

Mechanisms of Modal Activation of GluA3 Receptors^S

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ABSTRACT

AMPA receptors are the major excitatory neurotransmitter receptors in the central nervous system and are involved in numerous neurological disorders. An agonist-binding site is present in each of four subunits that form a functional channel. Binding consists of three steps: docking of agonist to the bilobed ligand binding domain (LBD), closure of the LBD, and increased stability of the closed-lobe conformation through interlobe hydrogen bonding. We describe GluA3 single channel currents activated by nitrowillardiine (NO₂W) and chlorowillardiine (CIW) in the presence of cyclothiazide, in conjunction with crystal structures of GluA2 and GluA3 LBDs bound to fluorowillardiine (FW), CIW, and NO₂W. When bound to NO₂W or CIW, the GluA3 channel opens to three conductance levels with comparable open probabilities and displays modal behavior

similar to that obtained with glutamate and FW as agonists (Poon et al., 2010). At lower concentrations, CIW evoked an alternate kinetic behavior, consisting of high open probability in lower conductance states. The structure of CIW bound to GluA3 LBD exhibits a unique partially open hydrogen bonding structure that may be associated with these alternative kinetics. NO₂W evoked longer open times than seen for other agonists in high and very high modes. The structure of GluA2 LBD bound to NO₂W exhibits fully closed lobes with additional interlobe interactions mediated by the nitro group. Beyond differences in efficacy between full and partial agonists, the complexities of the single channel behavior of AMPA receptors may also be associated with small interactions that modify the stability of various degrees of closure.

Introduction

AMPA receptors, a class of ionotropic glutamate receptors expressed in CNS neurons and glial cells, are involved in rapid excitatory synaptic transmission and plasticity (Derkach et al., 2007; Citri and Malenka, 2008) and in neurological diseases (Heath and Shaw, 2002; Hynd et al., 2004; Liu and Zukin, 2007). They are formed as tetrameric combinations of GluA1–4 subunits (Dingledine et al., 1999). A single subunit consists of an extracellular N-terminal domain, a ligand-binding domain (LBD), pore-forming transmembrane domains, and a cytoplasmic C-terminal domain. AMPA receptors can also bind allosteric modulators, such as cyclothiazide, which block desensitization. In contrast to NMDA receptors, whose activa-

tion is highly cooperative (Banke and Traynelis, 2003; Kussius and Popescu, 2009), AMPA receptor conductance increases in discrete steps as agonist concentration is increased (Rosenmund et al., 1998; Smith and Howe, 2000), consistent with each subunit binding an agonist molecule and contributing to channel activation (Dingledine et al., 1999; Traynelis et al., 2010). Detailed single-channel studies have begun to address the molecular mechanisms of AMPA receptor-channel activation (Smith and Howe, 2000; Jin et al., 2003; Poon et al., 2010; Prieto and Wollmuth, 2010).

Characterization of AMPA receptor structure and function has led to initial models of channel activation. Specifically, the closure of the LBD upon agonist binding has been directly correlated to activation of the gating mechanism (Armstrong and Gouaux, 2000). Early studies correlated the degree of LBD closure in crystal structures to the amplitude of whole-cell currents at saturating agonist concentrations. Full agonists exhibited the most closure and, in comparison, partial agonists exhibited partial closures (Jin et al., 2003). However, NMR studies and crystal structures of mutant GluA2 LBDs have suggested that the stability of the closed lobe may also contribute to agonist efficacy. Agonist binding consists of

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ABBREVIATIONS: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; LBD, extracellular ligand-binding domain of GluA2 and GluA3; FW, (S)-5-fluorowillardiine [(S)-(-)- α -amino-5-fluoro-3,4-dihydro-2,4-dioxo-1(2H)pyridine-propanoic acid]; NO₂W, (S)-5-nitrowillardiine [(S)-(-)- α -amino-5-nitro-3,4-dihydro-2,4-dioxo-1(2H)pyridine-propanoic acid]; CIW, (S)-5-chlorowillardiine [(S)-(-)- α -amino-5-chloro-3,4-dihydro-2,4-dioxo-1(2H)pyridine-propanoic acid]; VL, very low; L, low; M, medium; H, high; VH, very high; LL, log likelihood; MIL, maximum interval likelihood; NMDA, N-methyl-D-aspartic acid; P_c , closed probability; P_o , open probability; BrW, bromowillardiine; IW, iodowillardiine.

at least three steps: 1) binding to lobe 1, 2) closure of the lobes and contacts with lobe 2, and 3) interlobe contacts (including H-bonds) to stabilize the closed lobe form (Abele et al., 2000; Armstrong and Gouaux, 2000; Cheng et al., 2005; Robert et al., 2005; Weston et al., 2006; Zhang et al., 2008a; Fenwick and Oswald, 2010). Lobe closure is required for channel gating, but the degree of lobe closure that can trigger gating is not clear (i.e., does gating require a full closure?), and the role of auxiliary interlobe contacts has not been completely resolved. In addition, Prieto and Wollmuth (2010) suggest that cooperativity arising from intersubunit contacts may play a role in gating and modal behavior.

Long single-channel recordings can capture details of subtle channel behaviors that were analyzed using state models that describe measurable protein transitions during activation steps and that were, in turn, correlated to changes in the LBD. Our previous studies found that glutamate (full agonist) and FW (strong partial agonist) activated the GluA3 channel to three similar conductance levels, glutamate-activated channels favoring higher conductance states, and at least five modes of channel activity, defined by open probability, were observed (Poon et al., 2010). These modes were seen for both glutamate and FW, with the full agonist, glutamate, favoring those modes that had higher open probability. Given the complexity observed with a full agonist and a strong partial agonist, two slightly weaker partial agonists were tested to determine whether the modal behavior was similar to the stronger partial agonist and whether further complexities in channel gating could be observed. The studies described here show a larger spectrum of activity using two additional partial agonists in the willardiine family, nitrowillardiine (NO₂W), and chlorowillardiine (CIW). Although the crystal structures we obtained of FW and CIW bound to the LBD of GluA3 and for NO₂W and CIW bound to GluA2 are similar to most published AMPA LBD structures, several details differ that may be related to the responses elicited by NO₂W and CIW. Details that vary upon binding of different ligands are the orientation of the two lobes, the orientation of the peptide bond between Asp655 and Ser656, and the orientation of the side chain of Met712. These interactions within the LBD may contribute to differences in single channel activity of GluA3 receptors when bound to either strong or weak partial agonists.

Materials and Methods

Cell Culture. Human embryonic kidney 293 cells were stably transfected with the flip form of GluA3-G (S. M. Holley and L. M. Nowak, unpublished observations), and clonal stable cell lines were established. The receptor is the flip variant and has a G in the R/G editing site. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1 μ g/ml blasticidin, pH 7.4. The addition of the antibiotic blasticidin in the culture media selects for the growth of cells that express GluA3 channels. Cells were passaged every 3 to 4 days and used within 48 h after passage. All cultures were maintained in a 37°C incubator with 5% CO₂.

Acquisition of Single-Channel Currents. All experiments were performed at room temperature (~23°C). Pipettes were pulled from thick-walled borosilicate glass with filament (Sutter Instrument Company, Novato, CA) and fire-polished to a resistance of 15 to 20 M Ω . Solutions were buffered at pH 7.4 and filtered using a 2- μ m sterile filtering system (Corning Life Sciences, Lowell, MA). The bath

buffer was a Ca²⁺/Mg²⁺-free Dulbecco's phosphate-buffered saline with 1.0 mM added MgCl₂ (Invitrogen, Carlsbad, CA). The pipette solution contained 150 mM NaCl, 2 mM KCl, and 10 mM HEPES/NaOH, pH 7.4. Stock solutions of NO₂W or CIW (Tocris Bioscience, Ellisville, MO, or Ascent Scientific, Princeton, NJ), dissolved in the pipette solution, were kept frozen in aliquots at -20°C until the day of the experiment. Cyclothiazide (Tocris Bioscience, Ellisville, MO, or Ascent Scientific, Princeton, NJ) was dissolved in methanol (50 mM stock solution) and stored at -20°C. Cyclothiazide was added (100–150 μ M) to all agonist-containing pipette solutions, and the solutions were used for less than 2 h.

Single-channel currents were amplified at a gain of 100 mV/pA with an EPC-7 amplifier, low-pass filtered to 10 kHz by using an external eight-pole Bessel filter, and digitized at 20 kHz by using pClamp 7 software (Molecular Devices, Sunnyvale, CA). Single-channel currents were recorded for 2 to 10 min in the cell-attached mode with pipette holding potentials of +80 to +120 mV. Data were converted from abf to qdf format with the use of QuB software (<http://www.qub.buffalo.edu>) for analysis.

