. With regard to affinity, GluR6-WT had an EC50 for kainate of 455 ± 104 μM and the EC50 values were not significantly different for any of the mutants tested, A518T 596 ± 135 μM, A689S 215 ± 34 μM, N721T 239 ± 14μM, A518T/A689S 484 ± 148 μM, T/S/T 299 ± 71 μM (Fig. 2). With regard to KA efficacy, only A518T (23 ± 1% maximal glutamate current) and T/S/T (76 ± 9% maximal glutamate current) differed significantly from GluR6WT (49 ± 5% maximal glutamate current).

We also examined how kainate-evoked desensitization was affected by these mutations. Kainate-induced desensitization kinetics were compared at a dose of 300 μM (Fig. 4A and B). The τ desensitization for GluR6-WT was 3.50 ± 0.33 ms (n = 6), similar to a previous report.12 The τ desensitization values were 1.93 ± 0.40 ms (n = 5) for A518T, 4.58 ± 0.07 ms (n = 4), ms for A689S and 2.53 ± 0.32 ms for N721T. Current decay was best fit with a single exponential function. Both A518T/A689S and T/S/T were best fit with a double exponential function. In the case of A518T/A689S, approximately 60% of the current decay occurred during the fast phase and 40% during the slow phase. The fast phase had a τ of 3.10 ± 0.56 ms, like wild-type, and the slow phase had a τ of 10.32 ± 0.33 ms (n = 4). The weighted desensitization time constant for A518T/A689S was 5.74 ± 0.80 ms. Similar to the double mutant, T/S/T also displayed kainate-mediated desensitization kinetics that were best fit with a double exponential function. T/S/T had a fast phase during which approximately 78% of the current decay was observed and a slow phase where 22% of the current decay was observed. The τ value for the fast component was 2.53 ± 0.32 ms and the value for the slow component was 15.47 ± 1.42 ms (n = 4), T/S/T yielding a weighted τ desensitization of 5.22 ± 0.54 ms.

AMPA actions in A518T, A689S and N721T. AMPA normally does not bind to GluR6, so we further examined whether AMPA-evoked responses emerged in the A518T, A689S or A518T/A689S GluR6.
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A689S mutant receptors. In no case did either wild-type, nor the mutant receptors respond to direct application of AMPA up to 3 mM. Moreover, preincubation of GluR6-WT with up to 3 mM AMPA did not significantly inhibit 3 mM glutamate evoked peak currents (n = 4) (Figs. 2 and 5A and C). However, AMPA behaved as a full antagonist of 3 mM glutamate responses in the case of A518T and A689S, having IC50 values of 619 ± 200 µM and 768 ± 105 µM, respectively. The antagonist action of AMPA was even stronger in the double mutant, A518T/A689S, having an IC50 of 150 ± 29 µM. At 200 µM AMPA, the A518 mutation allowed 28.6 ± 4.7% inhibition (n = 4), A689S allowed 25.2 ± 2.7% inhibition (n = 4), and the A518T/A689S double mutation allowed 57.3 ± 3.5% inhibition (n = 4) of glutamate-evoked currents (Fig. 5), indicating the mutation effects were additive. Likewise, at 1 mM AMPA, the single mutants displayed approximately 50% inhibition of glutamate responses, significantly less than A518T/A689S, which was fully inhibited at this dose (p < 0.005). We also examined the ability of 200 µM AMPA to inhibit 300 µM glutamate responses (not shown), reasoning that as a competitive antagonist AMPA should produce further inhibition of responses elicited by a lower concentration of the agonist. Indeed, the double mutant displayed 80.4 ± 2.3% inhibition (n = 4) inhibition, while the single mutants, A518T and A689S displayed 50.0 ± 6.6% (n = 4) and 52.3 ± 5.3% (n = 4) inhibition of the 300 µM glutamate response, respectively. In all instances this was significantly greater than AMPA inhibition of 3 mM glutamate responses (p < 0.05).

