A Binding Site Tyrosine Shapes Desensitization Kinetics and Agonist Potency at GluR2

A MUTAGENIC, KINETIC, AND CRYSTALLOGRAPHIC STUDY

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Binding of an agonist to the 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)-propionic acid (AMPA) receptor family of the glutamate receptors (GluRs) results in rapid activation of an ion channel. Continuous application results in a non-desensitizing response for agonists like kainate, whereas most other agonists, such as the endogenous agonist (S)-glutamate, induce desensitization. We demonstrate that a highly conserved tyrosine, forming a wedge between the agonist and the N-terminal part of the bi-lobed ligand-binding site, plays a key role in the receptor kinetics as well as agonist potency and selectivity. The AMPA receptor GluR2, with mutations in Tyr-450, were expressed in Xenopus laevis oocytes and characterized in a two-electrode voltage clamp setup. The mutation GluR2(Y450A) renders the receptor highly kainate selective, and rapid application of kainate to outside-out patches induced strongly desensitizing currents. When Tyr-450 was substituted with the larger tryptophan, the (S)-glutamate desensitization is attenuated with a 10-fold increase in steady-state/peak currents (19% compared with 1.9% at the wild type). Furthermore, the tryptophan mutant was introduced into the GluR2-S12S ligand binding core construct and co-crystallized with kainate, and the 2.1-Å x-ray structure revealed a slightly more closed ligand binding core as compared with the wild-type complex. Through genetic manipulations combined with structural and electrophysiological analysis, we report that mutations in position 450 invert the potency of two central agonists while concurrently strongly shifting the agonist efficacy and the desensitization kinetics of the AMPA receptor GluR2.

The ionotropic glutamate receptors mediate most of the fast excitatory neurotransmission in the central nervous system. These receptors consist of three structural but also pharmacological and physiological distinct receptors classes; N-methyl-D-aspartic acid receptors, kainate receptors, and AMPA receptors (for review, see Refs. 1–5). The AMPA receptor class consists of four members, GluR1 through GluR4, where the subunits associate as two dimers (6–10) in a homomeric or heteromeric tetrameric receptor complex (11, 12). The kinetic properties of the receptor activity are highly dependent on the agonist. The full agonist (S)-glutamate and the partial agonist kainate induce markedly distinct responses on the AMPA receptors. (S)-Glutamate elicits large and almost completely desensitizing currents on GluR2 flip and flop, although kainate elicits non-desensitizing currents, also on both splice variants (13).

High resolution x-ray structures of isolated ligand binding cores from GluR2 (Fig. 1A) in complex with different agonists and antagonists (e.g. Refs. 6 and 14–16) have provided a detailed picture of the network of interactions between the receptor and the ligand. Comparisons of the structural and functional information suggest that full agonists like (S)-glutamate induce a tight (20°) closure of the two ligand binding domains, thereby resulting in the largest separation of the linker connecting the transmembrane region and the binding core. Partial agonists like kainate but also antagonists induce lower domain closure and a subsequent smaller separation of the linker region (6, 14, 15, 17).

Both (S)-glutamate and kainate interact with the receptor through a number of direct hydrogen bonds together with a number of water-mediated hydrogen-bond interactions (6). Most notably are the highly conserved interactions between the α-carboxyl group and Arg-485 together with those between the α-amino group and Pro-478, Thr-480, and Glu-705 in GluR2 (6). Examination of the crystal structures of co-complexes with different agonists and the ligand binding core of GluR2 suggests that two residues, Tyr-450 and Leu-650, with direct hydrogen bonding to the agonist are located at critical positions for the domain closure. Both Tyr-450 and Leu-650 appear in the crystal structure as wedges between the agonist and domain 1 and domain 2, respectively (Fig. 1).

The importance of Leu-650 on the receptor kinetics has been studied in a number of different systems. Substituting Leu-650 with a threonine decreases the potency of (S)-glutamate, AMPA, and quisquulate 8–70-fold, but conversely, the mutation increases the potency of kainate by 3-fold (18). Madden et al. (19) recently reported a Fourier transform infrared difference spectroscopy study on the soluble GluR4 ligand binding core with the GluR4(L651V) mutation (equivalent to GluR2(Leu-650)) (19). Notably, they revealed that receptor kinetics...