Analysis of Single-Channel Data. Only data from patches with one channel were used for analysis. A segment of the record without channel activity was used to define the baseline in QuB. As described previously (Poon et al., 2010), we tested the number of open conductance levels by idealizing the data assuming up to four different conductance levels, filtering at frequencies ranging from 1 to 10 kHz, and altering the dead time using a range from 50 to 200 μ s. The results from different combinations of criteria were then analyzed, and the criteria that provided the best fits to the amplitude histogram, dwell time histograms, and the largest log likelihood (LL) units were used for all subsequent analyses. Three open conductance levels provided the best fits to the data, and the addition of a fourth open conductance level consistently exhibited a lower LL, despite the increase in free parameters (Poon et al., 2010). All data were initially idealized at a dead time of 150 μ s using the segmental-k-means algorithm (Qin et al., 1996) to an initial simple linear model consisting of one closed and three open classes ($C \leftrightarrow O1 \leftrightarrow O2 \leftrightarrow O3$) starting with all rates set to 100 s⁻¹. The data were analyzed with or without an additional 5-kHz filtering (effective filter of 4.5 kHz). A dead time of 200 μ s was imposed on patches that had additional filtering. Maximum interval likelihood (MIL) analysis (Qin et al., 1996) was applied on this initial model to determine the fit to the dwell time histograms. States were then added one at a time to each closed and open class, reidealized, refitted using MIL, and retained if the fits to the histograms improved and/or if the LL units increased by >10. If the addition of a state increased the LL by 10, but the rates to the new state were either close to zero or unrealistically fast, the additional state was omitted. This process was repeated again with rates starting at 10 s⁻¹ to confirm that the final model and the final rates were not affected by the starting values. Our final model, similar to that in Poon et al. (2010), consists of three closed states and two open states in each of the open conductance levels (Figs. 1A and 3). Loops that connected closed states to the intermediate or large conductance levels in the model were tested but in most cases worsened the fit to the dwell time histograms and/or gave unrealistic rates, so loops were not considered in our final model. Based on the transition matrices (Fig. 1B), transitions between the closed and the intermediate or large conductance levels were infrequently observed. Therefore, the final model does not include these transitions.

Modal behavior was determined by segmenting the data based on the closed probability as described in Poon et al. (2010). Patches with long closures were modeled using four or five closed states. The critical time (t_{crit}), which describes the minimum closed duration between two bursts (Magleby and Pallotta, 1983), was used to discard long closures. The resulting clusters of openings and closings were reidealized to a segmental-k-means model with one closed and three open conductance classes to determine the closed and open probabilities of each segment. The select function in QuB was then used to sort the segments into modes. The X-means algorithm (Pelleg

and Moore, 2000) places each segment into the appropriate mode using the closed time probability (P_c ; used in this step because of the multiple conductance levels). The number of modes was determined by sorting the segments into similar clusters based on the value of x until the assignments stabilized. The sorted modes were analyzed individually using the model described above. In a few records having only three closed states and one mode, the model in Fig. 1A was applied to the entire record. The dwell times of similar modes were averaged, but each mode was modeled separately. Because the number of events in each group was necessarily less than the total number of events in the record, the LL was normalized to the number of events (LL per event), and each group was compared with the whole record. Based on the analysis using the x -means algorithm described above, five distinct modes (VL, very low; L, low; M, medium; H, high; VH, very high) were observed, as detailed in Poon et al. (2010), for glutamate and FW.

Once the record was divided into modes, an “efficacy factor” (Jin et al., 2003; Poon et al., 2010; Prieto and Wollmuth, 2010) was determined based on a binomial distribution. Once bound with two to four agonist molecules, the channel can display openings to up to three conductance levels. The four levels (closed plus three open) can be viewed as four trials (n), and the level of a particular opening is defined as the number of successes (k). The probability of success (“efficacy factor”) can then vary between agonist, at different concentrations, and, in this case, in different modes.

Protein Preparation and Purification. GluA2 LBD consists of residues Asn392 to Lys506 and Pro632 to Ser775 of the full rat flap form of GluA2 subunit (Hollmann and Heinemann, 1994), a “GA” segment at the N terminus, and a “GT” linker connecting Lys506 and Pro632 (Armstrong and Gouaux, 2000). A similar construct of the flip form of GluA3 LBD was prepared as described previously (Ahmed et al., 2009b). pET-22b(+) plasmids were transformed in *Escherichia coli* strain Origami B (DE3) cells and were grown at 37°C to OD₆₀₀ of 0.9 to 1.0 in Luria-Bertani medium supplemented with the antibiotics (ampicillin and kanamycin). The cultures were cooled to 20°C for 20 min and isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5 mM. Cultures were allowed to grow at 20°C for 20 h. The cells were then pelleted and the LBD purified using a nickel-nitrilotriacetic acid column, followed by a sizing column (Superose 12, XK 26/100). The final purification step used an HT-SP-ion exchange-Sepharose column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Glutamate (1 mM) was maintained in all buffers throughout purification. After the last column, the protein was concentrated and stored at 4°C in 20 mM sodium acetate, 1 mM sodium azide, and 10 mM glutamate at pH 5.5.

Crystallography. For crystallization trials, the ligand was exchanged by successive dilution and concentration, and the protein

was subsequently concentrated to 0.2 to 0.5 mM using a Centricon 10 centrifugal filter (Millipore, Billerica, MA). Crystals were grown at 4°C using the hanging drop technique, and the drops contained a 1:1 (v/v) ratio of protein solution to reservoir solution. The reservoir solution contained 14 to 15% polyethylene glycol 8000, 0.1 M sodium cacodylate, pH 6.5, 0.1 to 0.15 M zinc acetate, and 0.25 M ammonium sulfate. The final agonist concentration was 2 mM for FW, NO₂W, and CIW.

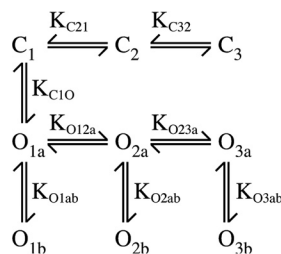
Data were collected at the Cornell High Energy Synchrotron Source beam line A1 using a Quantum-210 Area Detector Systems charge-coupled device detector. Data sets were indexed and scaled with HKL-2000 (Otwinowski and Minor, 1997). Structures were solved with molecular replacement using Phenix (Adams et al., 2002). Refinement was performed with Phenix (Adams et al., 2002), ccp4 (Collaborative Computational Project Number 4, 1994), and Coot 0.6.1 (Emsley and Cowtan, 2004) was used for model building.

Results

Single Channel Behavior of GluA3 Activated by NO₂W and CIW

NO₂W and CIW Activate GluA3 Channels in the Same Manner as Glutamate and FW. Members of the willardiine family of partial agonists share the same overall structure but differ in the substituent in the 5-position. They produce a graded level of whole-cell activation dependent on the size and electronegativity of the substituent, the electro-negativity seemingly affecting ligand potency and the size apparently affecting efficacy (Patneau et al., 1992). Efficacy followed the pattern FW > NO₂W > CIW (Patneau et al., 1992). Despite differences in efficacy, our single-channel studies, using agonist concentrations ranging from >EC₅₀ to saturating levels, show that the willardiine compounds all activate the homomeric GluA3 receptor to three conductance levels. NO₂W and CIW activated GluA3 channels to three open levels with conductances of 16 ± 0.7 , 29 ± 1.6 , and 40 ± 1.4 pS for NO₂W ($n = 4$) and 15 ± 0.9 , 25 ± 1.4 , and 38 ± 1.4 pS for CIW ($n = 4$), values similar to those reported by Poon et al. (2010) for glutamate and FW. A representative trace showing single-channel currents in 100 μM NO₂W is presented in Fig. 2A. Amplitude histograms for 500 μM NO₂W and 500 μM CIW are shown in Fig. 2, B and C, respectively, for receptors activating in a VH mode (see *Analysis of Single-Channel Data* for a detailed explanation of the modal behav-

A



B

| 500 μM CIW | C | O ₁ | O ₂ | O ₃ |
|----------------|------|----------------|----------------|----------------|
| C | - | 8.95 | 1.13 | 0.04 |
| O ₁ | 8.70 | - | 25.20 | 0.53 |
| O ₂ | 1.37 | 24.81 | - | 14.36 |
| O ₃ | 0.05 | 0.66 | 14.21 | - |

| 500 μM NO ₂ W | C | O ₁ | O ₂ | O ₃ |
|-----------------------------|------|----------------|----------------|----------------|
| C | - | 7.99 | 0.41 | 0.01 |
| O ₁ | 8.22 | - | 25.14 | 1.24 |
| O ₂ | 0.19 | 25.64 | - | 14.96 |
| O ₃ | 0.00 | 0.97 | 15.23 | - |

Fig. 1. A, the final kinetic model used to analyze the single channel data. The notation for the equilibrium association constants is: $K_{C21} = K_{C2 \rightarrow C1} / K_{C1 \rightarrow C2}$, $K_{O12a} = K_{O1a \rightarrow O2a} / K_{O2a \rightarrow O1a}$, $K_{O1ab} = K_{O1a \rightarrow O1b} / K_{O1b \rightarrow O1a}$, etc. B, transition matrices showing the percentage of transitions between conductance levels (i.e., transitions between sublevels). Transitions between nonadjacent states are rare compared with transitions between adjacent states. These data are used to constrain the final kinetic model such that only transitions between adjacent states are included (C to O₂, C to O₃, and O₁ to O₃ are not included in the model).

ior). As shown in Table 1, the population of each conductance level is similar for NO₂W and CIW in VH mode.

