Disruption of Interdomain Interactions in the Glutamate Binding Pocket Affects Differentially Agonist Affinity and Efficacy of N-methyl-D-aspartate Receptor Activation

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In ionotropic glutamate receptors, agonist binding occurs in a conserved clam shell-like domain composed of the two lobes D1 and D2. Docking of glutamate into the binding cleft promotes rotation in the hinge region of the two lobes, resulting in closure of the binding pocket, which is thought to represent a prerequisite for channel gating. Here, we disrupted D1D2 interlobe interactions in the NR2A subunit of N-methyl-D-aspartate (NMDA) receptors through systematic mutation of individual residues and studied the influence on the activation kinetics of currents from NR1/NR2 NMDA receptors heterologously expressed in HEK cells. We show that the mutations affect differentially glutamate binding and channel gating, depending on their location within the binding domain, mainly by altering $k_{eff}$ and $k_{on}$, respectively. Whereas impaired stability of glutamate in its binding site is the only effect of mutations on one side of the ligand binding pocket, close to the hinge region, alterations in gating are the predominant consequence of mutations on the opposite side, at the entrance of the binding pocket. A mutation increasing D1D2 interaction at the entrance of the pocket resulted in an NMDA receptor with an increased open probability as demonstrated by single channel and whole cell kinetic analysis. Thus, the results indicate that agonist-induced binding domain closure is itself a complex process, certain aspects of which are coupled either to binding or to gating. Specifically, we propose that late steps of domain closure, in kinetic terms, represent part of channel gating.

Ionotropic glutamate receptors (iGluRs) are the major mediators of excitatory neurotransmission in the brain and play important roles both in neuronal development and synaptic function. They are tetrameric protein complexes in which each subunit is composed of (i) an extracellular amino-terminal domain, (ii) a ligand binding domain (LBD), sharing sequence homology to bacterial periplasmic binding proteins, (iii) a membrane-associated domain with the transmembrane helices M1–M4 that forms the ion pore, and (iv) an intracellular carboxyl-terminal domain.

The family of iGluRs is divided into the subclasses of AMPA, kainate, and NMDA receptors that differ, among other things, in their ligand specificities. Despite the pharmacological differences, crystal structures have been obtained for LBDs of subunits from all three subclasses of iGluRs and have revealed a conserved clam shell-like architecture of two lobes (D1 and D2) around a central cleft that harbors the ligand binding site. Lobe D1 is formed from residues preceding the first transmembrane domain (M1) and the C-terminal residues of the extracellular loop between transmembrane domains 3 and 4, whereas the N-terminal part of this loop forms lobe D2.

LBD structures in complex with different ligands have shed light on the molecular mechanism of ligand binding and, in the absence of structural information on the transmembrane domain, have led to the formulation of hypotheses on how agonist binding may be coupled to channel gating. Agonists differ from antagonists in that they promote closure of the LBD clam shell through rotation of lobe D2 toward D1. In a largely mechanical model of receptor activation, this motion is thought to generate conformational strain within the tetrameric receptor complex that transduces to the membrane regions and, eventually, causes channel opening. Several studies in AMPA and kainate iGluRs relating structure to agonist efficacy show that the degree of domain closure induced by different agonists correlates with their functional activity. Interestingly, there is evidence that the glycine binding domain of the NR1 subunit of the NMDA receptor does not exhibit a similar correlation between domain closure and agonist efficacy. Recent structure-function data show that engineering sterical restrictions within the glutamate-binding pocket of the NR2B subunit of the NMDA receptor is correlated to the degree of agonist efficacy, implicating that the action of glutamate and glycine on the NR2 and NR1 subunits, respectively, may induce different structural mechanisms underlying agonist efficacy.

However, although crystallographic data have uncovered the difference between agonist- and antagonist-bound LBD struc-
Role of Binding Domain Residues in NMDA Receptor Activation

tures, kinetic studies are essential to elucidate the sequence and timing of conformational changes following agonist binding and their relationship to gating. Recently, one such study was performed for GluR2 AMPA receptors and has demonstrated that destabilizing the closed conformation of the binding domain through single amino acid mutations at the D1D2 interface reduces receptor open probability (14). Kinetically, the results place LBD closure as an intermediate step of receptor activation between ligand binding and channel opening, with domain closure being a prerequisite for channel opening but kinetically separate from it. At the same time, domain closure also contributes to apparent ligand affinity, because only the open state of the binding domain is permissive for dissociation of the ligand. The kinetic scheme proposed by Robert et al. (14), however, is derived from the effects of mutations at only one position in the binding domain. It is, therefore, based on the assumption that binding domain closure is itself a concerted step that can be monitored accurately by looking at single positions along the D1D2 interface. Here, we provide a more systematic exploration of the contributions of interdomain contacts in an iGluR LBD to ligand binding and channel gating. To this end, we introduced mutations into the NR2A subunit of NMDA receptors, targeting several D1D2 interactions predicted to stabilize the closed conformation of the binding domain and determined the kinetics of ligand binding and channel opening of the resulting receptors expressed in HEK293 cells. Our study reveals differential kinetic effects of the mutations depending on their location in the binding domain, arguing for the existence of distinct kinetic steps in the conformational rearrangements necessary for binding domain closure.