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The atomic coordinates and structure factors (code 2ANJ (GluR2-S12S/Y450W-kainate)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Table I.

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GluR2-S1S2(Y450A) and GluR2-S1S2(Y450W) were also constructed by the standard overlap polymerase chain reaction method using the wild-type GluR2-S1S2 construct as template (6). After PCR, the resulting fragments were digested with PstI and Asp718 and inserted into the wild-type construct, which had previously been digested with the same restriction enzymes. All mutations were verified by sequencing. Expression, refolding, and purification were done essentially as previously described (23, 24) except that a higher concentration of (S)-glutamate (10 mM) was used in the refolding buffer and in all subsequent steps.

Two-electrode Voltage Clamp—A female Xenopus laevis frog was anesthetized, and oocytes were isolated, processed, and injected the following day with 50 nl of in vitro transcribed cRNA. In vitro transcription was done using the mMESSAGE mMACHINE T7 kit from Ambion Diagnostics according to the supplied instructions. Oocytes were kept at 18 °C in Barth’s solution, and to increase survival some of the oocytes were kept in normal frog Ringer (NFR; 10 mM HEPES-NaOH, pH 7.4, 115 mM NaCl, 1.8 mM CaCl2, 2.5 mM KCl, 0.1 mM MgCl2) supplemented with 1% serum and 10 µg/ml penicillin, 10 µg/ml streptomycin. Recordings were done 2–12 days after injection in a low calcium Ringer solution (10 mM HEPES-NaOH, pH 7.4, 115 mM NaCl, 0.1 mM CaCl2, 2.5 mM KCl, 1.8 mM MgCl2). For further details, see Nielsen et al. 25. The pH in all solutions was routinely checked. We compensated for sodium ions in the (S)-glutamate solutions, and since it has been shown that external ions influence kainate receptor but not AMPA receptor kinetics (26), we argue that any alternations in ionic strength would not influence our results.

At the wild-type receptors we used 0.9 mM (S)-glutamate, 1 mM kainate, 2.5 mM (RS)-AMPA, and 1 mM (RS)-ATPA as saturating agonist concentrations. When characterizing the Tyr-450 mutants, we used 30 mM (S)-glutamate, 1 mM kainate, 1 mM (RS)-AMPA, and 1 mM (RS)-ATPA (see TABLE THREE and see Fig. 6). Data under two-electrode voltage clamp were acquired and analyzed as previously described (25, 27).

Rapid Application—Oocytes expressing GluR2 receptors were pre-tested in the standard two-electrode voltage clamp setup. Only oocytes giving responses >700 nA to 30 µM kainate at ~60 mV were selected for further experiments. Before recording the vitelline membrane was removed by incubating the oocyte in hyperosmotic medium (200 mM potassium aspartate, 20 mM KCl, 1 mM MgCl2, 5 mM EGTA-KOH, 10 mM HEPES-KOH, pH 7.4) or, alternatively, 2 × NFR. After 10 min of incubation, the vitelline membrane was separated from the oocyte using a pair of fine forceps.

As external recording solution we used NFR, and the internal solution was 100 mM KCl, 10 mM EGTA, and 10 mM HEPES, pH 7.4. All agonists were dissolved in NFR. Outside-out patches were excised from the oocyte-membrane using fire-polished borosilicate glass capillaries (outer diameter, 1.5 mm; inner diameter, 1.17 mm; with inner filament) supplied by Harvard apparatus LTD (Kent, UK). The patch pipettes displayed resistances of 2–4 megohms. Fast application of the agonists was obtained using a piezo-driven (Burleigh Instruments, Fishers, NY) double-barreled theta-glass tube (length, 100 mm; outer diameter, 2.0 mm; wall thickness, 0.3 mm; septum thickness, 0.117 mm) from Hilgenberg GmbH (Malsfeld, Germany). Agonist pulses were applied with a 3-s break between the individual sweeps. To visualize the interface between the two solutions, 10 mM sucrose was added to the agonist solutions except for (S)-glutamate. A 20–80% solution exchange was routinely measured by stepping from 100% into 60% NFR. Values ranged from 100 to 300 µs, which were faster than the rise time of the agonist-activated currents. All patches were clamped at −120 mV, and the currents were recorded with a HEKA EPC9 patch clamp amplifier.