The simple model derived from studies with glutamate and FW fit the data well (Fig. 1A). Figure 3 shows dwell-time histograms for the 500 μM NO₂W and CIW data. The closed histogram was fit by the sum of three exponentials, and the open histograms for each conductance level were fit by two exponentials, as reported for glutamate and FW activation of the GluA3 receptor (Poon et al., 2010). Based on state transition matrices (Fig. 1B), the channel preferentially transitions between adjacent conductance levels when bound to NO₂W and CIW. These results suggest the mechanism of activation with NO₂W and CIW, like FW, is similar to that of glutamate.

Although the frequency of the channel to enter a specific mode is dependent on the agonist concentration, the activity within a mode was the same and independent of agonist concentration. For this reason, and as done previously (Poon et al., 2010), the probability of populating each conductance level was averaged for the VH mode for each agonist at a saturating concentration to obtain a more global picture of the occupation of specific conductance

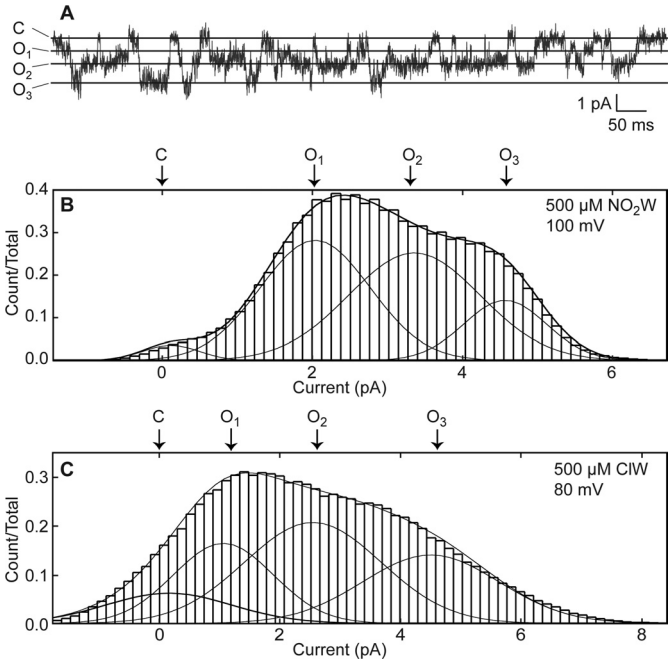


Fig. 2. A, a 500-ms representative segment of a GluA3 channel activated by 100 μM NO₂W opens to three different conductance levels. B and C, amplitude histograms and Gaussian fits for an entire record obtained with 500 μM NO₂W at 100 mV (B) and 500 μM CIW at 80 mV (C). For both the NO₂W and CIW records shown, the channel exhibited VH mode. The idealization was done using a dead time of 200 μs (see *Materials and Methods*).

TABLE 1

Average population of each conductance level in VH mode shown is the averaged probability of the GluA3 channel visiting each conductance level in the VH mode for glutamate, FW, NO₂W, and CIW (*n* = 3 for each agonist)
The last row (ΣP_{γ}) reports the sum of the *P* for each row multiplied by the conductance normalized to the value for glutamate. The conductances for glutamate and FW were taken from Poon et al. (2010).

| | 5 mM Glutamate | 200 μM FW | 500 μM NO ₂ W | 500 μM CIW |
|-----------------------|----------------|-----------|--------------------------|------------|
| <i>P_C</i> | 6 ± 3% | 9 ± 1% | 12 ± 3% | 13 ± 2% |
| <i>P_{O1}</i> | 6 ± 3% | 22 ± 5% | 37 ± 5% | 31 ± 3% |
| <i>P_{O2}</i> | 51 ± 6% | 47 ± 2% | 33 ± 4% | 42 ± 3% |
| <i>P_{O3}</i> | 36 ± 12% | 22 ± 7% | 17 ± 2% | 14 ± 4% |
| ΣP_{γ} | 1.0 | 0.81 | 0.79 | 0.73 |

levels for NO₂W and CIW compared with FW and glutamate (Table 1). All three willardiines have very similar open probabilities in all conductance levels. CIW and NO₂W populate the highest conductance level to a lesser extent than glutamate (*p* < 0.05). NO₂W and CIW spend a similar percentage of time in the largest conductance level (*p* > 0.05). The population in the intermediate conductance level is lower for NO₂W than for FW (*p* < 0.05). The rank order of the population of the largest conductance level is CIW ≤ NO₂W ≤ FW < glutamate. The slightly higher efficacy of FW compared with NO₂W seems to be caused by a higher open probability in O₂. These findings correlate well with the efficacy measured in whole-cell recordings (Patneau et al., 1992) and also suggests that efficacy differences of partial agonists in whole-cell studies can be attributed to the differences in open probability in each of the conductance levels (Jin et al., 2003; Poon et al., 2010).

NO₂W and CIW Induce Modal Behaviors. Modal gating of AMPA receptors has been previously described in detail for GluA3 bound to FW or glutamate and was correlated to the hydrogen bonding of the flipped peptide conformation of Asp655 and Ser656 to Tyr452 and Gly453 (Poon et al., 2010). A 180° flip of the Asp655/Ser656 peptide bond (GluA3) upon binding of agonist can result in the formation of H-bonds between Ser656 (lobe 2) and Gly453 (lobe 1), and between Asp655 and Tyr452 through a water molecule. Modal gating has also been reported for other AMPA (Prieto and Wollmuth, 2010) and for NMDA receptor subtypes (Popescu and Auerbach, 2003; Zhang et al., 2008b). Silberberg et al. (1996) also observed time-dependent changes in *P_o* for the Ca²⁺-activated K⁺ channels from *Drosophila melanogaster* (dSlo) and referred to this behavior as “Wanderlust Kinetics.” We tested whether modal gating could also be observed for the lower efficacy partial agonists NO₂W and CIW. Patches with long closures were segmented according to the *t_{crit}* (for CIW: 1 mM, 79 ms; 500 μM, 82–226 ms; 100 μM, 49–170 ms; 50 μM, 50–280 ms; for NO₂W: 1 mM, 78 ms, 500 μM, 127–295 ms; 100 μM, 81–180 ms; 50 μM, 131–169 ms) and sorted into the closest *P_c* distribution using the *x* means method (Pelleg and Moore, 2000; Poon et al., 2010). These sorted segments were then reanalyzed using the model in Fig. 1A. NO₂W and CIW each exhibited five modes of behavior. Modes were obtained by determining whether the channel was open to any level using 1 – *P_c*, as in Poon et al., (2010), and they are represented here by *P_o* (where *P_o* = 1 – *P_c*) of 80 to 100% (VH mode), 60 to 79% (H), 40 to 59% (M), 20 to 39% (L), and <20% (VL) (Fig. 4). Patches were also recorded in which a specific mode of activity was retained throughout the record, independent of agonist concentration. The kinetic data ac-

quired from these patches were averaged with other similar P_o modes of behavior.

The single-channel behavior of CIW and NO_2W follows a binomial distribution, similar to FW and glutamate, where the probability of success is referred to as an “efficacy factor” (Jin et al., 2003). Figure 5 shows the efficacy factors in each mode (averaged over concentration) for the willardiine derivatives and glutamate. The efficacy factors remain constant for a given mode regardless of concentration and are similar for CIW and NO_2W in the V and VH modes. It seems that the efficacy factors for NO_2W are lower than those for other agonists in the M, L, and VL modes, but this may be due to the small sample size for NO_2W in these modes; compared with CIW, most of the M, L, and VL activity for NO_2W are of short duration. As expected, glutamate efficacy is higher than that for the willardiine derivatives.