EXPERIMENTAL PROCEDURES

In Vitro Mutagenesis—Single amino acid mutations of the NR2A subunit were introduced into the pCis WT vector (15) with the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. All mutations were verified by partial DNA sequencing.

Cell Culture and Transfection—HEK293 cells (ATCC CRL 1573) were cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. For transfection, cells were plated onto fibronectin-coated glass coverslips. After 24 h, cells were cotransfected with green fluorescent protein (in the pGreenLantern vector (Invitrogen)) and the NMDA receptor subunits NR1 and NR2A (both in the pCis vector) using the Effectene transfection kit (Qiagen) and a DNA ratio of 1:10:30 for subunits NR1 and NR2A (both in the pCis vector) using the Effectene transfection kit (Qiagen) and a DNA ratio of 1:10:30 (green fluorescent protein/NR1/NR2A). Transfected cells were incubated in the presence of 2 μM 3-(2-carboxyethyl)-4,6-dichloro-1H-indole-2-carboxylic acid and 200 μM to 1 mM D-2-amino phosphonovalerate (both from Tocris) for 24–48 h before electrophysiological recordings.

Flash Photolysis and Whole Cell Recording—Flash photolysis was used for fast application of glutamate in whole cell recording experiments, with NI- and MNI-caged glutamate (from Sigma and Tocris, respectively) as photolabile precursors. Both compounds are suitable for studies on NMDA receptors (16, 17), with MNI-caged amino acids being characterized by higher photorelease efficiency compared with the NI-based precursors (18). MNI-caged glutamate, therefore, was used preferentially with low affinity mutant receptors.

The setup for solution exchange and photorelease of NMDA receptor agonists was as described previously (17). Briefly, it consisted of a quartz tube (350-μm diameter) from which solutions emerged with a speed of 5–10 mm/s and a quartz fiber (365-μm diameter) positioned perpendicular to it that served as the laser light guide. For whole cell recordings, cells were lifted from the coverslip and positioned between solution outlet and optical fiber. This configuration allowed complete solution exchange around the cell within 100–200 ms and optional photorelease of l-glutamate from its caged precursors through a light flash (345 nm, 15 ns) from an excimer laser-pumped dye laser (Lambda Physik). The amount of ligand released in photolysis experiments was controlled by attenuating the laser light with neutral density filters. Typical light intensities applied to cells were in the range of 50–400 mJ/cm2. In this range, the concentration of released glutamate is a nearly linear function of light intensity, and absolute concentrations can be calculated after initial calibration as described (17). To determine EC50 values of WT and mutant NMDA receptors, dose–response curves of glutamate-induced currents were analyzed in independent experiments by a rapid solution exchange application system in the presence of 50 μM glycine as described previously (19).

All whole cell recordings were done at a holding potential of −60 mV in extracellular solution containing 140 mM NaCl, 5 mM KCl, 0.85 mM CaCl2, and 10 mM HEPES at pH 7.4. Recording pipettes had resistances of 1.5–3 MΩ and were filled with intracellular solution composed of 145 mM KCl, 10 mM EGTA, and 10 mM HEPES at pH 7.4. Data were low pass–filtered at 10 kHz and digitized at a sampling rate of 12 kHz.

Single Channel Recording—Single channel data for WT NR1/NR2A and NR1/NR2A(N668D) receptors transiently expressed in HEK293 cells were obtained from patches in the inside-out configuration as described previously (19). The membrane potential was clamped at −100 mV. The bath/internal solution in these experiments contained 141 mM potassium gluconate, 2.5 mM NaCl, 11 mM EGTA, and 10 mM HEPES at pH 7.4. The pipette/external solution contained 125 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 0.85 mM CaCl2, and 20 mM HEPES also at pH 7.4 and was supplemented with the agonists l-glutamate and glycine at concentrations of 100 mM and 20 μM, respectively. Data were filtered at 10 kHz, digitized at 48 kHz, and stored on digital audio tape (DTR 1204; Biologic, Clay, France). For analysis, data were replayed from tape and sampled at 10 kHz. Tables of channel opening and closing events were generated with the TAC software (Instrutech). Open and shut time distributions were calculated using the method of maximum likelihood and the pStat program of the pclamp 6.0 package (Axon Instruments).

Data Analysis and Statistics—Photorelease of ligands from NI-caged precursors occurs on a time scale of microseconds or below (τ= 150 ns for an NI acetate model compound (20)). This is orders of magnitude faster than activation kinetics with millisecond time constants reported for NMDA receptors (e.g., see Refs. 21 and 22). Consequently, the time course of currents in our flash photolysis experiments is independent of the rate of ligand release and instead reflects intrinsic receptor kinetics. To interpret the observed kinetics in terms of rate constants of
distinct steps of the activation process, we used the kinetic model shown in Scheme 1.

\[
\begin{align*}
2k_{\text{on}} & \quad \text{R} + 2L \rightarrow \text{RL} + \text{L} \\
& \quad \text{RL} \rightarrow \text{RL}_2 \\
& \quad k_{\text{off}} \quad 2k_{\text{eff}} \\
k_{\text{on}} & \quad \text{RL}_2 \rightarrow \text{RL}_2 \\
& \quad \text{RL}_2 \rightarrow \text{RL}_2 \\
& \quad k_{\text{op}} \\
& \quad k_{\text{cl}} \\
\end{align*}
\]

