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Mechanism of Inhibition of the GluA2 AMPA Receptor Channel Opening: The Role of 4-Methyl versus 4-Carbonyl Group on the Diazepine Ring of 2,3-Benzodiazepine Derivatives

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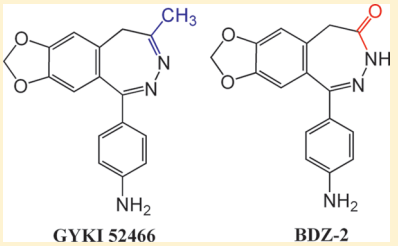
Supporting Information

ABSTRACT: 2,3-Benzodiazepine derivatives are synthesized as drug candidates for the potential treatment of various neurodegenerative diseases involving the excessive activity of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Here, we describe a rapid kinetic investigation of the mechanism of inhibition of the GluA2Q$_{flip}$ AMPA receptor channel opening by two 2,3-benzodiazepine derivatives, that is, the prototypic 2,3-benzodiazepine compound GYKI 52466 [(1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine)] and 1-(4-aminophenyl)-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one (BDZ-2). GYKI 52466 and BDZ-2 are structurally similar in that the 4-methyl group in the diazepine ring of GYKI 52466 is replaced by a carbonyl group, yielding BDZ-2. Using a laser-pulse photolysis technique with ~60 μs time resolution, we characterize the effect of the two compounds individually on the channel-opening process of the GluA2Q$_{flip}$ receptor expressed in HEK-293 cells. We find that BDZ-2 preferentially inhibits the open-channel state, whereas GYKI 52466 is more selective for the closed-channel state of the GluA2Q$_{flip}$ receptors. Each inhibitor binds independently to its own noncompetitive site, and the two sites do not interact allosterically. The significance of these results in the context of both the structure−activity relationship and the properties of the GluA2Q$_{flip}$ receptor channels is presented.



KEYWORDS: AMPA receptors, glutamate receptors, antagonist, kinetic mechanism, structure−activity relationship

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are one of three subtypes of ionotropic glutamate receptors (the other two being *N*-methyl-D-aspartic acid (NMDA) and kainate receptors). AMPA receptors mediate the majority of fast excitatory neurotransmission in the brain and are indispensible in brain development and activities such as memory and learning.[1,2] Excessive receptor activity, however, leads to calcium-mediated cellular toxicity, a causative factor in the pathogenesis of some acute and chronic neurological disorders such as stroke and amyotrophic lateral sclerosis (ALS). Thus, developing AMPA receptor antagonists to regulate excessive receptor activity has long been pursued as a therapeutic strategy for the treatment of these neurological disorders and diseases.

In an extensive effort to develop AMPA receptor inhibitors as potential drugs, 2,3-benzodiazepine derivatives, also known as GYKI compounds, have turned out to be one of the best classes of antagonists to date. In contrast to other classes of inhibitors such as quinoxalinedione compounds (e.g., NBQX and ZK200775),[3,4] 2,3-benzodiazepine derivatives are more selective toward AMPA receptors. Unlike 1,4-benzodiazepines, 2,3-benzodiazepine compounds do not possess sedative and anxiolytic activity, nor do they bind to the benzodiazepine site on γ-aminobutyric acid (GABA) ion channel receptors.[5,6] Furthermore, because 2,3-benzodiazepine compounds do not compete for the agonist binding site on AMPA receptors, they are thought to be noncompetitive inhibitors.[7] In vivo studies show that 2,3-benzodiazepine compounds are effective as anticonvulsants in seizure models[8−10] and as neuroprotective agents in both focal[11] and global ischemia.[12]

Despite a wealth of knowledge on the synthesis and pharmacological profiles of 2,3-benzodiazepine compounds, the detailed mechanism of action of these compounds on the AMPA receptor channel opening is poorly understood. To date, a structure−activity relationship for these compounds has not yet been defined on the time scale of the receptor channel opening or when the receptors are in the functional state, rather than a desensitized or ligand-bound, but channel closed state. In an attempt to address these deficiencies, we investigate the mechanism of action for these compounds using a laser-pulse photolysis technique with a photolabile precursor of glutamate or the caged glutamate (i.e., γ-*O*-(α-carboxy-2-nitrobenzyl)glutamate).[13] This technique provides an ~60 μs time resolution[13,14] and enables us to measure the rate of the receptor channel opening, separate from the rate of the ensuing desensitization reaction.[15] Consequently, we are able to measure the effect of a 2,3-benzodiazepine compound on the AMPA receptor channel-opening process within the μs-to-ms time domain.[14] An effect of an inhibitor on both the channel-opening rate constant, k_{op}, and the channel-closing rate constant, k_{cl} (or the lifetime of the channel =