The equilibrium constants associated with the model (Fig. 1A) also show differences among modes. The K_{eq} for both CIW and NO_2W favor channel opening to the smallest conductance level in all modes except L and VL, and K_{C10} decreases from VH to VL (Table 2). K_{eq} is a forward equilibrium constant; a value greater than 1 indicates a relatively greater population of, for example, O_2 than O_1 . In the VH mode, the K_{O12a} for NO_2W and CIW is greater than 1 for the transition between the smallest to the intermediate conductance levels. This is in contrast to transitions to the highest conductance level, which are less than 1 (K_{O23a}). For both agonists, K_{eq} to each open state (K_{C10} , K_{O12a} , and K_{O23a}) decreases as the modal behavior descends in activity (i.e., P_o decreases).

CIW Causes Alternate Kinetic Behaviors at Lower Concentrations of Agonist. Channel activity with CIW generally follows the same modal pattern as with glutamate; however, an anomaly was detected in the H mode. CIW seems to exhibit two populations of activity: one with a higher percentage of time in the largest conductance level (P_{O3} of 10–13%; $n = 6$) and another with a very low P_{O3} (0.17 to 2%;

$n = 5$). In Fig. 5A, two efficacy factors are shown for CIW in H mode, with the closed circle and dotted line reflecting the alternate kinetic behavior. Figure 5B displays an example of CIW activation of channels in H mode with a low P_{O3} . In the presence of both CIW and NO_2W , channels were also observed that open largely to the smallest conductance level, with occasional transitions to the middle conductance (Supplemental Table 1). FW was shown previously to produce similar behavior (Poon et al., 2010). The alternative kinetics produced by CIW that give rise to the lower efficacy observed in H mode in Fig. 5 may or may not be a result of the same processes that produce sustained openings to only the lowest conductance level. In any event, it seems clear that partial agonists can be locked into lower conductance states over relatively long periods (up to minutes).

NO_2W Produces Long Dwell Times. Similar to previous results with glutamate and FW, the dwell times in the open states of all conductance levels measured with NO_2W and CIW are longer in the VH modes and shorten with a decrease in modal activity (Table 3). The longest dwell time (the second time constant, τ_{O1b} , τ_{O2b} , and τ_{O3b}) in each of the conductance levels in the VH mode for NO_2W is longer than that observed for CIW ($p < 0.05$). τ_{O1b} , τ_{O2b} , and τ_{O3b} for NO_2W ranged from 4 to 45 ms for all three conductance levels. τ_{O1b} , τ_{O2b} , and τ_{O3b} for CIW ranged from 2 to 12 ms. Comparing this with previous findings (Poon et al., 2010), τ_{O1b} , τ_{O2b} , and τ_{O3b} for glutamate were similar to CIW, ranging from 2 to 6 ms, whereas FW ranged from 5 to 28 ms. The order of τ_{O1b} , τ_{O2b} , and τ_{O3b} in decreasing length is $\text{NO}_2\text{W} > \text{FW} > \text{CIW} > \text{glutamate}$.

Summary. The single channel analysis of GluA3 activated by NO_2W and CIW displays modal behavior as seen previously for FW and glutamate (Poon et al., 2010). In addition, CIW displays a lower efficacy at times in the H mode, and NO_2W exhibits relatively long dwell times in the VH mode.

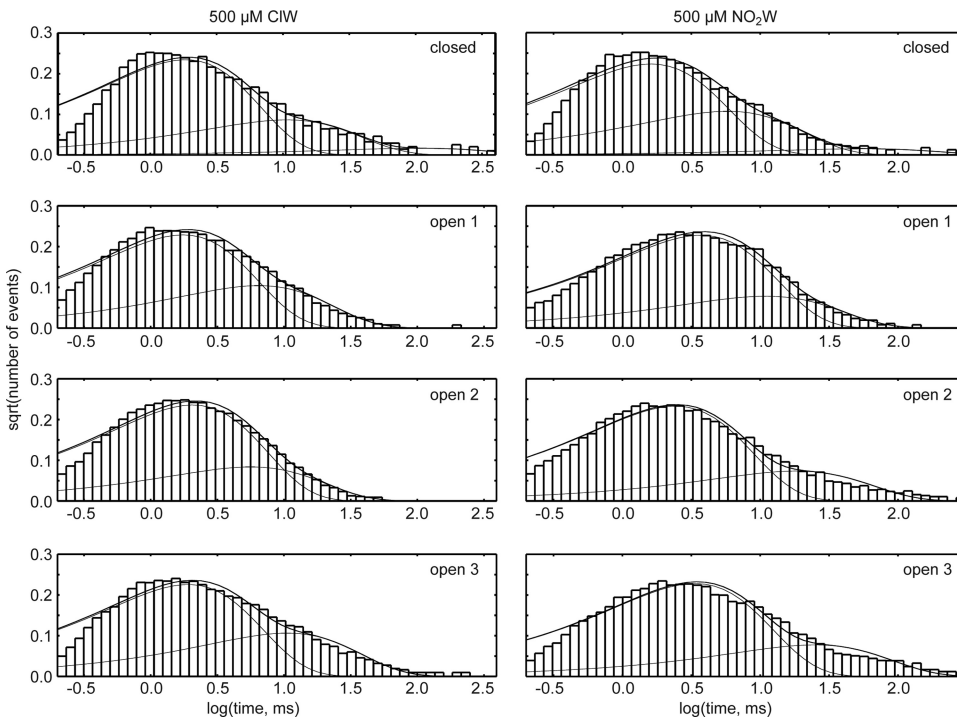


Fig. 3. Dwell time histograms for the same records used to generate Fig. 2, B and C. MIL (Qin et al., 1996) was used to fit the data to the model shown in Fig. 1A.

Structures of GluA2 and GluA3 Bound to FW, NO₂W, and CIW

Lobe Closure Variability. When bound to various agonists, the GluA3 and GluA2 LBD structures are quite similar, with small differences. The structure of GluA2 LBD bound to NO₂W is shown in Fig. 6A, and structural statistics are given in Supplemental Table 2. In all cases, the LBD dimer interface, seen in the structure of the tetrameric protein (Sobolevsky et al., 2009), was observed. The dimer interface is essentially the same for each of the agonists bound to the LBD, and it is similar to the dimer interface observed with cyclothiazide derivatives bound (Ptak et al., 2009). FW bound GluA3 is 2.6° more open relative to glutamate (Protein Data Bank code 3DP6, B protomer, glutamate bound to GluA2 (Ahmed et al., 2009b; Table 4). The structure of the GluA2 LBD bound to FW has been reported previously (Jin et al., 2003) and is similar overall to the GluA3 LBD structure reported here. The FW-bound GluA2 structure is 4° more open relative to glutamate.

The structures of CIW bound to the LBDs of GluA2 and GluA3 were determined. Despite the somewhat smaller size of the chlorine relative to the nitro moiety, the average lobe opening relative to the most closed glutamate-bound form of GluA3 for CIW bound to GluA2 LBD (4.1°, 2.4°, 2.2°; average $2.9 \pm 1.0^\circ$) is larger than NO₂W bound to GluA2 LBD but the distributions overlap. The lobe opening for GluA3 LBD bound

to CIW (3.7°; one copy per asymmetric unit) is within the range observed for GluA2.

Three crystals of NO₂W bound to GluA2 LBD, each with three structures per asymmetric unit, were analyzed. Despite the fact that the nitro substituent of NO₂W is larger than the fluorine of FW, six of the nine structures in the asymmetric unit for NO₂W bound to GluA2 LBD are more closed than observed for FW bound to either GluA2 or GluA3 LBD. In fact, six of the nine structures were more closed than one of the structures of GluA2 bound to glutamate (Protein Data Bank code 3DP6, A protomer, 1.75°). The most striking aspect of the NO₂W structure is the variation observed. The range of lobe opening relative to the most closed glutamate-bound structure is more than 3° (0.7–3.9°; average, $2.0 \pm 1.7^\circ$).