Although we could not obtain functional responses from these three mutants at the doses of AMPA we tested (Supplementary...
Figure 3. Summary of Glutamate mediated kinetics. Deactivation, desensitization and recovery time constants ($\tau$) are given in Table 1. (A) Overlay of outside-out patch currents comparing deactivation (following 1 ms pulses) and desensitization (during 25 ms pulses) for GluR6-WT, A689T, A689S, A518T/A689S, N721T and T/S/T. Patches were clamped at -70 mV and stimulated by ultrafast application and removal of 10 mM glutamate. Current decays were fit by a single exponential function using a least-squares algorithm, best fitting $\tau$ values for the individual fits are shown. (B) Recovery kinetics were assessed using a twin pulse protocol whereby a 10 ms conditioning pulse of 10 mM glutamate was applied to desensitize receptors and an equivalent test pulse was applied at various times afterwards. Each plot is an overlay of currents from 18 (WT, A518T, A689S, A518T/A689S) or 12 (N721T, A518T/A689S/N721T) individual stimulus pairings delivered at increasing interstimulus intervals. Note that the N721T and A518T/A689S/N721T recovery protocol is 10 seconds long, as opposed to the 5 second length in the other traces, in order to accommodate for slowed recovery. (C) Deactivation time constants ($\tau$) for wild-type and mutant receptors following 1 ms pulses of 10 mM glutamate. Values are mean ± SEM; N values indicated in bars. Asterisks denote a significant difference from wild-type (*p < 0.05). (D) Desensitization time constants ($\tau$) for wild-type and mutant receptors in the continued presence of 10 mM glutamate. Values are mean ± SEM; N values indicated in bars. (***p < 0.001) (E) Average recovery time course for wild-type (closed circles) and mutant receptors (A518T: open circles, A689S: closed square, A518T/A689S: open square, N721T: closed triangle, A518T/A689S/N721T: open triangle); time constants ($\tau$) are given in Table 1. (When calculating time constants, the recovery of each individual patch was fit with the recovery equation outlined in the methods.) Each data point represents the average recovery at a given interpulse interval ± SEM. Each data point is the average of at least four independent observations.
Table 1  Effects of natural substitutions on agonist responses

|                | GluR6       | A518T      | A689S      | A518T/A689S | N721T      | T/S/T      |
|----------------|-------------|------------|------------|-------------|------------|------------|
| Glu EC50 (µM)  | 1040 ± 60   | 606 ± 60   | 937 ± 140  | 506 ± 50*   | 1300 ± 62  | 513 ± 100* |
| τDeact (ms)    | 1.56 ± 0.16 | 1.88 ± 0.13| 1.79 ± 0.19| 2.32 ± 0.17 | 1.33 ± 0.12| 1.21 ± 0.06|
| 10 mM GLU (n = 6) | (n = 6)     | (n = 8)    | (n = 5)*   | (n = 4)     | (n = 5)*   | (n = 5)*   |
| τDesens (ms)   | 3.35 ± 0.23 | 3.43 ± 0.34| 3.18 ± 0.13| 3.17 ± 0.13 | 1.63 ± 0.10| 1.50 ± 0.04|
| 10 mM GLU (n = 11) | (n = 10)   | (n = 24)   | (n = 25)   | (n = 9)**   | (n = 5)**  | (n = 5)**  |
| τRecovery (s)  | 2.16 ± 0.20 | 2.35 ± 0.35| 1.35 ± 0.05| 4.27 ± 0.28 | 6.29 ± 0.31| 15.08 ± 0.70|
| 10 mM GLU (n = 8) | (n = 5)     | (n = 11)*  | (n = 4)**  | (n = 4)*    | (n = 4)**  | (n = 4)**  |
| KA EC50 (µM)   | 455 ± 105   | 596 ± 135  | 215 ± 34   | 484 ± 148   | 239 ± 14   | 299 ± 71   |
| KA/Glu         | 49 ± 5%     | 23 ± 1%    | 53 ± 3%    | 46 ± 7%     | 50 ± 6%    | 76 ± 9%    |
| (n = 5)        | (n = 4)     | (n = 4)    | (n = 4)    | (n = 4)     | (n = 4)    | (n = 4)    |
| τDes (ms)      | 3.50 ± 0.33 | 1.93 ± 0.45| 4.58 ± 0.07| 5.74 ± 0.79 | 2.28 ± 0.52| 5.22 ± 0.54|
| 0.3 mM KA (n = 5) | (n = 4)**  | (n = 4)    | (n = 4)*   | (n = 4)     | (n = 4)    | (n = 4)    |
| AMPA IC50 (µM) | 619 ± 120   | 768 ± 105  | 150 ± 29   | 1.44 ± 10^3 | 6.30 x 10^4| 2.38 x 10^4|
|                | *** †       | *** †      | *** †      | *** †       | *** †      | *** †      |