SCHEME 1

Here, R denotes the receptor, and L denotes the ligand that is photoreleased during the course of the experiment. \( k_{\text{on}} \) and \( k_{\text{off}} \) are the rate constants for ligand binding and dissociation, and \( k_{\text{op}} \) and \( k_{\text{cl}} \) are those for channel opening and closing. The model takes into account that two molecules of glutamate have to bind to the NMDA receptor. It assumes that their binding sites are equal (i.e. that they can be occupied and freed in any order, and, for simplicity, that they are independent of each other (i.e. there is no cooperativity between them)). Because all flash photolysis experiments were done in the presence of saturating concentrations of the co-agonist glycine, steps for glycine binding could be omitted from the kinetic scheme, assuming that the glycine binding sites are always occupied, independent of the glutamate concentration. In order to determine kinetic properties of WT and mutant receptors, the model was translated into a Berkeley Madonna (Macey and Oster) equation file. The software was used to run a least squares fit procedure with a Levenberg-Marquardt minimization algorithm and the four rate constants in the model as free parameters to give the best match between sets of experimentally observed currents at different ligand concentrations and theoretical currents predicted by the model. Theoretical currents were calculated for each iteration of the fitting routine with a numerical integration method (4th order Runge-Kutta, 100-μs time interval), using the current values of the rate constants. From the rate constants, the dissociation constant and open probability were calculated as \( K_D = k_{\text{off}}/k_{\text{on}} \) and \( P_o = k_{\text{op}}/(k_{\text{op}} + k_{\text{cl}}) \).

We used numerical integration of the differential equations, because analytical integration of the full set of differential equations by using preequilibrium simplification methods was not possible in this system. The reason for this was that the high affinity of the NMDA receptor for glutamate results in a small ligand dissociation rate constant, \( k_{\text{off}} \), which is in the same range as \( k_{\text{on}} \) and \( k_{\text{op}} \). Thus, it cannot be assumed that the ligand binding equilibrium is fast in comparison with the channel-opening equilibrium, a simplification that has been used in the past for analyzing the kinetics of other ligand-gated ion channels with lower affinities for the ligand (e.g. see Ref. 23).

Desensitization was omitted from the simplified kinetic scheme used for determination of the channel opening rates. This simplification is justified, because desensitization in NMDA receptors is 100–1000-fold slower than channel activation, as shown in supplemental Table 1. Furthermore, the rate of desensitization is not significantly changed by the mutations studied here, except by T494N (2-fold larger desensitization time constant). Therefore, a maximum of 6% of receptors desensitize during the time window of the current activation used for analysis of the channel opening kinetics.

Rate constants were determined, and \( K_D \) and \( P_o \) were calculated independently for each cell recorded from. Values are presented here as means ± S.E. One-way analysis of variance with a Tukey-Kramer post-test was used to test for differences between rate constants of WT and mutant receptors after logarithmic transformation. Differences were considered significant at \( p < 0.05 \), highly significant at \( p < 0.01 \), and extremely significant at \( p < 0.001 \).

**Molecular Modeling and Prediction of Effects of Mutations**

Since this work was carried out before publication of the crystal structure of the ligand binding domain of the NR2A subunit (9), candidate residues for mutagenesis were chosen based on a modeled structure. This model of the NR2A LBD with bound L-glutamate was generated based on the published (10) crystal structure of the GluR2 binding domain (Protein Data Bank entry 1FTJ) using the Sybyl 6.9 software (Tripos Associates) with residue numbering as in Ref. 24. The modeling procedure was analogous to that described previously for the binding domain of NR2B (25). Together with models of the NR2B binding domain with and without glutamate, this model was used to identify amino acid residues that may contribute to binding domain closure and that, therefore, represent candidate residues for mutagenesis and kinetic analysis of the resulting mutated receptors. Specifically, we were looking for residues that are involved in energetically favorable interactions between D1 and D2 across the binding cleft that are stronger in the ligand-bound than in the apo state. Such residues should help in stabilizing the closed conformation of the binding domain, and their mutation should change the equilibrium between the open and closed state of the binding domain. If binding domain closure is a prerequisite for channel gating, we hypothesized that a strong interaction between such residues might also change the equilibrium between the open and closed states of the ion channel. Although model-based, all of our predicted D1D2 interactions were later shown to be essentially correct with the advent of the crystal structure of the NR2A LBD (Protein Data Bank entry 2A5S).

To evaluate the effects of mutations, model structures of some of the mutated binding domains were obtained from the NR2A WT model with bound glutamate by manual exchange of the respective residues and subsequent dynamization and minimization of the structures. Differing from Ref. 25, dynamization was run for 10,000 fs (with 1-fs intervals) at low simulated temperature (20 K, 20-fs coupling factor) on the core of the binding domain (defined by an 8-Å radius around Glu\(^{394}\), Lys\(^{465}\), Asn\(^{668}\), and Asp\(^{712}\) and including about a quarter of total atoms in the model). In this way, overall structure remained largely preserved, whereas regions around mutations were allowed to settle into relatively unstrained conformations.