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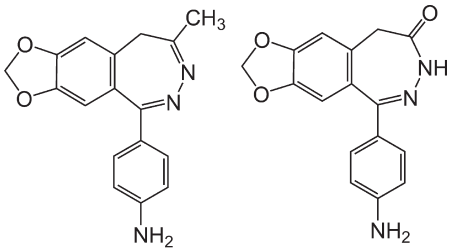

Figure 1. Chemical structures of GYKI 52466 and BDZ-2.

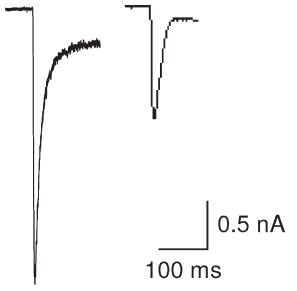

Figure 2. Representative whole-cell current responses from GluA2Q$_{flip}$ channels expressed in HEK-293 cells to 3 mM glutamate in the absence (left) and presence (right) of 60 μM GYKI 52466. The whole-cell current was recorded at −60 mV, pH 7.4, and 22 °C.

$1/k_{cl}$), reflects whether that inhibitor binds to and inhibits both the closed- and open-channel states. The presence of additional kinetic steps following the initial inhibitor binding may be also revealed. Extending this study to structurally similar 2,3-benzodiazepine compounds thus enables us to characterize the structure−activity relationship defined within the μs-to-ms time domain. Such a structure−activity relationship will be useful for designing more effective 2,3-benzodiazepine inhibitors with predictable properties.

In the present study, we focus on two 2,3-benzodiazepine compounds, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466)[7] and 1-(4-aminophenyl)-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one (BDZ-2).[16] GYKI 52466 is considered the prototype of this class, from which hundreds of derivatives have been synthesized. GYKI 52466 is also used frequently as the reference compound in evaluating other 2,3-benzodiazepine derivatives.[6] GYKI 52466 and BDZ-2 are structurally related in that BDZ-2 has a 4-carbonyl group whereas GYKI 52446 has a 4-methyl group on the diazepine ring (Figure 1). In order to replace the 4-methyl group of GYKI 52466 with a carbonyl group, the double bond between N3 and C4 of the diazepine ring has to be saturated as well (Figure 1). In other words, the structural change from GYKI 52466 to BDZ-2 is a replacement of the azomethine moiety with a ε-lactam moiety. Therefore, a potential difference in the inhibitory properties of these two compounds should be ascribed to the change of the structure.

RESULTS AND DISCUSSION

We transiently expressed the GluA2Q$_{flip}$ homomeric channels in human embryonic kidney (HEK-293) cells and investigated