Values are the mean ± SEM. NA = not active, †p < 0.05, ††p < 0.005, †††p < 0.001 versus GluR6-WT by ANOVA and Bonferroni multiple comparison post hoc. The double mutant A518T/A689S and the triple mutant A518T/A689S/N721T had kainate-mediated desensitization kinetics that were best fit with double exponential decay, values reported represent weighted averages. For AMPA EC50 † indicates p < 0.05 compared to A518T/A689S, †† indicates p < 0.005 compared to T/S/T. Fig. 1), we could not dismiss the possibility that the concentrations of AMPA being utilized were simply desensitizing the A518T and A689S mutations without causing activation, and the cost of testing higher concentrations of AMPA was prohibitive. In order to address this issue we also tested the N721T mutation alone and in conjunction with the A518T/A689S mutations. Previous studies have demonstrated that this point mutation in GluR6 is sufficient to allow activation and desensitization. 12 We expected that preincubating N721T and T/S/T in AMPA would desensitize these receptors while allowing AMPA behave functionally as an antagonist. We hypothesized that in their desensitized state the mutants would have a much higher affinity for AMPA than displayed by A518T, A689S, and A518T/A689S. We also speculated that the triple mutant T/S/T would have a higher affinity for AMPA than N721T alone. N721T had an IC50 of 0.24 ± 0.02 nM. Accepting a competitive interaction, Ki estimates from the Cheng-Prusoff equation were calculated to be 104 ± 20 µM for A518T, 183 ± 25 µM for A689S, and 22 ± 4 µM for the A518T/A689S double mutant. N721T had a Ki value of 435 ± 13 pM and T/S/T had a Ki value of 41 ± 4 pM. Altogether, these data suggest the A518T and A689S substitutions facilitate interactions with AMPA in the ligand-binding site. Clearly however, AMPA binding remained weak in the mutant receptors lacking the N721T mutation and failed to engage channel opening under our conditions. It is possible that the overall lack of effect observed on glutamate and kainate affinity could be caused by global differences between AMPA and KA receptors or unexpected conformational changes caused by these point mutations. In order to address this issue and further test the dimer interface model we made additional aliphatic mutations.

Additional A518 mutations disclose steric effects on agonist binding.  Results of mutations and functional studies, together with recent crystal structure data, strongly suggest that A518 and A689 residues are located in the GluR6 ligand-binding pocket. Nonetheless, our data also indicate that the natural alanine substitutions in GluR6 have only a modest impact on glutamate affinity and receptor kinetic properties. To further assess what role if any A518 plays in ligand binding and gating of GluR6, we made the residue smaller by mutation to glycine, removing the methyl side chain. GluR6-A518G was expressed in HEK293 cells and tested in outside-out patch-clamp recordings (Fig. 6A and B). In this case, we observed a far more profound shift in the dose-response curve compared to wild-type. The EC50 for activation of the A518G mutant was 21.6 ± 3.9 mM (Fig. 6C), and this value likely underestimates of the true EC50 due to the limitations of testing higher glutamate concentrations. Likewise, the kinetics of deactivation, desensitization and recovery kinetics were all significantly different from GluR6-WT when comparing 100 mM glutamate evoked currents (Fig. 6A, B and E–G). The τdesensitization was 0.75 ± 0.07 ms for A518G (n = 4) compared to 1.79 ± 0.15 ms for GluR6-WT (n = 6), the τdeactivation was 2.14 ± 0.16 ms (n = 18) compared to 3.63 ± 0.32 ms (n = 8) for GluR6-WT, and the τrecovery was 0.45 ± 0.08 s for A518G (n = 4) versus 2.34 ± 0.15 s for wild-type (n = 7).