In addition, published interaction propensity values (26) were used to estimate the impact of mutations on the strength of the corresponding D1D2 interaction. These values are based on a set of 1073 known protein structures and a distance cut-off of 4.5 Å used to define an interaction.

**RESULTS**

**Interactions across the NR2A D1D2 Interface That Stabilize the Closed Conformation of the Binding Domain**

From our NR2 binding domain models, we could identify two main regions in which agonist-induced D1D2 interactions are...
Role of Binding Domain Residues in NMDA Receptor Activation

FIGURE 1. Amino acid residues involved in glutamate-induced cross-lobe interactions in the NR1/NR2A binding domain. A, schematic illustration of the location of the mutated residues (yellow balls) and their proposed interaction partners (gray balls) in the binding domain (modeled structure in the closed conformation with S1 in blue and S2 in green). B, sequence comparison between NMDA receptor subunits NR2A, NR2B, and NR1 and the AMPA receptor subunit GluR2. Mutated residues are indicated by their numbers from A, and their interaction partners are indicated by asterisks. C and D, detailed views of the cross-lobe interaction regions 1 and 2, respectively, showing the orientation of the residues highlighted in A relative to each other and to glutamate in the binding site.

formed. These are located on opposite sides of the binding pocket, facing the ammonium group or the two carboxyl groups of the agonist, respectively. The first region comprises residues Glu394 and Thr494 in D1 (yellow balls 1 and 4 in Fig. 1A) and Tyr711 and Asp712 in D2 (gray balls) of NR2A. Residues that contribute to the domain interactions in the second region are Lys465 in D1 and Asn668 in D2 (yellow balls 2 and 5 in Fig. 1A). Our model and the now published crystal structure of the NR2A LBD agree in that they both suggest differential roles of the agonist in the formation of the interactions in these two regions. Domain interactions in the first region directly involve the bound agonist. The ammonium group of L-glutamate, for example, interacts with Thr494, which, in turn, forms a hydrogen bond with Asp712. Via a water molecule (present in the crystal structure), the agonist also interacts with Glu712, which itself contacts Tyr711. Thus, L-glutamate brings together the D1/D2 interaction partners Glu394/Tyr711 and Thr494/Asp712 in a complex network of hydrogen bonds (Fig. 1C). In contrast, the agonist does not form direct contacts to any of the interacting residues in the second region. Two prominent interactions in this region are single hydrogen bonds between Lys465 and Asn668 in D2 (Fig. 1D) and between Lys465 and Glu672 (not shown). Glu672 is located within helix F of the binding domain, and Asn668 is located immediately N-terminal of it. This helix carries two residues, Ser670 and Thr672, that are critical for binding the carboxyl groups of L-glutamate (24) and moves mutation, H466A, that we predicted to impair ligand binding. The imidazole ring system of His466 provides a hydrophobic environment for the glutamate backbone, which should contribute significantly to stability of the ligand-receptor complex (Fig. 1D). For the NR2B subunit of NMDA receptors, a role for the homologous histidine residue, His488, in ligand binding, but not in gating, is suggested by the finding that its mutation to alanine increases the EC50 of the resulting receptors for glutamate by more than 400-fold (13) but does not affect the efficacy of the partial agonist NMDA relative to L-glutamate (13).

Kinetic Effects of the Mutations—For a first characterization of the NR2A mutants, we determined glutamate dose-response curves and calculated the respective EC50 values and Hill coefficients (nH) by using a conventional capillary application system. Of the seven substitutions introduced, six significantly affected the EC50 value of glutamate (supplemental Table 1). The respective Hill coefficients were in the range of 1.02–1.35 (i.e. not different from the value obtained with the WT protein) (nH = 1.27 ± 0.19; supplemental Table 1). Together these data corroborate earlier reports showing that mutations introduced at the D1D2 interface of iGluRs affect apparent glutamate affinity (13, 14, 27, 28). To assess the effects of the mutations on receptor activation, rate constants for glutamate binding and dissociation as well as for channel opening and closing of the mutant receptors were compared with the corresponding values of WT receptors. Using our whole cell approach and kinetic
Role of Binding Domain Residues in NMDA Receptor Activation

A

B

FIGURE 2. Descriptive quality of the kinetic model. A, example whole cell currents (solid lines) elicited from one cell by photorelease of three different concentrations of L-glutamate from its caged precursor. B, magnification of the first 20 ms of traces in A. Superimposed on the traces in A and B are the currents (dashed lines) predicted for the same concentrations by the kinetic model and the parameter values determined for this cell. Predicted currents describe adequately only the rising phases of currents, because neither desensitization nor decrease in agonist concentration during the course of recordings is accounted for by the kinetic model used.

model of receptor activation, we found the following values for the WT parameters ($n = 8$ cells): $k_{on} = 5 \times 10^7 \pm 0.6 \times 10^7 \text{M}^{-1} \text{s}^{-1}$, $k_{off} = 160 \pm 30 \text{s}^{-1}$, $k_{op} = 380 \pm 70 \text{s}^{-1}$, $k_{cl} = 250 \pm 40 \text{s}^{-1}$. From these parameters, the open probability $P_O$ can be calculated as $0.59 \pm 0.08$, and the dissociation constant $K_D$ can be calculated as $3.3 \pm 0.5 \mu M$. These values and our relatively simple kinetic model are sufficient to describe adequately the lag and the rising phases of receptor currents induced by a range of agonist concentrations in our experiments (Fig. 2). The value determined here for $P_O$ is in reasonably good agreement with that recently reported for NR1/NR2A receptors (29), despite the different kinetic models used for analysis.