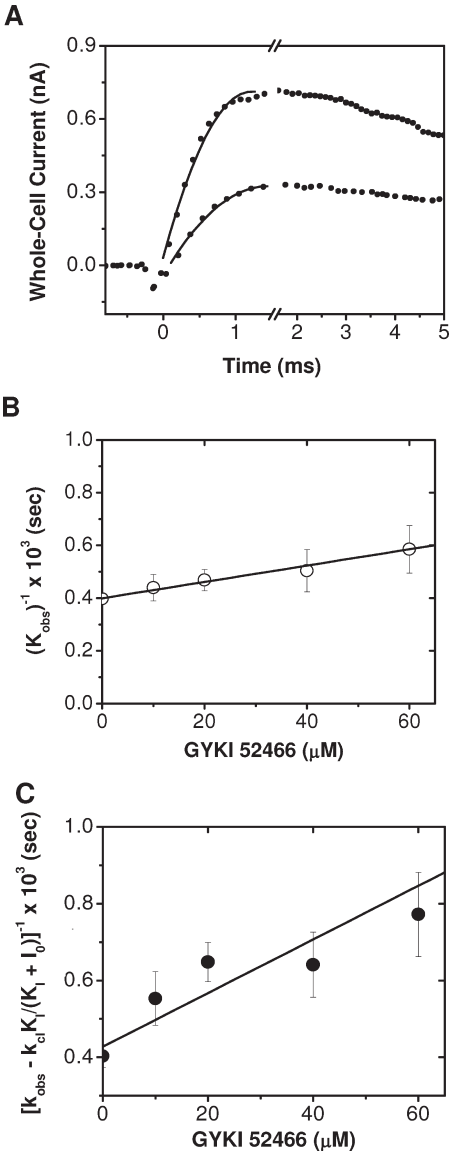

Figure 3. (A) Representative whole-cell current traces from the laser-pulse photolysis experiment showing that GYKI 52466 inhibited both the rate and amplitude of the opening of the GluA2Q$_{flip}$ channels (lower trace with 20 μM GYKI 52466) as compared to the control (upper trace). (B) Effect of GYKI 52466 on k_{cl}, measured over a range of concentrations of GYKI 52466 and at 100 μM of photolytically released glutamate. From the slope of this graph plotted using eq 4, a K_I of 128 ± 30 μM was obtained. (C) Effect of GYKI 52466 on k_{op}, obtained at 350 μM of photolytically released glutamate concentration. From this plot by eq 5, a K_I of 61 ± 11 μM was determined.

the mechanism of inhibition of this channel by GYKI 52466 and BDZ-2. Shown as an example (Figure 2), GYKI 52466 inhibited, as expected, the amplitude of the glutamate-induced whole-cell current. At all concentrations of inhibitors we used, the rate of channel desensitization was unaffected, consistent with the results from earlier studies of this class of inhibitors.[7,14,17] We therefore focused our investigation of the inhibitory effect on the rate of the channel opening and the current amplitude. Because the experimental data on GYKI 52466 and BDZ-2 are qualitatively the same, the figures and plots presented are mainly from GYKI 52466.

Table 1. Inhibition Constants Calculated from the Rate and the Amplitude Measurements

	laser-pulse photolysis technique					
	rate measurement		amplitude measurement		solution-flow technique	
inhibitor	K_I^* (μM) (closed-channel)	$\overline{K_I}^*$ (μM) (open-channel)	K_I (μM) (100 μM glu)	K_I (μM) (250−350 μM glu)	K_I (μM) (closed-channel)	$\overline{K_I}$ (μM) (open-channel)
GYKI 52466	61 ± 11	128 ± 30	15 ± 1.0	16 ± 1.0	14 ± 1.0	30 ± 2.0
BDZ-2	48 ± 5.0	194 ± 20	25 ± 1.0	23 ± 1.0	25 ± 1.0	7 ± 1.0

Using the laser-pulse photolysis technique, we characterized the effect of GYKI 52466 and BDZ-2, one at a time, on the channel-opening rate process of GluA2Q$_{flip}$. As shown (Figure 3A), both the time course and amplitude of the whole-cell current from the GluA2Q$_{flip}$ receptor channels, as triggered by the binding of photolytically released glutamate, were decreased in the presence of GYKI 52466. This indicated that the compound inhibited the opening of the GluA2Q$_{flip}$ receptor channels. The observed rate constant in the absence and presence of an inhibitor, k_{obs} and k_{obs}' respectively, was found to obey a first-order rate law for over 95% of the rising phase (Figure 3A). Such a monophasic rate process was observed without exception, from the lowest (100 ± 10 μM) to the highest (350 ± 20 μM) concentrations of photolytically released glutamate, and at every concentration of inhibitors (i.e., up to 60 μM) used in this study. In addition, the single-exponential rate process for the whole-cell current rise has been consistently observed in the channel opening rate process of not only GluA2Q$_{flip}$ in the absence[15] and presence of other inhibitors,[14,18] but other homomeric AMPA receptor channels as well.[19−21] These results therefore suggested that the rate of current rise in the laser-pulse photolysis measurement reflected the channel-opening, rather than the ligand-binding rate process[14,15] (see also a minimal mechanism for the channel opening and inhibition in Figure 5). On the other hand, the whole-cell current decay or the falling phase of the current (Figure 3A) was due to receptor desensitization. The rate of channel desensitization (Figure 3A) at any given glutamate concentration in the absence and presence of an inhibitor was at least 10-fold slower than the current rise, suggesting that the channel-opening rate process was measured virtually free from a relatively slower rate of desensitization.[14,15,19] Taken together, k_{obs}' reflected the effect of an inhibitor on the rate of the channel-opening process.