FIGURE 1. Structure of ionotropic glutamate receptors and ligands. A, an individual subunit is highlighted on the left and is formed by a single polypeptide chain beginning with the N-terminal UVBP (leucine-isoleucine-valine-binding protein)-like domain, which is followed by S1 forming the majority of domain 1 (D1) of the ligand binding core (red). The amino acid chain then transverses domain 2 (D2) of the ligand binding core and forms M1, the P-loop, and M2. Subsequently, the rest of domain 2 (D2, blue) is formed by S2, ending up in the last transmembrane D2 forms M1, the P-loop, and M2. The resting of domain 2 (red) is followed by S1 forming the majority of domain 1 (D1) of the ligand binding core—(blue). The amino acid chain then transverses domain 2 (D2) of the ligand binding core and forms M1, the P-loop, and M2. Subsequently, the rest of domain 2 (D2, blue) is formed by S2, ending up in the last transmembrane D2 forms M1, the P-loop, and M2.

Experimental Procedures

Glutamate Receptor Ligands and Reagents—(S)-Glutamate, kainate, and all other reagents were purchased from regular commercial sources.

Mutagenesis—Mutations in the full-length GluR2 receptor were introduced by the standard overlap polymerase chain reaction method using mutagenic primers designed with the desired mutations. We used GluR2Qo as template for the reactions, and the mutants were later subcloned into GluR2Qo(LA83Y), GluR2Q1, and GluR2QQI(LA83Y) using the restriction sites for Apal and MunI. All recordings were performed on receptors un-edited in the Q/R site (21). Numbering of GluR1 and GluR2 is according to the mature protein and corresponds to numbering previously reported (22, 23).
sampled at 100 kHz, and filtered at 2.9 kHz using the acquisition program Pulse (both from HEKA electronic GmbH, Lambrecht, Germany).

Fast application data were analyzed in Pulse-fit (HEKA) by fitting to the exponential equation $A_t = A_{\text{max}} e^{-t/\tau} + I_{\text{SS}}$, where $A_t$ is the measured current at time $t$, $A_{\text{max}}$ is the maximal current amplitude, $I_{\text{SS}}$ is the steady-state current, and $\tau$ is the desensitization time constant. The traces were exported from Pulse-fit and drawn in SigmaPlot 3.0 (Jandel Scientific, SPSS Science Inc., Chicago, IL). All traces displayed in the figures are individual raw sweeps.

Crystallization—Protein solutions of 8 mg/ml GluR2-S1S2(Y450W) in 10 mM Hepes pH 7.0, 20 mM NaCl, 1 mM EDTA supplemented with 10 mM (S)-glutamate and kainate, respectively, were used for crystallization. Crystals were grown at 6°C in 10–20% polyethylene glycol 8000, containing 0.1 M cacodylate, pH 6.5, 0.1 M Li$_2$SO$_4$ using the hanging-drop method with a 1:1 (v/v) ratio of protein and precipitant solution. Small crystals suitable for data collection appeared when the protein was co-crystallized with a 1:1 (v/v) ratio of protein and precipitant solution. Small crystals containing kainate, whereas only very small needle-like crystals unsuitable for data collection appeared when the protein was co-crystallized with (S)-glutamate.

X-ray Data Collection and Structure Determination—Before data collection crystals of the GluR2-S1S2(Y450W)-kainate complex were transferred through reservoir solution containing additional 15% glycerol and cryo-cooled using liquid nitrogen. Synchrotron data to 2.1 Å resolution were collected on the beamline I711 at MAX-Lab, Lund, Sweden. The HKL package (Denzo, Xds11, and Scalepack) (28) was used for autoindexing, integration, and scaling of the data (for statistics on data collection, see supplemental Table I).