These effects of A518G mutation further suggest that this residue is important for glutamate binding in the GluR6 ligand-binding pocket, although neither the natural alanine residue nor the glycine mutation would support hydrogen bond interactions with agonist or adjacent residues. Based upon these results, we hypothesized instead that A518 acts as a “steric wedge”, within the ligand-binding pocket. In this way, removing the methyl group with an alanine to glycine mutation would create additional space within the ligand-binding pocket that enhances mobility of the bound ligand and weakens its interactions with other residues. The ligand therefore binds less stably and can more readily escape. A similar observation was made in the NR2A subunit of NMDA receptors, where mutation of S670 to a glycine (the equivalent of A689 in GluR6) decreased the dwell time of glutamate in the binding pocket, leading to an increase in the deactivation rate of macroscopic currents, the reduction of single channel burst lengths, and a 100 fold reduction in glutamate affinity. 26 An alanine substitution at this same position in NR2A receptors, S670A, resulted in a less drastic two-fold shift in glutamate affinity. A caveat when interpreting these results is that glycine mutations
may cause kinks in the tertiary alpha helical structures of a protein. In order to address this issue, we tested additional A518 mutations to residues with progressively larger aliphatic side chains. In doing so, we hypothesized that the larger side chains would occlude the binding pocket to hamper ligand binding. The larger residues were also expected to reduce the extent of domain closure and so to slow rate of desensitization.

Three larger point mutants were produced that included A518V, A518I and A518F; of these only A518V could be functionally characterized as the larger substitutions produced nonfunctional channels. Like A518G, the glutamate dose response for A518V was right-shifted by several orders of magnitude. Testing up to 100 mM concentrations, the glutamate EC$_{50}$ was at least 13.5 ± 2.5 mM (Fig. 6C). Deactivation, desensitization and recovery kinetics were also tested at 100 mM glutamate for A518V and all were significantly different from GluR6-WT. The $\tau$ deactivation was for A518V was 1.07 ± 0.32 ms (n = 6) compared to 1.78 ± 0.15 ms for GluR6-WT (n = 6) (Fig. 6A and F). The $\tau$ desensitization was 8.21 ± 0.52 ms (n = 16) compared to 3.63 ± 0.32 ms for GluR6-WT (n = 8) (Fig. 6G). Most remarkably, recovery was 0.20 ± 0.04 s for A518V (n = 6) compared to 2.34 ± 0.15 s for wild-type (n = 7) (Fig. 6B and E). As we anticipated, A518V mutation therefore appears to occlude the ligand-binding site, as indicated by the 10-fold reduction in glutamate affinity, nearly 2-fold faster deactivation, and nearly 12-fold faster recovery from desensitization. Moreover, the A518V mutation slowed desensitization by 3-fold, which is consistent with less domain closure in the mutant.

We also tested A518G and A518V using a 3 mM concentration of kainate in conjunction of the rightward shift we observed in the apparent affinity for glutamate. We measured kainate-induced desensitization kinetics (Fig. 6G), which were significantly faster for A518G, 1.56 ± 0.08 ms (n = 5) and nearly 4-fold slower for A518V, 13.5 ± 0.57 ms (n = 7) compared to the GluR6-WT desensitization rate of 2.57 ± 0.30 ms (n = 5). These changes paralleled the changes observed in glutamate-mediated desensitization.