Next we varied the ligand (i.e., glutamate) concentration in order to characterize the effect of an inhibitor on k_{op} and k_{cl}. This is because k_{obs} is a function of ligand concentration (see eq 2 in the Appendix in the Supporting Information) and in general the magnitude of k_{obs} is contributed by both the k_{cl} and k_{op} terms.[15,21] However, when ligand concentration is lowered, the k_{obs} expression or eq 2 can be reduced to $k_{obs} \approx k_{cl}$ (see Appendix). Previously we rationalized[14,15,19] that a glutamate concentration that correlates to ~4% of the fraction of the open-channel form satisfies the condition under which $k_{obs} \approx k_{cl}$. Specifically for GluA2Q$_{flip}$ channels, this correlates to 100 μM glutamate concentration.[14,15,19] Thus, if the channel-opening rate is measured at 100 μM glutamate concentration but at varying inhibitor concentrations, the inhibition constant for the open-channel state or $\overline{K_I}^*$ can be uniquely determined from k_{obs}' using eq 4. Following this rationale, we characterized the effect of GYKI 52466 on k_{obs} (Figure 3B) and determined $\overline{K_I}^*$ to be 128 ± 30 μM; for BDZ-2, $\overline{K_I}^*$ was found to be 194 ± 20 μM (see also the summary of these data in Table 1). Furthermore, the effect of an inhibitor on k_{op} was determined at a higher ligand concentration, where $k_{obs} > k_{cl}$. As such, the inhibition constant for the closed-channel state (i.e., K_I^* in this case) was estimated by the use of eq 5 (Figure 3C is an example of this experiment). Specifically, K_I^* was estimated to be 61 ± 11 μM for GYKI 52466 and 48 ± 5 μM for BDZ-2, respectively (these data are also summarized in Table 1).

GYKI 52466 and BDZ-2 inhibited both k_{op} and k_{cl} (Table 1), consistent with a noncompetitive mechanism for both compounds. By a noncompetitive model, GYKI 52466, for example, bound to its noncompetitive site on the receptor, and such a site was accessible through both the closed-channel and the open-channel states. Consequently, binding of GYKI 52466 to both the closed-channel and the open-channel conformations led to inhibition, which was manifested in an inhibitory effect of this compound on both k_{op} and k_{cl}. In contrast, if GYKI 52466 or BDZ-2 inhibited the channel competitively by displacing the glutamate from its binding site, only the effect on k_{op}, but not on k_{cl}, would be expected (i.e., there would be no $[\overline{K_I}^*/(\overline{K_I}^* + I)]$ term associated with k_{cl} in eq 3, and thus $1/k_{obs}$ as in eq 4 would be independent of inhibitor concentration). If GYKI 52466 inhibited the channel uncompetitively, commonly known as open-channel blockade, only the effect on k_{cl}, but not on k_{op}, would be expected (i.e., there would be no $[\overline{K_I}/(\overline{K_I} + I)]$ term associated with k_{op} in eq 3, and thus, in eq 5, $(k_{obs} - k_{cl}')$ would be independent of inhibitor concentration).

We also measured the effect of inhibition on the amplitude of the whole-cell current. This experiment served two purposes. First, the ratio of the whole-cell current amplitude in the absence and presence (A/A_I) allowed us to determine an inhibition constant independent from the rate measurement (see Methods, and eqs 6 and 7 in the Supporting Information). Second, we used a rapid solution flow approach as an independent method/control to measure the amplitude of the whole-cell current.[14] To measure the inhibition constant for the closed- and open-channel states separately, we used a low and a high glutamate concentration.[14,15] Specifically, the A/A_I ratio determined as a function of inhibitor concentration allowed us to calculate the K_I value, using eq 6, for the closed-channel, determined at 100 μM glutamate (where the majority of the receptor was in the closed-channel state), and the open-channel state, determined at 3 mM glutamate concentration (where the majority of the receptor was in the open-channel state).[14,15] The inhibition constants estimated for GYKI 52466 (Figure 4) and BDZ-2 are summarized in Table 1. For any particular compound, the inhibition constants estimated from the two methods, that is, laser and flow measurements, based on the ratio of the whole-cell current amplitude, were in good agreement (Figure 4 and Table 1). At comparable glutamate concentrations such as 100 and 250 μM (note that amplitude data at the 250 μM level was collected from the laser