The structure was solved by molecular replacement using the program CNS (29) and the wild-type GluR2-S1S2-Y450W-kainate structure (PDB code 1FTK without water molecules and ligand) as template followed by simulated annealing. Further refinement was done by repeated cycles of model building in O (30) and maximum-likelihood refinement and individual B-factor refinement using CNS until $R_{\text{free}}$ converged. Residues 390–506 from S1, a short linker consisting of the amino acids Gly and Thr, followed by residues 632–774 from S2 of the GluR2-S1S2(Y450W) construct as well as the ligand kainate could be fitted reliably into the electron density. The program Procheck (31) estimated 91.3% of the residues to be in the allowed region of a Ramachandran plot and 8.7% to be in the generously allowed region. 254 water molecules were located in the electron density in hydrogen-bonding distance from the protein molecule.

### RESULTS

The importance of the proposed steric interaction between agonists and the Tyr-450 in GluR2 was investigated by mutating the residue to the smaller alanine or the larger tryptophan.

**Mutations at Tyr-450 Affect Agonist Potencies Making GluR2(Y450A) Kainate-selective—**Homomeric receptors composed of GluR2o and GluR2i wild-type or mutant subunits were expressed in $X$. laevis oocytes, and dose-response relationships were established under a two-electrode voltage clamp (TABLE ONE, Figs. 2 and 3). These experiments revealed a 30- and 60-fold increase in $EC_{50}$ for (S)-glutamate at GluR2i(Y450W) and GluR2o(Y450W), respectively, as compared with the wild-type receptors. On the other hand, the

### TABLE ONE

**Potencies of (S)-glutamate and kainate on GluR2i/GluR2o and mutants**

Relative potencies of (S)-glutamate and kainate when characterized in the two-electrode voltage clamp setup on $X$. laevis oocytes expressing the respective receptors are shown. At least three individual oocytes were tested for each value.

| Receptor          | (S)-Glutamate EC$_{50}$ (min/max) | $n_H$ | Kainate EC$_{50}$ (min/max) | $n_H$ |
|-------------------|----------------------------------|-------|----------------------------|-------|
| GluR2i$^a$        | 30 μM (29/31)                    | 1.5   | 170 μM (160/190)           | 1.0   |
| GluR2i(L483Y)$^a$ | 10 μM (10/10)                    | 1.7   | 170 μM (160/190)           | 1.1   |
| GluR2o$^a$        | 5.7 μM (5.2/6.4)                 | 1.1   | 130 μM (120/140)           | 1.1   |
| GluR2o(L483Y)$^a$ | 9.0 μM (7.6/11)                  | 1.6   | 110 μM (98/120)            | 1.1   |
| GluR2i(Y450A)     | 12 mM (11/13)                    | 2.8   | 8.1 μM (7.0/9.4)           | 1.2   |
| GluR2i(Y450A,L483Y)| 9.0 mM (7.6/11)                  | 1.8   | 2.5 μM (2.1/2.8)           | 1.4   |
| GluR2o(Y450A)     | 17 mM (16/19)                    | 2.0   | 3.1 μM (2.9/3.3)           | 1.3   |
| GluR2o(Y450A,L483Y)| 8.3 mM (8.1/8.6)                 | 2.2   | 1.9 μM (1.7/2.0)           | 1.4   |
| GluR2i(Y450W)     | 880 μM (830/930)                 | 1.2   | 130 μM (120/140)           | 1.3   |
| GluR2i(Y450W,L483Y)| 190 μM (180/190)                 | 1.5   | 86 μM (73/100)             | 1.1   |
| GluR2o(Y450W)     | 340 μM (300/380)                 | 1.1   | 62 μM (61/63)              | 1.3   |
| GluR2o(Y450W,L483Y)| 67 μM (61/73)                    | 1.5   | 38 μM (32/46)              | 1.3   |

$^a$ EC$_{50}$ values for (S)-glutamate and kainate on GluR2i/o and GluR2i/o(L483Y) have been published previously (27).

![FIGURE 2. Traces from X. laevis oocytes under two-electrode voltage clamp.](http://www.jbc.org/)

Oocytes expressing the given receptors, and (S)-glutamate and kainate were applied in a series of concentrations, written above each response. The oocytes in panels A–D were clamped at $-70$, $-40$, $-80$, and $-80$ mV, respectively.
potency of kainate increased modestly, 2-fold or less for GluR2o(Y450W) and GluR2i(Y450W).