Additionally, the 10–90 rise times were computed for GluR6-WT, A518G, and A518V. At 1 mM glutamate, the average rise-time for GluR6-WT was 710 ± 67 µs (n = 5), A518G was 1600 ± 230 µs (n = 4), and A518V was 2425 ± 668 µs (n = 4) (Fig. 6D). At this dose, both A518G and A518V had significantly slower onset than wild-type (Fig. 9A). At 10 mM glutamate however, the average for GluR6-WT was 408 ± 44 µs (n = 6), A518G was 450 ± 35 µs (n = 6), and A518V was 770 ± 66 µs (n = 5) (Fig. 6D); only A518V was significantly slower than GluR6-WT at 10 mM glutamate. At higher concentrations (30 and 100 mM glutamate), the onset for A518G and A518V became increasingly faster and converged with the rate of onset of GluR6-WT currents. We take this to indicate that the rate-limiting process of channel opening seen at super-saturating agonist concentrations is unchanged even as the rate of agonist binding becomes somewhat slower in the mutant receptors.

**DISCUSSION**

The present study examined three natural substitutions in the GluR6 ligand-binding site (A518, A689 and N721) that replace highly conserved threonine and serine residues in other iGluR subunits. Results from reverse mutations demonstrate how these...
substitutions account for AMPA selectivity but otherwise have remarkably little impact on glutamate and kainate affinity or gating kinetics of GluR6. Results from alternative aliphatic mutations, A518G and A518V, which alter the volume of the binding pocket, provide support for a model linking the extent of binding domain closure with channel activation and desensitization. Mutations that permit more or less domain closure also cause faster or slower desensitization, respectively, most likely because of the associated stress on the dimer interface. Kinetic analyses further demonstrate that deactivation and recovery rates are strongly correlated, suggesting that agonist dissociation and relaxation from the agonist-bound closed cleft conformation are early events in a multi-step process of recovery from the desensitized state.

**iGluR gating and desensitization.** An understanding of glutamate receptor gating is evolving yet incomplete. The emerging model of GluR function is now informed by high-resolution crystal structures of the ligand-binding domain in conjunction with mutational analyses and biophysical characterization. The current model holds that agonist binding induces closure of the clamshell binding-domain, which pulls against the dimer interface connecting adjacent subunits to promote channel opening. In the continued presence of ligand, glutamate receptors undergo another conformational change, entering a desensitized state wherein the dimer interface is disrupted and the ligand-binding site is uncoupled from the channel pore. Once in the desensitized state, receptors must reverse these conformational changes to recover from desensitization before they can be reactivated by the agonist.

Insights into the conformational rearrangements associated with ligand binding, gating, and desensitization have come from crystallization of the GluR-S1S2 fusion proteins. The various iGluR S1S2 proteins form a conserved bi-lobed structure with the ligand-binding site located in a solvent accessible crevice between an upper lobe, which is connected to the dimer interface, and a lower lobe, which is connected to the transmembrane helices. The dimer interface is formed in part by a salt-bridge hydrogen bond network linking two adjacent binding domains in a head-to-tail fashion. Notably, crystallization of GluR2-S1S2 with different ligands indicated that full agonists, such as glutamate, allow the greatest extent of binding domain closure, whereas antagonists like DNQX produce very little domain closure and partial agonists like kainate or willardiines produce intermediate degrees of domain closure. Recently, the link between domain closure and agonist efficacy has become controversial because it appears not always to hold true when KARs and NMDARs were subsequently examined. There is also a relationship between greater domain closure and faster, more complete desensitization. Mutagenesis studies in the full-length receptors have confirmed the importance of dimer stability in maintaining the nondesensitized active state. Structure-activity studies in GluR2 correlated the extent of binding domain closure by various agonists with the relative amplitude of steady-state to peak currents, thus linking domain closure with desensitization, presumably involving destabilization of the dimer interface. In this case, similar studies comparing full and partial agonist actions in GluR6 demonstrated that the same was true for both AMPA and KARs, which are the fast-desensitizing iGluR subtypes.