Kinetic parameters for ligand binding and channel activation of the seven mutant receptors were determined in the same way as those of WT receptors. The results are compared in Table 1 and reveal quite different effects on receptor kinetics of the changes introduced into the two regions of the D1D2 interface. This becomes obvious when effects on binding and gating are compared separately (Fig. 3). Of the seven mutations, five affect the binding step of receptor activation (Fig. 3A); however, they do so to very different extents. In line with our expectations, the most drastic shift in binding rate, dissociation rate, and $K_D$ is seen with mutation H466A, reflecting the important contribution of histidine 466 to stabilization of glutamate in its binding site. According to the results, glutamate is highly destabilized in its binding pocket in the H466A mutant receptor, resulting in a 7-fold decrease in its residency time on the receptor. Highly significant effects on binding step parameters are also produced by mutations E394A, K465E, and N668K, whereas no significant changes in the binding equilibrium could be observed with the two more conservative mutations K465N and N668D. These results indicate that the contributions of residues 465 and 668 to affinity for glutamate are minor. Finally, a small increase in $K_D$ can be seen for the T494N mutation. Although the effect does not reach statistical significance, this is in agreement with findings for a mutation of the homologous residue Thr$^{408}$ to alanine in NR2B, which causes a more than 500-fold increase in $EC_{50}$ (13). Given the very conservative nature of the mutation to asparagine, which should alter interactions in the binding pocket only marginally (not shown, according to modeling), the result supports a critical role of threonine 494 for glutamate affinity of NMDA receptors. In fact, we had decided to introduce only a slight perturbation at this position, because the observation with NR2B had raised the possibility that, otherwise, we might not be able to study the resulting low affinity receptors with our approach, in which the range of glutamate concentrations that can be used is limited by the efficiency of photorelease from the caged precursor and its solubility in the recording solution.

In contrast to binding, gating is not significantly influenced by most of our mutations with the remarkable exception of N668D (Table 1, Fig. 3B). Gating parameters of WT receptors were reproduced best in E394A and T494N mutant receptors, and thus we could not find any indication of a contribution of the two residues located in region 1 to the channel activation step. This is in contrast to our findings for the residues of region 2. Here, N668D shows a dramatic and extremely significant decrease in the channel closing rate (10-fold compared with WT) (Table 1). As explained above, we expected residues 465 and 668 in WT receptors to form a hydrogen bond across the D1D2 interface in the ligand-bound state. Although, except for N668D, none of the mutations at these positions produced a statistically significant change in gating parameters in analysis of variance, we observed an apparent correlation between the predicted effects of our mutations on the 465-668 interaction and their influence on gating in the kinetic analysis. Substitution of asparagine 668 with a charged aspartate in N668D should increase the strength of the interaction with lysine 465, and this mutation strongly increases the $k_{op}/k_{cl}$ ratio as a measure of gating efficiency. In contrast, replacing Asn$^{668}$ with lysine results in two positively charged lysine side chains facing each other at the D1D2 interface, which should be an extremely unfavorable situation. In terms of gating, we find that, of the four mutations that directly affect position 465 or 668, N668K...
Role of Binding Domain Residues in NMDA Receptor Activation

TABLE 1

Kinetic parameters for WT and mutant NR1/NR2A receptors

|        | n  |  \( k_{on} \) |  \( k_{off} \) | \( K_D \) |  \( k_{op} \) |  \( k_{cl} \) | \( k_{op}/k_{cl} \) |  \( P_o \) |
|--------|----|--------------|---------------|----------|--------------|------------|----------------|--------|
| WT     | 8  | 50 \( \pm \) 6 | 160 \( \pm \) 30 | 3.3 \( \pm \) 0.5 | 380 \( \pm \) 70 | 250 \( \pm \) 40 | 2.0 \( \pm \) 0.5 | 0.59 \( \pm \) 0.08 |
| E394A  | 3  | 20 \( \pm \) 3* | 660 \( \pm \) 220* | 40 \( \pm \) 20* | 370 \( \pm \) 200 | 290 \( \pm \) 40 | 1.2 \( \pm \) 0.7 | 0.49 \( \pm \) 0.12 |
| K465E  | 4  | 15 \( \pm \) 6* | 280 \( \pm \) 20 | 30 \( \pm \) 8* | 540 \( \pm \) 140 | 500 \( \pm \) 200 | 1.6 \( \pm \) 0.7 | 0.53 \( \pm \) 0.10 |
| K465N  | 4  | 30 \( \pm \) 1 | 130 \( \pm \) 20 | 4.7 \( \pm \) 0.8 | 350 \( \pm \) 80 | 120 \( \pm \) 30 | 3.3 \( \pm \) 0.9 | 0.70 \( \pm \) 0.10 |
| H466A  | 3  | 8 \( \pm \) 2b | 1100 \( \pm \) 400b | 140 \( \pm \) 30b | 220 \( \pm \) 70 | 550 \( \pm \) 150 | 0.5 \( \pm \) 0.2 | 0.31 \( \pm \) 0.11 |
| T494N  | 3  | 35 \( \pm \) 5 | 220 \( \pm \) 30 | 6.2 \( \pm \) 1.0 | 330 \( \pm \) 40 | 170 \( \pm \) 20 | 2.0 \( \pm \) 0.1 | 0.67 \( \pm \) 0.01 |
| N668K  | 5  | 25 \( \pm \) 5* | 330 \( \pm \) 40* | 14 \( \pm \) 2* | 180 \( \pm \) 70 | 210 \( \pm \) 8 | 0.8 \( \pm \) 0.3 | 0.40 \( \pm \) 0.08 |
| N668D  | 5  | 30 \( \pm \) 5 | 80 \( \pm \) 20 | 2.4 \( \pm \) 0.5 | 190 \( \pm \) 40 | 25 \( \pm \) 6b | 10.9 \( \pm \) 4.3b | 0.87 \( \pm \) 0.04 |