When determining the dose-response relationships for the Y450A mutant, we observed a dramatic reduction in the potency for (S)-glutamate, with an almost 3000-fold shift at GluR2o(Y450A) and a 400-fold shift at GluR2i(Y450A). In contrast, kainate exhibited higher potency at the GluR2(Y450A) mutant, with a 21- and 42-fold decrease in EC50 at GluR2o(Y450A) and GluR2i(Y450A), respectively. Thus, kainate exhibits more than 1400- and 5400-fold higher potencies at GluR2i(Y450A) and GluR2o(Y450A) than (S)-glutamate.

Mutation of Leu-507 in GluR3 (corresponding to Leu-483 in GluR2) to any aromatic residue has been shown to block or reduce the conformational changes required for the receptor complex to enter the desensitized state (31). To investigate the contribution from desensitization, we also determined the potency of the agonists in the presence of the non-desensitizing conferring mutation (32). The largest effect was observed for (S)-glutamate on GluR2o(Y450W,L483Y), showing a 5-fold higher potency on this receptor as compared with GluR2o(Y450W). Introduction of L483Y in either of the GluR2(Y450A) mutants changed the potency of both agonists less than 3-fold, showing that differences in the desensitization contributed less to the large shift in the ligand potency than the mutations in position 450 (TABLE ONE, Fig. 3).

**Mutations at Tyr-450 Change the Desensitization Properties**—To determine the kinetic properties of the mutant GluR2 receptors, we used a piezo-driven rapid application setup. At this setup we applied agonists to outside-out patches excised from *X. laevis* oocytes with a solution exchange less than 300 μs. Given the reduced potency of (S)-glutamate on GluR2o(Y450W) (TABLE ONE), this mutant was activated with high concentrations of (S)-glutamate. 100 mM (S)-glutamate elicited desensitizing currents in response to rapid agonist application but with slower kinetics and a 10-fold higher steady-state/peak ratio compared with the wild-type receptor (Fig. 4). We determined the desensitization time constant, \( \tau \), to 4.5 ± 0.3 ms and the steady-state/peak ratio to 19 ± 2.3% on GluR2o(Y450W), compared with 2.0 ± 0.2 ms and 1.9 ± 0.4%,
respective, for the wild-type receptor (TABLE TWO). When (S)-glutamate was applied to outside-out patches containing the GluR2(Y450W) homomeric receptors, the responses were almost non-desensitizing, with a steady-state/peak ratio at 86 ± 0.04% (Fig. 4A).

In contrast to the non-desensitizing currents activated at wild-type GluR2i/o receptors (13), kainate elicited a strongly desensitizing current when applied to GluR2o(Y450A) (Fig. 5A). On this mutant, responses desensitized with a time constant at 3.0 ± 0.4 ms, comparable with that observed for (S)-glutamate at wild-type GluR2o (TABLE TWO). The steady-state/peak ratio for kainate was 9.8 ± 1.3%. Similar results were observed for the equivalent mutation in GluR1(Y446A), where kainate also induced a desensitizing current (τ = 1.6 ms, data not shown). We also tested (S)-glutamate on GluR2o(Y450A) but were not able to detect any currents, even at 100 mM. This may reflect the 2600-fold increase in EC_{50} for (S)-glutamate.