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**Figure 5.** Emergent actions of AMPA. (A) Representative responses of GluR6-WT, A518T, A689S and A518T/A689S to 200 μM AMPA, 3 mM glutamate or 3 mM glutamate after preexposure to 200 μM AMPA. Outside out patches were pulled from transiently transfected HEK293 cells, held at -70 mV and stimulated by 25 ms pulse of AMPA or glutamate. (B) Representative responses of N721T and A518T/A689S/N721T to 3 mM glutamate following preexposure to 10nM AMPA. (C) AMPA IC50 [molar] values for GluR6WT, A518T, A689S, A518T/A689S, N721T, and A518T/A689S/N721T. Preincubation with highest concentration of AMPA tested, 3 mM, did not inhibit glutamate evoked currents in GluR6WT. (ND, not determined)
Natural substitutions in GluR6. Our study examined both the pharmacological and functional implications of natural substitutions of GluR6 binding site residues, A518, A689 and N721. Residue A518 is equivalent to T501 in GluR2 (Fig. 1) and a conserved threonine in all other NMDA, AMPA and KAR subunits. Mutating this threonine to alanine in GluR3 or NR2A produced a rightward shift in the glutamate dose-response by two orders of magnitude. A similar observation was made with the chick kainate binding protein. Residue A689 is also predicted to occupy the ligand-binding site, however the equivalent residue in NMDA, AMPA and other KAR subunits (with the exception of GluR7) is a serine. Serine to alanine mutations at this position in AMPA and NMDA receptors reportedly has more modest effects on glutamate affinity, and A689S mutation in GluR6 has no affect on glutamate mediated desensitization or 3H-kainate binding. In contrast to previous studies examining the reverse mutations in other iGluRs, we found no effect of A518T mutation in GluR6 on glutamate affinity or response kinetics. Neither did the A689S mutation affect these measures. Only the double mutant A518T/A689S mutations had a significantly lower EC₅₀ for glutamate, suggesting the functional consequences of these substitutions in the GluR6 ligand-binding pocket are minimal with respect to glutamate-evoked responses.

Table 2 Effects of A518G/V mutations on agonist responses

|        | GluR6 | A518G | A518V |
|--------|-------|-------|-------|
| Glu EC50 (mM) | 1.04 ± 0.06 | 21.6 ± 3.9** | 13.5 ± 2.5** |
| tDeact (ms) 100 mM GLU | 1.79 ± 0.15 [n = 6] | 0.75 ± 0.07 [n = 4]* | 1.07 ± 0.32 [n = 6]* |
| tDesens (ms) 100 mM GLU | 3.63 ± 0.32 [n = 8] | 2.14 ± 0.16 [n = 18]* | 8.21 ± 0.52 [n = 16]** |
| tRecovery (s) 100 mM GLU | 2.34 ± 0.15 [n = 7] | 0.45 ± 0.08 [n = 4]** | 0.2 ± 0.04 [n = 6]** |
| tDesens (ms) 3 mM KA | 2.57 ± 0.30 [n = 5] | 1.56 ± 0.08 [n = 5]* | 13.5 ± 0.57 [n = 7]** |

Values are mean ± SEM, *p < 0.05, **p < 0.005, ***p < 0.001 versus GluR6-WT by ANOVA and Bonferroni multiple comparison post hoc.

Effects on AMPA binding and function. Whereas wild-type GluR6 does not bind AMPA, reversal of the natural substitutions had remarkable effect on AMPA binding in the mutants. Like GluR6-WT, the A518T, A689S and A518T/A689S were also not