* \( p < 0.05. \)
* \( p < 0.001. \)
* \( p < 0.001. \)

produced the lowest \( k_{op}/k_{cl} \) ratio. The effects on the 465–668 interaction of the mutations of Lys\(^{465} \) to either glutamate or asparagine should be of intermediate strength, and accordingly, the gating efficiency of the corresponding receptors resembles that of WT receptors more closely. In an attempt to determine more rigorously the extent of this potential correlation, we used a logarithmic plot of \( k_{on}/k_{on} \) and of \( k_{off}/k_{off} \) and consequently the values of \( k_{on} \) and \( P_o \) are constant and equal to WT receptors along the solid lines in A and B, respectively. Data points above these lines thus correspond to receptors with decreased affinity/open probability compared with WT, whereas those below it indicate receptors that have higher affinity/open probability than WT. Error bars represent S.E. values, and the S.E. for the lines of equal affinity and open probability is indicated by dashed lines.

Finally, it should be noted that, unexpectedly, substitution H466A also seems to have an impact on gating properties, although this effect does not reach statistical significance with the limited sample size for that mutation. Our binding domain models do not provide an obvious explanation for this finding other than that the mutation introduces a radical change into the glutamate binding pocket. Besides reducing affinity, this may also affect the exact orientation of the ligand in its binding site, which, in turn, may disturb the formation of other interactions important for gating, such as between the \( \gamma \)-carboxyl group and helix F. In any case, however, it is clear that the effect of H466A on gating is small compared with its very pronounced effect on affinity.

Comparison of WT NR1/NR2A and NR1/NR2A(N668D) Receptors at the Single Channel Level—Although the emphasis of this study was on comparison of the kinetic characteristics of mutated and WT receptors on the whole cell level, the unique properties conferred by mutation N668D (i.e. a gain of function with respect to gating efficiency) prompted us to verify this result by single channel analysis as an independent method. Also, among the mutations studied here, the N668D substitu-
Role of Binding Domain Residues in NMDA Receptor Activation

FIGURE 4. Correlation of interaction propensity of residues 465 and 668 with glutamate gating efficacy in WT and four mutant receptors. Propensity values of the residue pairs (indicated in parentheses) at positions 465 and 668 are plotted against the observed $k_{\text{op}}/k_{\text{cl}}$ ratio ± S.E. of the corresponding receptors. The dashed line represents the best linear fit to the data.

allow us to give an experimentally supported explanation for the apparent existence of the two classes of mutant receptors. An intriguing finding is the similarity between the kinetic characteristics of the two classes and those reported for WT NR1/NR2A receptors in the so-called H- and L-modes of gating (31). However, the H-mode of gating is normally not observed in the absence of Mg$^{2+}$ chelators in the extracellular recording solution, and switching between modes should occur in individual receptors (31), although there is a chance that we have missed such events due to relatively brief recording periods of around 1–5 min in our experiments.

DISCUSSION

To elucidate the mechanisms behind the coupling of ligand binding and gating in receptors of the iGluR family, several previous studies have used isolated ligand binding domains recovered as soluble proteins after bacterial expression (10–12, 32, 33), and structural and functional data for these LBD constructs have identified key residues for ligand binding as well as for interactions between LBD part-domains D1 and D2. For iGluRs of the AMPA receptor subfamily, the degree of agonist-mediated closure of the clam shell-like binding domain has been found to be correlated with agonist efficacy (10, 11), and the contributions of several amino acid residues to ligand binding and domain closure in an isolated LBD have been studied (32). More recently, the first kinetic study on a full-length AMPA receptor was carried out to evaluate the contribution of a specific D1D2 interaction to agonist binding and gating (14). Our study, however, is the first to investigate systematically such contributions for several interactions across the D1D2 interface.