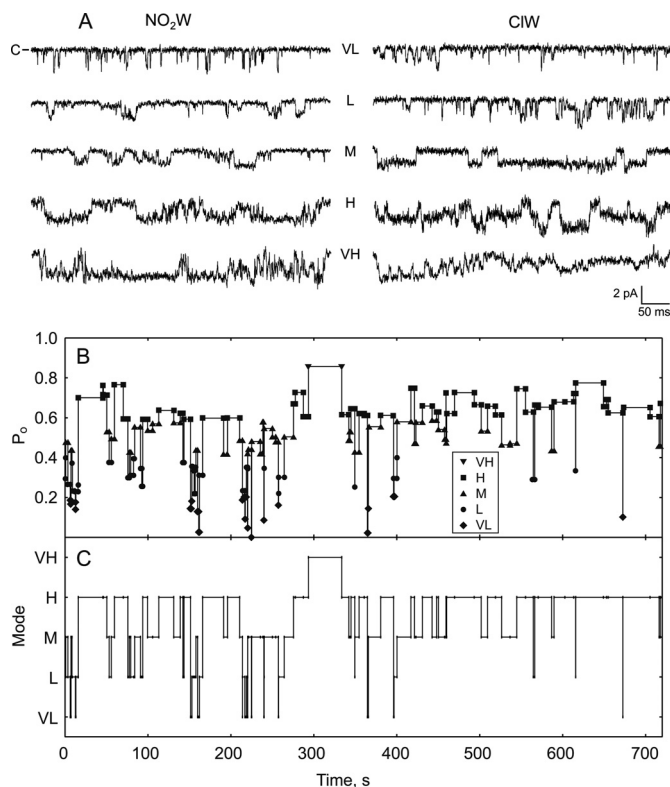


Fig. 4. A, representative 500-ms segments of single-channel records showing modal behaviors for CIW [50 μM (VL), 100 μM (L, M, H), and 500 μM (VH)] and NO₂W [50 μM (VL, L), 100 μM NO₂W (M, H), and 500 μM NO₂W (VH)]. The traces are additionally filtered at 1 kHz for display purposes. B, segment of a record with 100 μM CIW as the agonist showing the changes in P_o as a function of time (the segments of closed time greater than t_{crit} are removed from the record). C, the same record as B is shown with mode classification. Note that, as described by Poon et al. (2010), the transitions are most likely to occur between adjacent modes (e.g., VL to L, H to M, etc.).

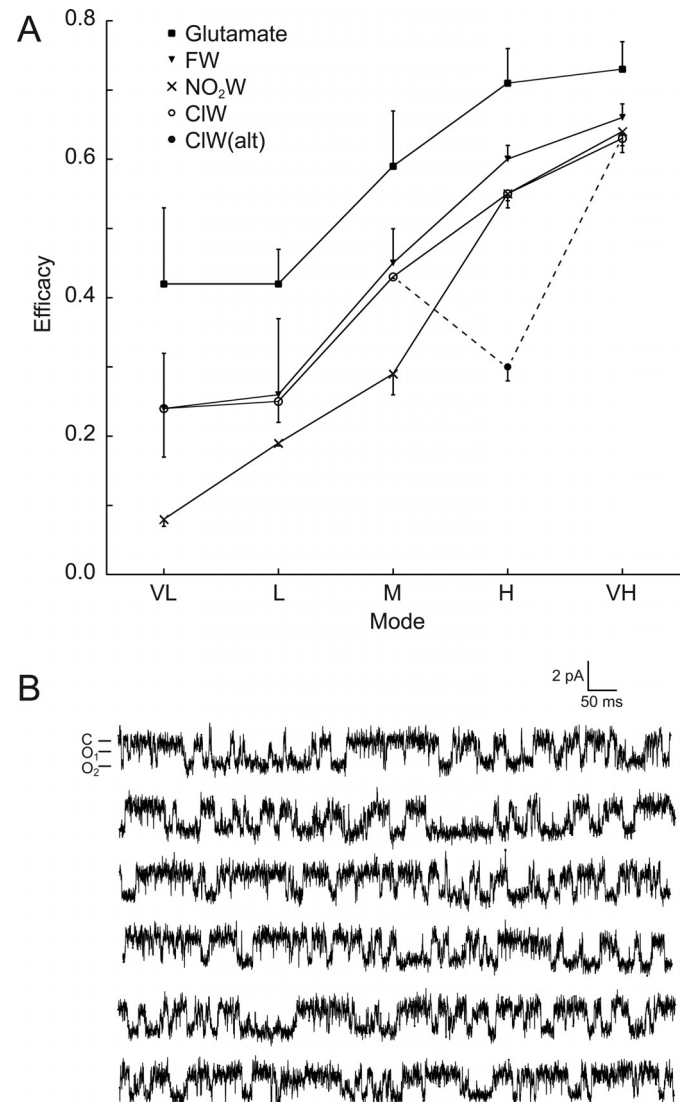


Fig. 5. A, the efficacy factor (P) was calculated as described in Poon et al. (2010) based on the amplitude histograms for records separated into specific modes. The concentrations used ranged from 50 to 1000 μM for CIW and NO₂W. The values for FW and glutamate were taken from Poon et al. (2010). The dotted line indicates alternative kinetic behavior exhibited at lower concentrations of CIW. The efficacy factor is synonymous with the probability of success in a binomial distribution. The higher the value, the more likely a gate will be open. B, example of the alternate kinetic behavior exhibited when CIW activates the channel.

Asp655/Ser656 Peptide Conformation. The flipped conformation of the Asp655/Ser656 peptide bond (this and all subsequent residues are given in GluA3 numbering; for GluA2, the corresponding peptide bond is Asp651/Ser652), which is typically seen in structures with full agonist bound (Armstrong and Gouaux, 2000), allows the formation of two H-bonds across the lobe interface: the carbonyl of Ser656 (lobe 2) forms an H-bond with the amide of Gly453 (lobe 1) and the carbonyl of Asp655 forms a water-mediated H-bond with the amide of Tyr452 (Fig. 6E). This conformation has been suggested to relate to modal behaviors; that is, the formation of these hydrogen bonds in individual subunits may stabilize its active form and the combination of those subunits that are or are not H-bonded may result in different modes of activation (Poon et al., 2010). The FW bound GluA3 LBD structure reveals that the Asp655/Ser656 peptide bond is in the flipped conformation (Fig. 6B). Although this conformation has not been observed crystallographically for the GluA2 LBD bound to FW, residual dipolar coupling measurements using NMR spectroscopy (Maltsev et al., 2008) suggested that FW-bound GluA2 can also assume a flipped conformation for the Asp655/Ser656 peptide bond. In the case of FW bound to GluA3, the lobes are slightly more separated than with glutamate so that neither the water-mediated Asp655-Tyr452 H-bond nor the direct H-bond between Ser656 and G453 is formed. Instead, an additional water molecule sits in the pocket, forming an H-bonding network that includes the backbone carbonyl of Ser656 and the backbone amides of Gly453 and Tyr452 (Fig. 6B). This may result in additional stability in this partially open form of the LBD.

The GluA3 LBD structure bound to CIW exhibits several interesting features (Fig. 6, C and D). Unlike other structures that are not fully closed but similar to the FW GluA3 LBD structure, the Asp655/Ser656 peptide bond is in the flipped conformation. The question then is whether the H-bonds typical of the flipped conformation are formed. The carbonyl of Asp655 probably does not participate in an H-bond with the amide of Tyr452, although the water molecule is in the appropriate position. However, an acetate molecule is present in the cleft that forms an interlobe H-bond with the backbone carbonyls of Asp655 and Lys451 (Fig. 6C). Although the carbonyl of Ser656 is not sufficiently close to the amide of Gly453 to make a direct H-bond, there exists an H-bonding network involving three water molecules that joins the carbonyl of Ser656 to the amides of both Tyr452 and Gly453 (Fig. 6C). This seems to suggest that a semistable partially open, flipped conformation exists. In contrast, all three structures of GluA2 LBD bound to CIW are in the unflipped conformation; however, in two of the three structures, the three water molecules are in similar positions (Fig. 6D). The side chain of Ser656 (Ser652 in GluA2) is rotated to form a water-mediated H-bond with Gly453. In all three copies, both the backbone carbonyl and the side chain of Asp655 interact with Lys660 (Fig. 6D).