Figure 6. Effects of unnatural A518 substitutions on glutamate potency and kinetics. (A) Overlay of outside-out patch currents comparing the kinetics of deactivation (1 ms pulses) and desensitization (25–50 ms pulses) of GluR6-WT, A689G and A518V. Patches were taken from transfected HEK293 cells, held at -70 mV and stimulated by ultrafast application and removal of 100 mM glutamate; best fitting τ values for the individual fits are shown. (B) Kinetics of recovery from desensitization for GluR6-WT, A518G and A689V. Recovery was measured using a twin pulse protocol. Each plot is an overlay of 18–20 individual pairings delivered at increasing interstimulus intervals. Note the time scales are different as indicated below the traces. (C) Glutamate concentration-response curves comparing GluR6-WT (n = 12), A518G (n = 10) and A689V (n = 12). Glutamate-evoked peak current amplitudes from are plotted relative to the maximal response at 100 mM glutamate and fit with the logistic equation (see Methods). (D) Glutamate response rise times for effective concentrations between 1–100 mM. Values represent the mean ± SEM from 4–7 patches. Asterisks denote significant differences from wild-type (*p < 0.05; **p < 0.01). (E) Plots of recovery over time for GluR6-WT at 10 mM and 100 mM glutamate, compared to A518G and A689V at 100 mM glutamate. (F) Deactivation time constants (τ) for wild-type and mutant receptors following 1 ms pulses of 100 mM glutamate. Values are mean ± SEM; N values indicated in bars. Asterisks denote significant differences from wild-type (*p < 0.05). (G) Desensitization time constants (τ) for wild-type and mutant receptors in the continued presence of 100 mM glutamate or 3 mM kainate. Values are mean ± SEM, n values indicated in bars. Asterisks denote significant differences from wild-type (*p < 0.05; **p < 0.001). EC₅₀, τdeactivation, τdesensitization and τrecovery values are given in Table 2.
activated by AMPA. Unlike GluR6-WT however, AMPA was able to bind to all of these mutants as a competitive antagonist, thus blocking functional responses to glutamate with micromolar affinities. The third residue we examined was N721, which replaces a conserved threonine in AMPA receptors. Previous studies found that mutating N721 to serine or threonine allows GluR6 to respond weakly to AMPA.\textsuperscript{12,27} This is in part due to the relief of a steric clash between the asparagine residue and the isoxazole ring in AMPA.\textsuperscript{7} We found the N721T mutant, which is both activated and desensitized by AMPA, has at least 100-fold higher affinity for AMPA than A518T, A689S or A518T/A689S mutants. The triple mutant, A518T/A689S/N721T had still 5-fold higher affinity compared to N721T mutation alone. These results indicate that the natural substitutions together, and not the N721 substitution by itself, contribute to AMPA selectivity at AMPA versus kainate receptors.

**Effects on kainate responses.** When comparing the crystal structures for the S1S2 fusion protein, it was observed that both glutamate and kainate produce nearly the same extent of domain closure in GluR6 (23.3 Å versus 26.6 Å), while kainate produces much less closure than glutamate in the GluR2 structure (12.3 Å versus 20 Å).\textsuperscript{7,20} Based upon the domain closure and dimer interface model, these differences in domain closure could account for the differences in kainate-mediated desensitization between AMPA and KA receptors. The volume of the GluR6 binding crevice is 255 ± 15 Å\textsuperscript{3} compared to 218 ± 4 Å\textsuperscript{3} for GluR2.\textsuperscript{7} Replacing the alanines in GluR6 by conservative serine and threonine mutations would reduce the volume of the GluR6 binding pocket, which we expected would reduce the extent of domain closure and slow kainate-mediated desensitization. In fact, despite clear effects on AMPA affinity, these point mutations had only minimal effects on kainate-mediated desensitization. Notwithstanding the modest slowing by A518T/A689S and T/S/T mutations, all of the mutants remained rapidly desensitizing to kainate. Notably, kainate efficacy was increased in the triple mutant T/S/T, which confirms the natural substitutions are in a position to impact kainate binding and domain closure. There was also a trend towards lower EC\textsubscript{50} values for kainate in A689S, N721T and T/S/T, although these were not significant after multiple comparisons. Results therefore indicate that differences in kainate-mediated desensitization are largely unrelated to substitutions or volume differences in the ligand-binding crevice itself but more likely involve additional residues in GluR6 that are not conserved in GluR2. Two such residues include V685 and T741, which in GluR2 are L671 and M729. In GluR2, these residues wedge against the isopropenyl group, inhibiting full domain closure of GluR2 around kainate, and the V685L and T741M mutations in GluR6 were previously found to slow kainate-mediated desensitization.\textsuperscript{7,12} The current results suggest the non conserved residues we studied in GluR6 may not account for differences in binding domain closure observed between AMPA and KARs. Neither do they contribute much to differences in kainate mediated desensitization.