To examine if the kainate-induced desensitization at GluR2(Y450A) involves a mechanism similar to the intersubunit interface “sliding” proposed for (S)-glutamate and other agonists inducing a tight domain closure (17, 33), we tested the double mutant GluR2o(Y450A,L483Y). GluR2o(L483Y) is proposed to stabilize the dimer interface, thereby preventing interdomain sliding (33). Application of 1 mM kainate evoked a non-desensitizing response from GluR2o(Y450A,L483Y) in outside-out patches (Fig. 5B, TABLE TWO), suggesting that the structural changes involved in the kainate-induced desensitization at GluR2o(Y450A) pursue a mechanism similar to the wild-type receptor. These observations suggest that kainate, as a partial agonist on wild-type GluR2, might act as a full agonist on GluR2o(Y450A). To estimate the agonist-specific efficacy on these tyrosine mutants, we compared the currents induced by kainate (1 mM) to AMPA (1 mM), ATPA (1 mM), and (S)-glutamate (30 mM), all full agonists that induce a 20° domain closure at the GluR2 ligand binding domain (TABLE THREE). Application of 30 mM glutamate at GluR2o(Y450A,L483Y) activated a current of 31% of the maximal kainate-induced current. We were not able to dissolve AMPA and the derivative ATPA to concentrations that would give a saturating response at GluR2(Y450A). However, the response activated by 1 mM AMPA at GluR2o(Y450A,L483Y) was less than 0.3% of the maximal kainate response and 1% of the (S)-glutamate response (Fig. 6), indicating that the structural rigidity of AMPA and ATPA, as compared with (S)-glutamate, makes isoxazole compounds more sensitive to the Y450A mutation.

**Structural Analysis of Ligand Binding in GluR2—**To get more detailed structural information, we introduced mutations equivalent to Y450A and Y450W into the soluble ligand binding core of GluR2-S1S2J.

### TABLE TWO

| Kinetics of wild-type and mutant GluR2i and GluR2o | (S)-Glutamate | Kainate |
|---------------------------------------------------|---------------|---------|
| | τ | ss/peak | | τ | ss/peak |
| GluR2i | 9.8 ± 0.79 | 6.9 ± 1.7 | Non-des | Non-des |
| GluR2o | 2.0 ± 0.19 | 1.9 ± 0.39 | Non-des | Non-des |
| GluR2o(Y450A) | No response | No response | 3.0 ± 0.37 | 9.8 ± 1.3 |
| GluR2o(Y450A,L483Y) | — | — | Non-des | Non-des |
| GluR2o(Y450W) | 4.5 ± 0.26 | 19 ± 2.3 | Non-des | Non-des |

| | a | b |
|------------------|---|---|
| Data are from Holm et al. (27); 10 mM (S)-glutamate and 1 mM kainate. |
| Data are from Koike et al. (13); 1 mM kainate. |
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TABLE THREE
Agonist efficacy on wild-type and mutant forms of GluR2o

Agonists were applied in saturating concentrations (see “Experimental Procedures”) to two-electrode voltage-clamped oocytes expressing the different receptor types. The (S)-glutamate-elicited response was set at 100%. Values in parentheses represent S.E. from 4–9 oocytes.

| Receptor/agonist            | (S)-Glutamate | Kainate | AMPA | ATPA |
|----------------------------|---------------|---------|------|------|
| GluR2o                     | 100           | 390 (28)* | 86 (4.3) | 180 (14)* |
| GluR2o(L483Y)              | 100           | 31 (3.0)* | 150 (22) | 98 (1.0)* |
| GluR2o(Y450A)              | 100           | 440 (24)  | 5.2 (0.72) | 12 (4.5) |
| GluR2o(Y450A,L483Y)        | 100           | 320 (33)  | 1.0 (0.23) | 1.8 (0.40) |
| GluR2o(Y450W)              | 100           | 190 (5.8) | 19 (1.3)  | 33 (1.3)  |
| GluR2o(Y450W,L483Y)        | 100           | 47 (2.5)  | 68 (3.7)  | 34 (2.5)  |

* Selected values for kainate and ATPA have previously been published (27).

FIGURE 6. Y450A has dramatic effects on agonist efficacy. Saturating agonist concentrations (see “Experimental Procedures”) were applied to X. laevis oocytes expressing the given receptors and analyzed under two-electrode voltage clamp. The two sets of traces are scaled after their individual (S)-glutamate response. Dotted lines indicate the size of the (S)-glutamate response.

Otherwise, hydrogen-bonding contacts between the protein and kainate were essentially unchanged (TABLE FOUR).