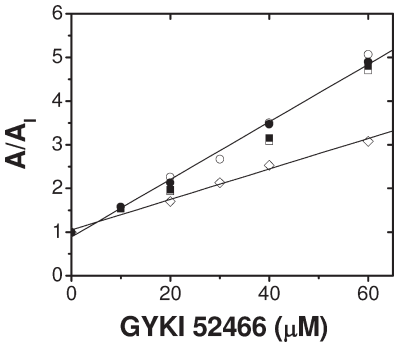

Figure 4. Effect of GYKI 52466 on the amplitude of the whole-cell current as plotted using the A/A_I ratio. This graph shows the different binding affinities at 100 μM, 250 μM, and 3 mM glutamate using both regular solution flow measurements and laser-pulse photolysis. The 3 mM glutamate (◇) has a $\overline{K_I}$ of 30 ± 2.0 μM. At 100 μM glutamate, the solution flow measurement (○) has a K_I of 15 ± 1.0 μM, while the laser-pulse photolysis (●) shows a K_I of 15 ± 1.0 μM. At 350 μM glutamate, the cell flow measurement (□) has a K_I value of 16 ± 1.0 μM, while the laser-pulse photolysis (■) shows a K_I of 16 ± 1.0 μM.

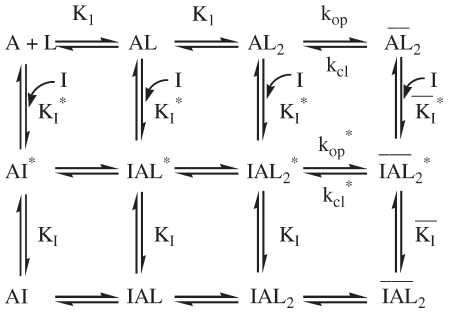

Figure 5. Minimal mechanism of the inhibition of the GluA2Q$_{flip}$ receptor by GYKI 52466 and BDZ-2.

experiment only), the A/A_I ratios determined from the two methods were roughly identical (i.e., the data in columns 3−5 in Table 1). This result was expected because the fractions of receptors in the open-channel form were ∼4% and ∼8%, respectively.[15] In fact, K_I estimated under these conditions (different glutamate concentrations, and different techniques as shown in Table 1) ranged from 14 ± 1.0 to 16 ± 1.0 μM for GYKI 52466, and from 23 ± 1.0 to 25 ± 1.0 μM for BDZ-2. On the other hand, at 3 mM glutamate concentration where ∼95% of the channels were supposedly in the open-channel conformation, the $\overline{K_I}$ value for the open-channel conformation was found to be 30 ± 2.0 μM for GYKI 52466 and 7.0 ± 1.0 μM for BDZ-2. It should be noted, however, that the laser-pulse photolysis of the caged glutamate to deliver 3 mM photolysized glutamate was not practical, and thus, there were no rate or amplitude data from the laser measurement at this concentration.

Comparison between K_I for the closed-channel state and $\overline{K_I}$ for the open-channel state, as calculated from the amplitude data (Figure 4 and Table 1), led us to conclude that GYKI 52466 had ∼2-fold higher potency for the closed-channel state of the GluA2Q$_{flip}$ receptor, whereas BDZ-2 exhibited more than 3-fold potency for the open-channel state. Furthermore, BDZ-2 was a better inhibitor because it inhibited GluA2Q$_{flip}$ channels more strongly than GYKI 52466 did ($\overline{K_I}$ = 7 μM for BDZ-2 vs K_I = 14 μM for GYKI 52466). Specifically, BDZ-2 lost the potency to the closed-channel state by about 2-fold, yet it gained the potency for the open-channel state, as compared to GYKI 52466, by more than 4-fold (Table 1).