**Aliphatic mutations support the dimer interface model.** Besides the conservative mutations studied, we also examined additional aliphatic mutations to the A518 site in an attempt to further study the relationship between binding domain closure and desensitization. We hypothesized that increasing the side chain length would partially occlude the ligand-binding pocket, thereby reducing glutamate affinity and reducing the extent of domain closure. The domain closure and dimer interface model predicts that less domain closure would produce less stress on the dimer interface and thereby slow the rate of desensitization. Conversely, decreasing side chain length by glycine mutation would increase the extent of domain closure and accelerate desensitization. Results were in good agreement with these predictions of the model. The A518V mutant had a 10-fold higher EC\textsubscript{50} for glutamate and desensitized 3 times slower than GluR6-WT, whereas the A518G mutant had a 10-fold higher EC\textsubscript{50} for glutamate and desensitized 2 times faster than wild-type. The results obtained from these mutations therefore provide additional support for the model linking the extent of domain closure with destabilization of the dimer interface and subsequent desensitization.

**Recovery is a multi-step process.** Two other effects of the aliphatic A518 mutations were noteworthy. One was their faster deactivation, which is an estimate of k\textsubscript{off}. The other was their faster recovery from desensitization. Overall, the effects of low-affinity mutations were strongly correlated for deactivation and glutamate EC\textsubscript{50} and also for deactivation and recovery from desensitization. The former is not surprising because k\textsubscript{off} is the rate of agonist dissociation, which is a component of both deactivation and EC\textsubscript{50}. The latter suggests that k\textsubscript{off} is also a component of recovery; i.e., that ligand must dissociate before resensitization and reactivation can occur. The faster deactivation and recovery rates in A518G and A518V correspond to faster dissociation of glutamate. This correlation also holds for A518T/A689S, which deactivated slower, recovered slower, and had a higher affinity for glutamate. This finding is also consistent with observations from other mutations correlating stability of the agonist-bound conformation with recovery rates.\textsuperscript{12,19}

Unlike the A518V, A518G and A518T/A689S mutants however, N721T affinity or deactivation rate did not correlate with recovery kinetics. Both N721T and T/S/T mutants recovered slowly from desensitization even as their deactivation rates were not slower than GluR6-WT. The reason is unclear, but we note that these mutants also desensitized very fast such that the brief 1 ms pulses in this case were poor measures of deactivation. Also, there is reason to suspect that N721T mutation negates an interaction with a water molecule, which creates more space in the binding pocket and allows for greater domain closure and faster desensitization without impacting glutamate affinity. The GluR6 crystal structures include several water molecules in the binding site of the glutamate bound state, and the amino group of N721 makes hydrogen bond contacts with one of them.\textsuperscript{7} The absence of this water molecule and hydrogen bond interactions between residues. If so, then recovery is a multi-step process that involves the sequential reopening of the binding cleft, dissociation of agonist, and reintegration of contacts stabilizing the dimer interface; which are in agreement with previous studies of AMPA and KAR recovery.\textsuperscript{10,35}

**Implications and future directions.** In summary, our findings confirmed that A518, A689 and N721 are located within the ligand-binding domain and determined their roles in GluR6 function. Mutating these residues to the conserved threonine and serine...
residues present in other GluRs minimally increased the stability of the glutamate bound conformation of GluR6, while predominantly impacting recovery kinetics and AMPA selectivity. We conclude that the role of the A518 side chain within the ligand-binding domain is that of a “steric wedge” that defines the size of the binding pocket without directly binding ligand or other principle ligand-binding residues. Importantly, the data also suggest there are two functionally important processes related to strength of agonist binding. One is a relationship between binding domain closure and desensitization. The other is a relationship between binding domain opening, agonist dissociation, and recovery from desensitization. These findings are inconsistent with the reaction schemes and predictions of AMPA receptor kinetic models, which should therefore be refined to more adequately describe KAR gating.36

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