The nine structures of NO₂W bound to GluA2 show considerable variability. In the most closed structure, the Asp655/Ser656 peptide bond is in the flipped conformation (Fig. 6E), with the H-bonds to Tyr452 and Gly453 formed as observed for full agonists. For the remainder, the bond is not flipped, and in some cases, two conformations of the side

TABLE 2

Equilibrium constants for modes derived from single channel records equilibrium association constants were determined using the model defined in Fig. 1 for NO₂W and CIW as the agonist

The equilibrium constants were averaged across all concentrations in a given mode. All constants are unitless, and errors are given as S.E.M. The number of patches analyzed is given in parenthesis after the mode.

| Mode | K_{C32} | K_{C21} | K_{C10} | K_{O1ab} | K_{O12a} | K_{O2ab} | K_{O23a} | K_{O3ab} |
|-------------------|------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| NO ₂ W | | | | | | | | |
| VH (12) | 3.5 ± 0.8 | 1.9 ± 0.3 | 3.7 ± 0.6 | 0.40 ± 0.07 | 1.2 ± 0.1 | 0.42 ± 0.08 | 0.44 ± 0.07 | 0.55 ± 0.10 |
| H (15) | 4.8 ± 1.5 | 1.5 ± 0.2 | 2.0 ± 0.1 | 0.31 ± 0.04 | 0.80 ± 0.04 | 0.30 ± 0.08 | 0.30 ± 0.03 | 0.37 ± 0.04 |
| M (4) | 2.3 ± 0.4 ^a | 1.6 ± 0.3 | 1.2 ± 0.1 | 0.18 ± 0.04 | 0.31 ± 0.05 | 0.20 ± 0.04 | 0.18 ± 0.04 | 0.33 ± 0.02 |
| L (3) | 1.8 ± 0.3 | 1.1 ± 0.1 | 0.6 ± 0.1 | 0.55 ± 0.42 | 0.19 ± 0.04 | 0.23 ± 0.07 | 0.16 ± 0.01 | 0.24 ± 0.02 |
| CIW | | | | | | | | |
| VH (11) | 2.0 ± 0.3 | 2.3 ± 0.2 | 3.7 ± 0.5 | 0.54 ± 0.27 | 2.3 ± 0.8 | 0.17 ± 0.03 | 0.29 ± 0.05 | 0.30 ± 0.06 |
| H (12) | 5.9 ± 2.6 | 1.7 ± 0.1 | 2.1 ± 0.1 | 0.18 ± 0.03 | 0.62 ± 0.08 | 0.21 ± 0.04 | 0.19 ± 0.04 | 0.40 ± 0.09 |
| M (2) | 2.3 ± 0.3 | 1.2 ± 0.1 | 1.2 ± 0.1 | 0.17 ± 0.02 | 0.40 ± 0.09 | 0.13 ± 0.02 | 0.21 ± 0.06 | 0.31 ± 0.03 |
| L (4) | 1.6 ± 0.2 | 0.92 ± 0.07 | 1.0 ± 0.1 | 0.17 ± 0.03 | 0.26 ± 0.03 | 0.14 ± 0.02 | 0.12 ± 0.02 | 0.34 ± 0.03 |
| VL (4) | 2.3 ± 0.6 | 1.0 ± 0.1 | 0.34 ± 0.09 | 0.19 ± 0.03 | 0.26 ± 0.11 | 0.10 ± 0.04 | 0.09 ± 0.03 | 0.33 ± 0.06 |

^a One outlier with a K_{C32} of 36 was removed because it was based on a limited number of events.

TABLE 3

Dwell times for modes derived from single channel records dwell times were determined using the model defined in Fig. 1 for NO₂W and CIW as the agonist

The dwell times were averaged across all concentrations in a given mode. The entries in bold are significantly different ($P < 0.05$) between NO₂W- and CIW-activated channels. For example, the τ_{O3b} for NO₂W is significantly longer than the τ_{O3b} for CIW. The number of patches analyzed is given in parenthesis after the mode.

| Mode | τ_{C1} | τ_{C2} | τ_{C3} | τ_{O1a} | τ_{O1b} | τ_{O2a} | τ_{O2b} | τ_{O3a} | τ_{O3b} |
|-------------------|-------------|-------------|-------------|--------------|------------------|--------------|------------------|--------------|------------------|
| NO ₂ W | | | | | | | | | |
| VH (12) | 1.5 ± 0.2 | 6.7 ± 1.0 | 27 ± 3 | 2.1 ± 0.3 | 9.3 ± 2.1 | 2.1 ± 0.3 | 13 ± 4 | 2.3 ± 0.3 | 16 ± 3 |
| H (15) | 1.6 ± 0.2 | 7.6 ± 0.8 | 33 ± 4 | 1.5 ± 0.1 | 5.8 ± 0.6 | 1.5 ± 0.2 | 5.8 ± 1.4 | 1.3 ± 0.1 | 6.2 ± 0.5 |
| M (4) | 1.5 ± 0.2 | 7.0 ± 0.9 | 31 ± 7 | 2.1 ± 0.7 | 6.0 ± 1.8 | 1.1 ± 0.2 | 3.9 ± 1 | 0.9 ± 0.03 | 4.7 ± 0.9 |
| L (3) | 2.2 ± 0.2 | 9.4 ± 0.3 | 36 ± 5 | 1.3 ± 0.2 | 5.9 ± 2.2 | 0.8 ± 0.1 | 3.3 ± 0.7 | 0.8 ± 0.1 | 4.3 ± 1.4 |
| CIW | | | | | | | | | |
| VH (11) | 0.9 ± 0.1 | 4.1 ± 0.3 | 30 ± 5 | 1.1 ± 0.2 | 4.5 ± 0.7 | 1.8 ± 0.2 | 5.1 ± 0.6 | 1.6 ± 0.2 | 7.2 ± 1.0 |
| H (12) | 1.3 ± 0.2 | 5.5 ± 0.7 | 32 ± 5 | 1.6 ± 0.2 | 5.5 ± 1.0 | 1.5 ± 0.2 | 5.9 ± 1.4 | 1.0 ± 0.1 | 5.6 ± 1.0 |
| M (2) | 1.2 ± 0.3 | 6.0 ± 0.04 | 27 ± 5 | 1.0 ± 0.2 | 3.2 ± 0.6 | 0.8 ± 0.1 | 2.6 ± 0.6 | 0.9 ± 0.1 | 4.5 ± 1.0 |
| L (4) | 1.4 ± 0.2 | 7.1 ± 0.8 | 35 ± 6 | 1.3 ± 0.1 | 4.2 ± 0.2 | 1.2 ± 0.2 | 3.9 ± 0.6 | 1.0 ± 0.1 | 4.6 ± 0.2 |
| VL (4) | 1.6 ± 0.3 | 6.9 ± 1.1 | 23 ± 5 | 0.7 ± 0.2 | 2.4 ± 0.6 | 0.7 ± 0.1 | 2.9 ± 0.5 | 0.9 ± 0.2 | 5.5 ± 1.5 |

chain of Asp655 are apparent (Fig. 6F). The side chain of Asp655 can form a salt bridge with the side chain of Lys660. This is seen in the conformer with the flipped peptide bond, but only one of the two conformations of the Asp655 side chain can form this salt bridge in the two unflipped structures. In the case of the unflipped structure, the side chain of Lys660 can interact with both the side chain and backbone carbonyl of Asp655 (Fig. 6F). However, in the flipped form, the carbonyl of Asp655 forms an interlobe H-bond with Tyr452, so that only the Asp655 side chain is available for interaction with Lys660. In addition, the nitro group of NO₂W contributes interactions that may stabilize the closed-lobe conformation; NO₂W can form an H-bond with the side chain of Thr690 and the side chain hydroxyl of Tyr452 (Fig. 7A).

Although these structures capture either the flipped or unflipped conformations of Asp655-Ser656, it is very likely that both of these states are visited in solution. Based on the structures reported here, it seems that the flipped conformation of the Asp655/Ser656 peptide can exist in partially closed LBD structures and is stabilized by water molecules occupying the partially open space. NMR results (Maltsev et al., 2008) suggest the GluA2 LBD exhibits an equilibrium between the flipped and unflipped conformations, which means either form could be crystallized. The differences in the flipped region between GluA2 and GluA3 are unlikely to be functional differences; rather, both conformations are most likely present in both subtypes.

Met 712 Side-Chain Conformation. Met712, located in helix I, has been seen to adopt different conformations depending on the agonist that is bound (Armstrong and Gouaux, 2000; Jin et al., 2002; Jin and Gouaux, 2003). In glutamate-bound and FW-bound GluA2 and GluA3 structures, M712 is in an extended conformation. For NO₂W, comparison of the structure with that of the FW-bound and glutamate-bound GluA2 and GluA3 structures indicates that the larger size of NO₂W is accommodated by a change in the

rotameric state of Met712 at the β carbon (Fig. 7B) and the exclusion of one water molecule (relative to the CIW-bound form) from the binding pocket (Fig. 7C). This places the side chain in a position that is intermediate between the glutamate-bound form and the form reported previously for BrW and IW (Jin and Gouaux, 2003).

The rotamer of the side chain of Met712 in the CIW-bound GluA3 LBD structure is similar to willardiine partial agonists such as BrW and IW. However, in the structure of GluA2 bound to CIW, two of the protomers have a conformation of Met712 similar to that of the NO₂W structure, and one has density corresponding to both the NO₂W conformation and to the conformation seen with CIW bound to GluA3. As noted above, for CIW, the rotation of the side chain allows an additional water molecule to enter the binding pocket to interact with the side-chain hydroxyls of Tyr407 and Tyr736 (Fig. 7C).

Summary. Although the overall structures of the GluA2 and GluA3 LBD are similar for all agonists tested, variability in lobe closure, the state of the peptide flip region, and some side chain rotamers can vary. In addition, NO₂W makes additional contacts that are not observed in other willardiines. These differences may be associated with the functional differences observed in single channel recording.