However, a close examination of the inhibition constants for the same inhibitor showed a discrepancy in the constants between the rate and the amplitude measurements (see the laser-pulse photolysis data in columns 1−4 in Table 1). For instance, at a glutamate concentration of 100 μM, we found a K_I* of 61 μM as *determined by the rate analysis* of GYKI 52466 data. Yet a K_I of 15 μM was calculated from the amplitude of the whole-cell current traces from the same laser-pulse photolysis measurement (Figure 3A). This represented a 4-fold difference. By the same comparison, there was a 2-fold difference for BDZ-2. In fact, an inhibition constant obtained from the amplitude measurement for either inhibitor, whether such a constant was pertinent to the open-channel or the closed-channel state, was always smaller than the corresponding value obtained from the rate measurement.

Such a discrepancy can be ascribed to a minimal mechanism of inhibition (Figure 5). By this mechanism, the initial binding of GYKI 52466 or BDZ-2 is assumed to form a loosely bound intermediate with the receptor ($\overline{IAL_2}$*) at the first step. Such an intermediate is partially capable of conducting ions resulting in a partial inhibition of receptor activity. In the second step, the intermediate isomerizes rapidly into a tighter complex ($\overline{IAL_2}$) which is no longer capable of conducting ions. The two-step inhibition process involving the formation of the initial, loose intermediate is pertinent to both the closed- and open-channel states.

By this mechanism (Figure 5), the mode of action of GYKI 52466 and BDZ-2 can be sufficiently explained. First, both the rate and the amplitude measurements in the laser-pulse photolysis experiment with GYKI 51466 and BDZ-2 were associated with the channel-opening process. Therefore, a smaller K_I value or a stronger inhibition observed from the amplitude measurement, an equilibrium measure, suggested that an observed, larger magnitude of the inhibition constant from the rate measurement was only part of the overall inhibition. An additional step, following the formation of the initial loose intermediate of GYKI 52466 with the receptor, was required to turn over the initial, partially conducting intermediate into a totally inhibitory complex, thereby yielding additional inhibition. This means that, in the rate measurement, only one step or precisely the slower step, that is, the initial step in the mechanism (Figure 5), was observable. In fact, the evaluation of the inhibition constants using the rate data was based on a one-step process (eqs 4 and 5 in the Appendix in the Supporting Information). These equations allowed us to estimate the inhibition constants, which were larger than those obtained from the amplitude measurement, and which we assigned to the initial step as in this minimal model of inhibition (Figure 5).

Furthermore, in the presence of an inhibitor, the rate of the channel opening was slowed as compared with the control (Figure 3A). A slower rate was assumed to reflect the first step or the step involving the formation of the intermediate (Figure 5). This assumption was based on the fact that throughout the concentration range for both glutamate and the inhibitors, only a single exponential rise for the opening of the channel was observed. This observation also suggested that the rates for the two steps are significantly different. If the second step involving isomerization reaction was slow or comparable to the first step, we would expect a full or close to full inhibition such