The complex crystallizes as a dimer, as previously observed in other studies (6, 14, 33). As a measure of the D2–D2 domain separation in the dimer upon binding of an agonist, we have calculated the distance between Cα atoms of the two Pro-632 residues located in the vicinity of the Gly-Thr linker residues. This linker replaces the M1 and M2 transmembrane regions (Fig. 1A). Gouaux and co-workers (17) have observed an apparent linear correlation between dimer separation and domain closure. In agreement with this, we observe a longer distance between the two Pro-632 of 35.4 Å in the mutant compared with 33.1 Å in the wild-type construct (6).

The Asp-651—Ser-652 peptide bond has been shown to adopt distinct conformations (6). This has been proposed to be yet another mechanism by which the ligand binding core accommodates agonists of varying chemical structure. In the present structure the conformation of this peptide bond is similar to that observed in the wild-type structure (6). Generally, ligands allowing more than 18° domain closures can accommodate a flipped conformation of the Asp-651—Ser-652 peptide bond, and some of the agonists even stabilize both flipped and unflipped conformations (6).

DISCUSSION

The ligand binding site of the glutamate receptors is formed between two domains connected by a flexible linker, which enables different ligands to stabilize various degrees of domain closure (5). Using a mutagenesis approach, we show that the domain closure also can be affected by mutations in critical residues located within the ligand binding pocket and that these mutations exhibit dramatic effects on agonist potency and desensitization kinetics.

The Y450W Mutation Alters Domain Closure—Surprisingly, the structure of GluR2-S1S2J(Y450W) with kainate bound revealed a 15° closure, which is 2° tighter than the GluR2-S1S2J-kainate complex (6). The substituted tryptophan is angled 6° compared with Tyr-450 to compensate for the reduced space caused by the tighter closure (Fig. 7C). The tighter domain closure results in a slight reduction in the length of several of the kainate-protein interactions (TABLE FOUR), contributing to the increased potency of kainate at the Y450W mutant as compared with the wild-type receptor. In contrast, the Y450W mutation reduces the potency of (S)-glutamate 30–50-fold. In the S1S2J-(S)-glutamate complex Tyr-450 (OH) forms a hydrogen bond with Glu-402 (OE2) (O–O distance 3.6 Å), thereby stabilizing the interdomain interaction between Glu-402 and Thr-686. Tyr-450 (OH) can only weakly stabilize Glu-402 (OE2) (O–O distance 3.6 Å) in the kainate complex (6, 23). Because the Y450W mutation disrupts the hydrogen bond between Tyr-450 (OH) and Glu-402 observed in the GluR2-S1S2J-(S)-glutamate complex, we speculate that this might affect the potency of (S)-glutamate to a greater extent than for kainate (TABLE ONE).

The 2.2-fold increase in time constant (7) and the 10-fold increase in the steady-state/peak current observed for the (S)-glutamate-elicited currents on GluR2o(Y450W) (when compared with GluR2o) together with the almost non-desensitizing current on GluR2i(Y450W) (TABLE TWO) suggest that (S)-glutamate stabilizes a less tight domain closure on the mutant as compared with the wild-type receptor.

There is a strong correlation between the domain closure induced by an agonist and the efficacy (5, 6, 14), whereas the correlation to the desensitization kinetics and the steady-state/peak ratios also depend on other properties of the ligand-receptor interaction than the domain closure (19, 27). For agonists of similar structural scaffolds, as e.g. willardines or isoxazole derivatives, there is a correlation showing that derivatives that stabilize a tighter domain closure also show faster desensitization and lower steady-state/peak currents. However, marked differences are observed between different structural classes, where a domain closure of 15° induced by kainate at GluR2o(Y450W) results in non-desensitizing currents, whereas closure by bromo-willardine (15°) and iodo-willardine (15°) induces desensitizing currents (17).

The structural analysis also indicated that Leu-650 (Fig. 7B) might exhibit a similar action as a wedge between domain 2 and the ligand (18). Indeed, the mutant GluR2i(L650T) also increased the domain closure to 15° in the kainate complex and showed a similar effect on the potency for (S)-glutamate and kainate as observed for GluR2i(Y450W) (18).