Discussion

Efficacy of Partial Agonists. Although recognizing that the lobe closure observed in a crystal may or may not reflect the frequency and extent of lobe closure in an intact receptor, Jin et al. (2003) postulated that partial agonism arose from partial lobe closure, and efficacy was dependent on the degree of closure. Although this is consistent with the initial crystal structures, more recent work has supported a model in which the stability of full lobe closure determines efficacy (Robert et al., 2005; Zhang et al., 2008a), such that partial

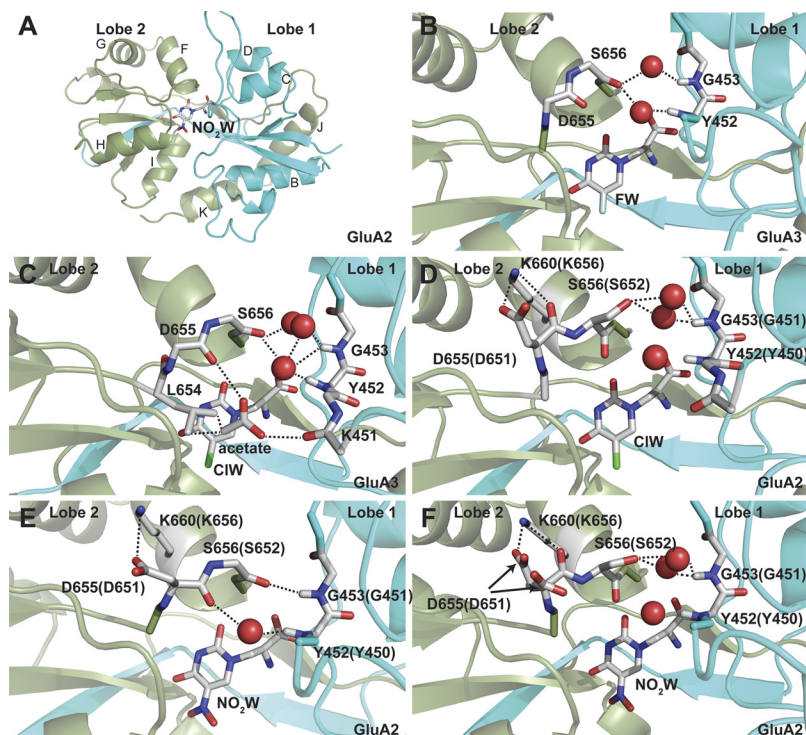


Fig. 6. A, GluA2 LBD bound to NO₂W (P2₁2₁2, $R = 20.7$, $R_{\text{free}} = 25.9$, 2.1-Å resolution). The N-terminal portion of the LBD is shown in cyan and the segment between M3 and M4 is shown in green. B, FW bound to GluA3 LBD (P222₁, $R = 19.6$, $R_{\text{free}} = 25.5$, 2.85-Å resolution). The Asp655/Ser656 is in the flipped conformation, however, and two waters mediate a hydrogen bonding network involving the backbone carbonyl of Ser656 and the backbone amides of Gly453 and Tyr452. C, CIW bound to GluA3 LBD (P222₁, $R = 22.3$, $R_{\text{free}} = 27.8$, 2.4-Å resolution). The Asp655/Ser656 is in the flipped conformation; however, an acetate molecule is present that forms an interlobe H-bond with the backbone carbonyls of Asp655 and Lys451, as well as three water molecules that join the carbonyl of Ser656 to the amides of both Tyr452 and Gly453. The acetate is a component of the crystallization buffer. D, CIW bound to GluA2 LBD (P22₁2₁, $R = 18.2$, $R_{\text{free}} = 20.3$, 1.7-Å resolution). The Asp655(Ser652) is in the unflipped conformation. E, NO₂W bound to GluA2 LBD. The Asp655(Ser652) is in the appropriate position to form hydrogen bonds to Gly453(Gly451) and Tyr452(Tyr450). F, NO₂W bound to GluA2 LBD. The Asp655(Ser652) is in the unflipped conformation. For both CIW and NO₂W, the side chain of Asp655(Ser652) forms a salt bridge with Lys660(Lys656). For all GluA2 structures, residue numbering is given as that for GluA3, with the GluA2 numbering in parenthesis.

closures probably represent intermediate states of low energy in the crystal. The evidence includes a demonstration that partial agonists can exist in a wide range of lobe orientations (Maltsev et al., 2008) that are in equilibrium (Fenwick and Oswald, 2008; Maltsev et al., 2008; Ahmed et al., 2009a) and that even the lowest efficacy agonists can access a fully closed state (A. H. Ahmed, S. Wang, H.-H. Chuang, and R. E. Oswald, manuscript in preparation). The crystal structures presented here show that FW-, CIW-, and NO₂W-bound LBD access a range of lobe orientations, some of which are fully closed. All structures show multiple side chain orientations near the lobe interface. Although multiple side-chain conformations do not directly imply multiple lobe orientations, they suggest the possibility of dynamics near the lobe interface. The side chain of Asp655 can make a salt bridge with Lys660, and the presence or absence of this salt bridge may influence the orientation of the Asp655/Ser656 bond and interlobe H-bonding (S. M. Holley, A. H. Ahmed, J. Srinivasan, S. E. Murthy, R. E. Oswald, and L. M. Nowak submitted). These results suggest that partial agonists exist in a range of lobe orientations and the differences in the population of conductance levels are less likely to be associated with a fixed degree of lobe closure. The question is how often these states are visited for each agonist. The fact that GluA2 can be crystallized in the fully closed state when bound to NO₂W may suggest that this state is of low energy. Why then is the efficacy of FW greater than NO₂W, albeit to a small degree, whereas the single-channel open probabilities are very similar? There are three potential structural differences between the bound forms of FW and NO₂W: 1) the orientation of Met712, 2) additional interactions with lobe 2 through the nitro group in NO₂W, and 3) possible differences in H-bonding across the lobe interface.

Considering the orientation of Met712, the rotameric state in both the CIW and NO₂W structures of GluA2 differs from that of previous structures (Jin and Gouaux, 2003). Although the significance of this change in rotameric state is difficult to assess, Met712 is part of helix I, which undergoes a bending

motion when bound to weak partial agonists, such as IW (Fenwick and Oswald, 2008). At the other end of helix I, a disulfide bond connects helix I to the C-terminal portion of the LBD, which in the intact protein is the beginning of the linker to the M4 helix. The M4 helix of one subunit is associated with the ion channel gating region of an adjacent subunit (Sobolevsky et al., 2009), and this slight change in Met712 could alter gating kinetics. In fact, mutations in the M4 segment of NMDA receptors alter function (Ren et al., 2003).

With residual dipolar coupling measurements using NMR spectroscopy, Maltsev et al. (2008) estimated that the proportion of the flipped conformation of the Asp655/Ser656

TABLE 4

Summary of structures of the LBD of GluA2 and GluA3

The degree of lobe opening was determined using the DomainSelect function in DynDom (Hayward and Lee, 2002). The reference structure is the B protomer of the glutamate-bound form of GluA2_o (Protein Data Bank code 3DP6; Ahmed et al., 2009b). Lobe opening is shown relative to the reference structure for each protomer. Either one or three protomers were observed in the asymmetric unit. Three crystals of GluA2 bound to NO₂W were analyzed with three protomers each. In cases in which more than one structure was obtained, the mean and S.D. is given followed by the range of lobe openings. The flipped versus unflipped conformation of the Asp655/Ser656 (GluA3 numbering) peptide bond is relative to the apo form as described by Armstrong and Gouaux (2000) and is indicated in the column labeled "peptide." The notation "f" or "u" indicates flipped or unflipped, and the number before the letter indicates the number of protomers in that conformation.

| Agonist | Protein Data Bank Code | Peptide | Lobe Opening | |
|-------------------|------------------------|---------|--------------|-----------|
| ° | | | | |
| GluA2 | | | | |
| Glu ^a | 3DP6 | 3f | 0.8 ± 0.8 | (0–1.7) |
| FW ^b | 1MY4 | 1u | | 4 |
| CIW | 3RTF | 3u | 2.9 ± 1.0 | (2.2–4.1) |
| NO ₂ W | 3RTW | 8u, 1f | 1.9 ± 1.2 | (0.7–3.9) |
| GluA3 | | | | |
| Glu ^a | 3DLN | 1f | 0.8° | |
| FW | 3RT6 | 1f | 2.6° | |
| CIW | 3RT8 | 1f | 3.7° | |

^a Ahmed et al. (2009b).

^b Jin and Gouaux (2003).