Specifically, we investigated the NR1/NR2A subunit combination of NMDA receptors and quantified rate constants for binding and gating steps based on whole cell recordings of currents in response to rapid application of L-glutamate. Theoretically, analysis of equilibrium dose-response relationships should provide similar quantitative information on underlying binding and gating parameters (34). However, the practical quality of such dose-response data is in most cases not adequate to separate the binding and gating effects of a receptor mutation, since changes in gating efficacy are assumed to be mirrored only in small changes of the respective Hill coefficient (34). Therefore, we chose to activate the receptors by jumping the glutamate concentration, because following the subsequent channel opening in real time and as a function of the glutamate concentration allows the direct and independent determination of the rate constants associated with the glutamate binding and the channel opening processes. In choosing a kinetic model for fitting experimental currents, we gave preference to a simple model, including only ligand binding and gating steps, in which all rate constants can be determined from whole cell recordings, over more complex ones that describe more adequately certain aspects of NMDA receptor activity at the single channel level but would require fixing of some of their parameters to estimated values. It is, therefore, an important question whether our simplified model can provide meaningful information about the binding and gating steps underlying real NMDA receptor activation in our experimental system. There are three
arguments that make us confident that this is the case. First, quality of fits to our experimental data were generally very good (see Fig. 2) for all agonist concentrations at all receptor variants investigated, indicating that, although simple, our model still provides an adequate description of whole cell data. Also the quality of fits was not improved by using more complex models for receptor activation, such as the one proposed in (35). Due to the lack of desensitization steps in the kinetic scheme, it is capable of describing adequately only the rising phase of current responses. However, since desensitization compared with channel opening is several orders of magnitude slower in wild type NMDA receptors, as well as in the mutant receptors studied here (supplemental Table 1), and because its mechanism is not a focus of this study, this does not compromise our results. Second, the parameter values that we determined for WT NR1/NR2A receptors are comparable with published ones (29) that were obtained from a more sophisticated activation scheme derived from single channel analysis and initially proposed by Banke and Traynelis (35). This well accepted model assumes that the NR1 and NR2 subunits undergo independent conformational changes before gating of the receptor. The channel opening rate determined from our model should correspond approximately to $k_{op}$, the forward reaction rate for the conformational change undergone by the NR2 subunit, which is rate-limiting for channel opening in the Banke and Traynelis model. The values are as follows: $k_{op} = 380 \pm 70 \text{ s}^{-1}$ and $k_{op} = 230 \pm 26 \text{ s}^{-1}$, which is in reasonably good agreement, given the complexity of the fitting procedures involved. Likewise, our value for the channel closing rate ($250 \pm 40 \text{ s}^{-1}$) is similar to the sum ($352 \text{ s}^{-1}$) of $k_{f-}$ and $k_{c-}$, the combined backward reaction rates for the confor-

TABLE 2

Comparison of single-channel shut and open times of WT and N668D receptors

|          | WT     | N668D (class I) | N668D (class II) | Literature data$^a$ |
|----------|--------|-----------------|------------------|---------------------|
| Shut times |        |                 |                  |                     |
| $\tau_1$ ($\mu$s) | NM$^b$ | $0.68 \pm 0.31$ | $0.45 \pm 0.09$ | $38.9 \pm 4.4$ |
| $\tau_2$ ($\mu$s) | $8.4 \pm 3.4$ | $8.9 \pm 6.4$ | $4.3 \pm 0.5$ | $0.54 \pm 0.04$ |
| Shutoff $\tau$ ($\mu$s) | $80.4 \pm 5.5$ | $47.0 \pm 14.0$ | $394 \pm 59$ |
| Open time |        |                 |                  |                     |
| $\tau$ ($ms$) | $2.17 \pm 0.14$ | $2.15 \pm 0.08$ | $53.9 \pm 27.2$ | $2.23 \pm 0.06$ |

$^a$ Data for WT NR1/NR2A receptors expressed in HEK cells taken from Ref. 30.

$^b$ NM, not measurable due to the time resolution of recordings.

$^c$ $\tau_1$ and $\tau_2$ are dependent on ligand concentration and not directly comparable between our recordings, for which we used 100 nM glutamate, 20 \mu M glycine, and those in Ref. 30, for which 300 nM glutamate and 10 \mu M glycine were used.

$^d$ Not determined.

$^e p < 0.05$. 

FIGURE 5. Single channel comparison of NR2A(N668D) and WT receptors. A, representative stretches of recordings from inside-out patches of mutant class II (upper two traces) and WT (lower two traces) receptors. B, shut time and open time distributions for single patches of mutant class II (top row) and WT (bottom row) receptors. Note the different x-scale in the open time distribution.
Role of Binding Domain Residues in NMDA Receptor Activation

We show here that cross-lobe interactions in two regions on the D1D2 interface, specifically lysine 465 and asparagine 668, in channel gating. Experimental evidence for this is (i) the clear effect of mutation N668D on the channel closing rate, also supported by the single channel data, and (ii) the strong correlation between the predicted (based on propensity values) strength of interaction between residues 465 and 668 in different mutant receptors and the gating efficiency of these receptors as determined with our concentration jump/whole cell current recording approach. With a detailed structural model of the WT LBD already at hand, it may seem to be an obvious strategy to use molecular modeling to calculate directly the effect of mutations on the stability of the closed LBD instead of using the indirect measure of propensity values. However, when attempting the molecular modeling approach (for details, see “Experimental Procedures”), we found that, due to their exposed location at the surface of the binding domain, the side chains of residues 465 and 668 are only weakly constrained in the models, and their predicted orientation and energetic contribution, therefore, is largely dependent on the exact starting conditions of the simulation, which makes it difficult to obtain results that are unbiased by expectations. We conclude that this problem outweighs the uncertainties associated with the use of propensity values, the most serious of them being to try to draw conclusions about a specific interaction in a given protein, such as the NMDA receptor, from an average interaction propensity in a set of other proteins. It should be noted that the set of propensity values used here was based on a distance cut-off of 4.5 Å, which is a reasonable maximum distance to define interactions, considering the width of the cleft between domains D1 and D2 in the closed conformation of the LBD. In the region around positions 465 and 668, the backbone-backbone distance between D1 and D2 is about 5–6 Å, which should prevent the two side chains from adopting conformations in which they are separated by more than the cut-off distance while still being exposed to the cleft. Indeed, we never observed a larger 465–668 side chain distance in any of our models of the mutated binding domains (data not shown), even for the unfavorable lysine-lysine pairing forced by the N668K exchange.