By introducing the Y450W mutation (S)-glutamate is converted to an almost non-desensitizing agonist on GluR2i(Y450W) and the steady state/peak ratio is increased 10-fold on GluR2o(Y450W) (TABLE TWO).
Similar kinetic properties on GluR2i and GluR2o, respectively, are induced by (S)-2-amino-3-(3-hydroxy-5-tert-butyl-4-isothiazolyl) propionic acid ((S)-thio-ATPA) (Fig. 1B), which induces an 18° domain closure (27). This suggests that similar kinetic properties can be obtained by different mechanisms, either by a tryptophan residue in position 450 or with a 5-position tert-butyl group combined with an isothiazol ring as in (S)-thio-ATPA.

The Y450A Mutation Renders AMPA Receptors Highly Selective for Kainate—The Y450A mutation exhibits a dramatic effect on the potency for both (S)-glutamate and kainate, decreasing the EC_{50} for kainate to low micromolar levels. The potency for (S)-glutamate is 23-fold higher than kainate on GluR2o, but kainate becomes 5500-fold more potent than (S)-glutamate on GluR2o(Y450A). (S)-Glutamate applied at 30 mM (a 10,000-fold higher concentration than the EC_{50} for kainate) evoked a current that was 22% of the maximal kainate current on GluR2o(Y450A). Similarly, the agonists AMPA and ATPA activated currents less than 3% of the maximal kainate current when applied to GluR2o(Y450A) at concentrations more than 300-fold higher than the EC_{50} for kainate, suggesting that kainate might be considered a selective agonist on AMPA receptors containing the Y450A mutation. Similar reductions in agonist potencies are observed for the N-methyl-D-aspartic acid receptors, where mutation of the equivalent phenylalanine to alanine in NR1 reduces the potency of glycine 6300-fold (34). The equivalent position encodes a His in NR2, and a mutation to Ala in NR2A reduces (S)-glutamate potency 220-fold (35), suggesting that an alanine at that position dramatically changes the structure of the binding site.

Kainate as a Desensitizing Agonist—Interestingly, when Tyr-450 is substituted with the smaller amino acid, alanine, kainate elicits a
strongly desensitizing current (Fig. 5A, TABLE TWO). It has been suggested by some researchers that kainate can induce a very fast desensitizing response (32); however, whether it is the same molecular mechanisms, which underlie the potential fast kainate-induced desensitization mechanisms, which underlie the potential fast kainate-induced desensitization and the desensitization observed for other agonists, remains unclear. Unfortunately, the inability to co-crystallize kainate with GluR2-S1S2J(Y450A) hampers a detailed structural understanding, although the increased potency and the desensitization suggest that kainate might stabilize a tighter domain closure than for the tryptophan mutant, i.e. >15°. Alternatively, the geometry of the ligand binding site can accommodate a transition into a desensitized state not achievable for the tryptophan mutant.

| Interactions of the ligand binding core of GluR2-S1S2J(Y450W) and wild-type GluR2-S1S2J, both in complex with kainate |
|--------------------------------------------------|
| Potential hydrogen bonds/ionics interactions (in Å) to ligand within 3.4 Å are tabulated. Wat1 makes further hydrogen bonds to Glu705N, Wat2 to Leu703O and Wat3 to Ser652O, Thr655N, and Lys656N (distances not listed). |

Y450W mutant | Wild type |
|--------------|-----------|
| O atom of kainate | 2.8 | 3.0 |
| Ser-654N | 2.9 | 3.0 |
| Ser-654O | 2.8 | 2.6 |

N atom of kainate

Pro-478O | 2.8 | 2.8 |
Thr-480Oy | 3.3 | 3.3 |
Glu-705Oy | 3.0 | 3.1 |

O61 atom of kainate

Thr-655Oy | 2.6 | 2.6 |
Wat1 | 2.7 | 2.6 |
Wat2 | 3.2 | 3.3 |

O62 atom of kainate

Ser-654N | 3.1 | 3.0 |
Thr-655N | 2.9 | 3.1 |
Thr-655Oy | 3.3 | |
Wat3 | 2.8 | 2.9 |

*Wild-type GluR2-S1S2J was crystallized in complex with kainate by Armstrong and Gouaux (6).

The structure of kainate is shown in Fig. 1B.

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