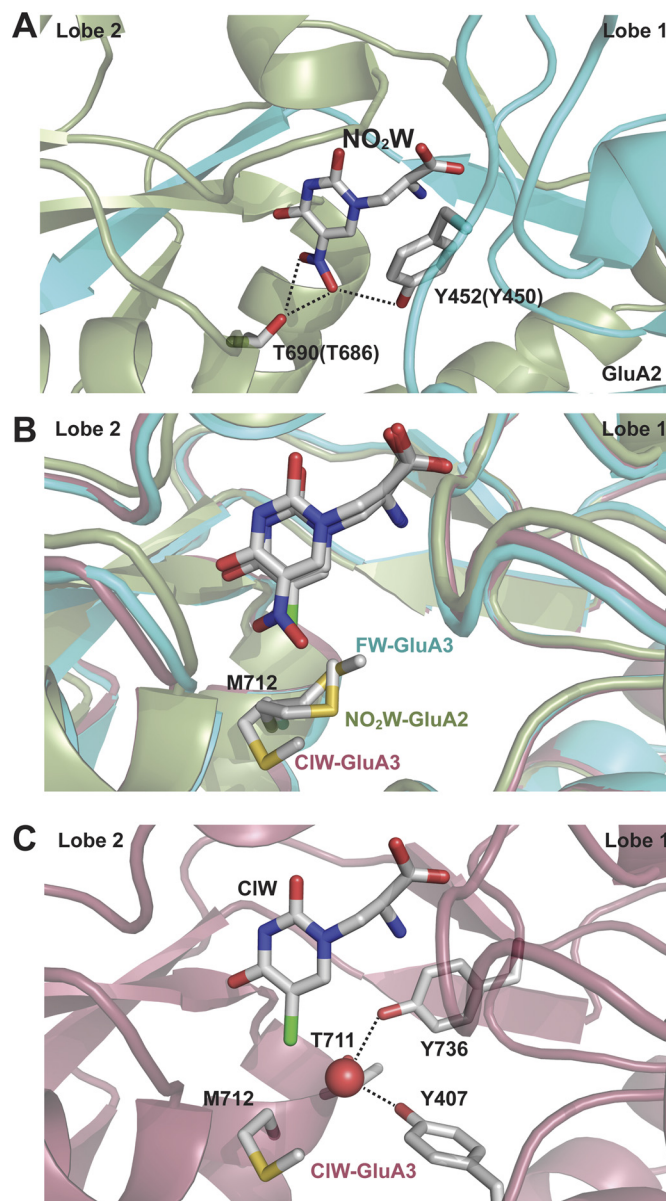


Fig. 7. A, the nitro group of NO₂W bound to GluA2 contributes to stability by making additional H-bonds to Thr686 and Tyr450 (GluA2 numbering; Thr690 and Tyr452 GluA3 equivalent). Residue numbering is that for GluA3, with the GluA2 numbering in parenthesis. B, the side chain of Met712 is in an extended state when bound to FW (cyan). There is a rotameric change at the β carbon when bound to NO₂W (green). The rotameric change is most dramatic with CIW bound (magenta). (C) The change in the rotameric state of Met712 when CIW is bound makes room for binding of an additional water molecule.

peptide bond varies with agonist: 0.54, 0.44, 0.46, 0.45, 0.28, and 0.29 for (S)-willardiine, FW, CIW, BrW, IW, and NO₂W, respectively. This would suggest that the Asp655/Ser656 bond is in a position to form H-bonds more often for FW and CIW than for NO₂W; however, as demonstrated by the GluA3 structures bound to FW and CIW, the flipped peptide bond does not always correspond to a fully closed lobe. The additional contacts made by NO₂W in lobe 2 (H-bonding to Thr690 and Tyr452) may stabilize lobe closure and compensate for the lower propensity to form a flipped conformation.

Modal Activation and the Peptide Flip Conformation. Modal activation of glutamate receptors has been reported for NMDA receptors (Popescu and Auerbach, 2003; Zhang et al., 2008b), GluA3 (Poon et al., 2010), and GluA1, GluA2, and GluA4 (Prieto and Wollmuth, 2010). Prieto and Wollmuth (2010) demonstrated a lack of binomial gating at subsaturating concentrations of glutamate in 200-ms records from outside-out patches, suggesting two gating modes. A cooperative interaction between subunits was postulated to change the affinity at lower concentrations of glutamate. In longer recordings, Poon et al. (2010) detected five distinct gating modes that were observed in both glutamate and FW. Although higher modes were more likely to be observed at higher concentrations, all modes were observed at all concentrations tested. Like glutamate and FW, activation of the channel by NO₂W and CIW exhibits five modes of activation (VL, L, M, H, and VH; Fig. 4). It is currently not possible to determine whether the two modes observed by Prieto and Wollmuth (2010) correspond to those described here. The difference may have arisen because the records reported here were obtained in cell-attached patch recordings and were considerably longer than those described by Prieto and Wollmuth (2010).

We previously postulated (Poon et al., 2010) that modal behavior could arise from locking a specified number of LBDs in the tetramer closed as a result of the flipping of the Asp655/Ser656 peptide bond. When full agonists are bound, the Asp655/Ser656 peptide bond flip results in the formation of two H-bonds between the carbonyl of Ser656 (lobe 2) and the amide of Gly453 (lobe 1) and between the carbonyl of Asp655 and the amide of Tyr452 through a water molecule. The fully closed, H-bonding pattern exists on the time scale of approximately 100 ms (Fenwick and Oswald, 2010), which is the time scale of modal gating. The number and arrangement of subunits bound to agonist and in the H-bond locked state could determine the mode. As described previously (Poon et al., 2010), the VH mode might be generated by full occupancy and three to four H-bonded subunits, and the lower modes would have progressively fewer H-bonds and a tendency toward lower occupation.

FW and CIW bound to GluA3 LBD show a variation on this H-bonding pattern. The peptide bond is flipped in the context of lobes that are slightly more open (~3° relative to the glutamate structure), making the H-bonds difficult to form. However, additional water molecules enter the cleft (in one case, an acetate molecule) and mediate the H-bonds between the two lobes. These hybrid H-bonded conformations have not been previously reported and may result in subtle functional effects. One possible outcome is the alternate kinetics observed with CIW in H mode. Assuming the modes are set by the occupancy and H-bonding state (Poon et al., 2010), this hybrid H-bonding state increases the number of possibilities.

If the hybrid H-bonding state has the same stability as the fully closed pattern but actually is a conformation that does not permit channel activation, then the H and VH modes that have extremely low probability for the largest conductance state may arise from a fully occupied tetramer with two to three subunits in the standard H-bonding conformation and one or two in the hybrid conformation.

Dwell Time Variation between Agonists. In the VH mode, NO₂W exhibits relatively long channel lifetimes, and the overall efficacy is slightly better than CIW. The size of the nitro group is larger than the chloro substituent, but the lobes are, on average, more closed for NO₂W than CIW. The fraction of bound NO₂W in the flipped conformation calculated from residual dipolar coupling measurements suggests NO₂W was 0.29 (similar to another low efficacy partial agonist, IW); whereas, the fractions for CIW and FW were 0.46 and 0.44, respectively (Maltsev et al., 2008). However, the additional contacts of NO₂W to the side chains of Thr690 and Tyr452 may help stabilize the fully closed state and contribute to longer openings in each conductance level. These interactions are not present in the other willardiine partial agonists and may compensate for the decreased population of the flipped conformation.

Conclusions

The structures of the willardiines bound to GluA2 and GluA3 LBD suggest that full lobe closures are possible, particularly with NO₂W. Structures of kainate bound to the LBD of a kainate receptor reveal 3° less closure than glutamate, yet kainate is a full agonist at kainate receptors (Mayer, 2005), further implying that crystal structures represent a subset of accessible conformational states. Partial agonists at NMDA receptors exhibit full lobe closures (Inanobe et al., 2005; Kussius et al., 2010). Although NMDA receptors have very different activation kinetics (slow activation, long openings) compared with AMPA receptors, the mechanism leading to a gating event may be conserved for all subtypes of glutamate receptors. Taken together with our previous results (Poon et al., 2010), these findings imply that agonist efficacy may be dependent upon the relative fraction of time spent in an ensemble of conformations that include fully open, fully closed, and intermediate degrees of closure. The stability of each conformer is in turn determined by multiple interlobe contacts that can differ between different agonists.

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Authorship Contributions

Participated in research design: Poon, Ahmed, Nowak, and Oswald
Conducted single channel recording experiments: Poon and Nowak
Analyzed single channel recording experiments: Poon, Nowak, and Oswald

Purified protein, prepared crystals, and collected X-ray data: Ahmed

Analyzed X-ray data: Ahmed and Oswald

Wrote or contributed to the writing of the manuscript: Poon, Ahmed, Nowak, and Oswald

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