We show here that cross-lobe interactions in two regions on opposite sides of the ligand binding pocket have very different effects on the kinetics of NMDA receptor activation. Of the residues investigated here, those that are involved in hinge region interactions (region 1; see Fig. 1C) are also interacting, either directly (Thr494) or indirectly via a water molecule (Glu394) with the agonist through its ammonium group, thus providing a direct explanation for the effect on ligand affinity of mutations in this region. Together with the absence of gating effects of these mutations, this result suggests that the formation of cross-lobe interactions near the hinge region is a process associated kinetically with ligand binding and separate from channel opening. Hinge region interactions may be necessary to create an optimal binding site for the ammonium group of glutamate and/or to lock the agonist into its binding site. This situation parallels that in AMPA receptors, for which it was recently shown that the effects of disrupting a cross-lobe interaction, that involves Glu102 (equivalent to Glu394 of NR2A) and Thr686 (not conserved in NR2A), can be explained as a result of destabilization of an additional agonist-bound closed state (14). This locked state differs from the initial state after ligand binding in that the agonist cannot dissociate from it and, thus, contributes significantly to the apparent affinity of AMPA receptors. Destabilization of the locked state by mutations is therefore seen as an increase in the ligand dissociation rate in a simplified kinetic model similar to the one used here (14). As pointed out in Ref. 14, the stability of the locked state, which is proposed to be the only state from which channel opening can occur, influences also the apparent open probability, although locking is kinetically distinct from gating. As one consequence of this, mutations that destabilize the locked state cause reduced efficacies of partial relative to full agonists. Although we have not performed any experiments with agonists other than glutamate in this study, previous work on NR2B (13) has demonstrated that mutation of Glu387 (homologous to Glu394 studied here) to alanine reduces efficacy of NMDA relative to glutamate (to 28% compared with 71% in WT). A reduction of relative efficacy (to 57%) was also seen with mutation of Tyr705 (equivalent to Tyr711 of NR2A) to alanine, and a smaller decrease, although not significant, was seen also for Thr488 (Thr494 in NR2A) to alanine. Combined with the data presented here, these results suggest that agonist-induced formation of D1D2 interactions near the hinge region may stabilize a hypothetical locked state in NMDA receptors as it does in AMPA receptors.

In contrast to the results obtained for region 1, our data for region 2 mutant receptors show that, in NR2A, formation of D1D2 interactions at the entrance of the binding pocket is kinetically coupled to gating and is of minor importance for ligand binding. Mutations at the homologous positions in NR2B (459 and 662), again, significantly decrease gating efficacy of NMDA relative to glutamate (13), but our kinetic analysis for NR2A attributes this to a direct effect on gating (i.e. changes in channel opening or closing rates). The residues corresponding to Lys465 and Asn668 are involved in D1D2 interactions also in NR1 (12), but the whole network of interactions on this side of the binding pocket is not conserved in AMPA receptor subunits. Interestingly, in AMPA receptors full and partial agonists have been shown to induce closure of the LBDs to different extents, but agonists of NR1 seem to cause full domain closure independent of their efficacy in triggering gating (12). This difference might be a consequence of the stronger D1D2 interactions in the vicinity of helix...
Role of Binding Domain Residues in NMDA Receptor Activation

F in NMDA receptors. The degree of LBD closure in AMPA receptors may be largely determined by interactions between agonist and residues of the binding domain. In NMDA receptors, however, such interactions might serve only to initiate movement of helix F, which then would “snap” into place, driven by the attractive forces between D1 and D2. In this model of NMDA receptor LBDs as molecular relays, the likelihood of switching to the closed conformation would be influenced by the nature of the agonist (i.e. its efficiency in inducing movement of helix F). In contrast, the likelihood of reopening would be determined by the network of interactions between D1 and D2. In agreement with this model, partial agonists of either NR1 or NR2 have been reported to shift components of single channel shut time (35, 37) but not open time histograms (i.e. to cause slower channel opening), whereas, complementarily, we find that a mutation, N668D, at the D1D2 interface of NR2A has no effect on channel opening but dramatically slows channel closing.

In summary, the data presented here suggest that agonist-induced activation of NMDA receptors proceeds through several kinetically distinct steps and that D1D2 interactions in opposing regions of the LBD with respect to the glutamate binding pocket contribute differentially to stability of the corresponding states.

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