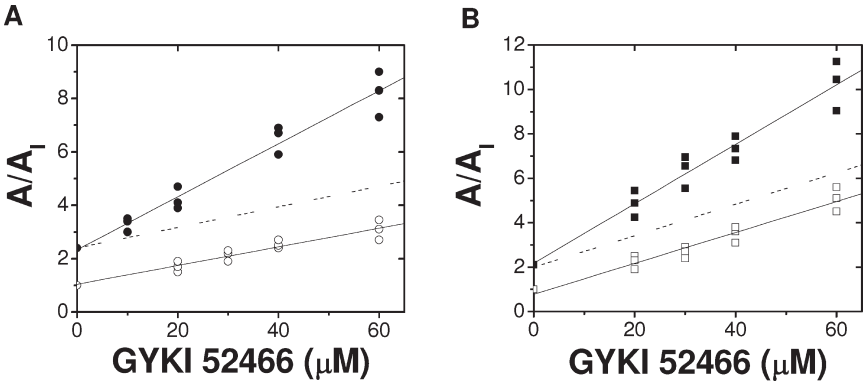

Figure 6. (A) Double inhibition of GYKI 52466 and BDZ-2 at the open-channel form using 3 mM glutamate. BDZ-2 was set at 10 μM. The open circle represents GYKI 52466 only, while the filled circle represents both GYKI 52466 and BDZ-2. The K_I of the double inhibition is 10 $\pm$ 1.0 μM compared to the 30 $\pm$ 2.0 μM of just GYKI 52466 (n = 3 cells). (B) Double inhibition of GYKI 52466 and BDZ-2 at the closed-channel state using 100 μM glutamate. BDZ-2 was fixed at 30 μM. The open square represents GYKI 52466 only, while the filled square represents both GYKI 52466 and BDZ-2. The K_I of the double inhibition is 8 $\pm$ 1.0 μM compared to the 15 $\pm$ 1.0 μM of just GYKI 52466 (n = 3 cells). The dashed lines indicate theoretical results from a one-site model (eq 8) using the K_I values obtained from the experiments.

that the inhibition constants calculated from the rate data would agree closely with those from the amplitude data.[22] The absence of this scenario suggests that the second step from both the closed-channel and the open-channel pathways (Figure 5) would have to be much faster than the initial step. As such, the decrease in k_{obs} in the presence of inhibitor was correlated to the increase in the inhibitor concentration. More precisely, $(1/k_{obs})$ increased linearly with increasing inhibitor concentration (Figure 3B and C), as predicted by eqs 4 and 5. Thus, the inhibition constants determined from the laser-pulse photolysis measurement of the channel-opening rate process were assigned to the first step associated with the initial inhibitor-receptor intermediates. Specifically, $\overline{K_I}^*$ and K_I^* correspond to the inhibitor−receptor intermediates involving the open- and the closed-channel conformations of the receptor (Table 1). It should be noted that a two-step inhibition mechanism like the one we propose for GYKI 52466 or BDZ-2 (Figure 5) is the same mechanism that we have proposed from the study of other 2,3-benzodiazepine derivatives.[14] Similar mechanisms involving two-step inhibition are also well documented for enzyme inhibitors[23,24] and inhibitors of the nicotinic acetylcholine receptor, such as cocaine[25] and MK-801.[26] For cocaine, the first step yields a corresponding complex thought to be equally if not more conductible, thus producing no inhibitory effect on k_{obs} at either low or high ligand concentration.[25] For MK-801, the second step, which yields the nonconducting inhibitor−receptor complex, is thought to occur only through the open-channel form.[26]

Given that GYKI 52466 and BDZ-2, two structurally similar compounds, clearly preferred different conformations of the same receptor (Table 1), we asked whether the two inhibitors bound to the same site or two distinct noncompetitive sites on GluA2Q_{flip}. To address this question, we carried out a double-inhibitor experiment (see Methods). Comparison of the ratio of the current amplitude in the absence and the presence of the two inhibitors (Figure 6) with the ratio in one inhibitor (Figure 4) showed that the slope from which the $K_{I,app}$ value was determined (using eq 8) was higher when both inhibitors were present. This result suggested that GYKI 52466 and BDZ-2 bound to two different sites on the same receptor, a conclusion verified at two different glutamate concentrations (Figure 6), reflecting the closed- and the open-channel states. Specifically, the double-inhibitor experiment yielded a $K_{I,app}$ of 10 $\pm$ 1 μM for the open-channel state and 8 $\pm$ 1 μM for the closed-channel state (Figure 6A). Yet the corresponding $K_{I,app}$ for GYKI 52466 *alone* was 30 and 14 μM for the open- and closed- channel states, respectively (Figure 4 and Table 1). As a control, we repeated the double-inhibitor experiment by keeping the concentration of GYKI 52466 constant while varying the concentration of BDZ-2 (Supporting Information Figure 1), and we observed the same phenomenon. As expected, if the two inhibitors bound to their respective sites independently, the total inhibitor concentration on the same receptor would be higher, thus yielding a stronger inhibition as opposed to a single inhibitor (eq 8). Conversely, if GYKI 52466 and BDZ-2 bound to the same site, an apparent inhibition constant calculated from the slope (eq 7) would be invariant irrespective of the mixture of GYKI 52466 and BDZ-2 (long, dashed line in both Figure 6A and B).

The results from the double-inhibitor experiment allowed us to further deduce the inhibition constant of GYKI 52466 for the closed- and open-channel states by using eq 8. The derivation of eq 8 was in fact based on the model by which the inhibition was contributed *independently* by the binding of the two sites. Granted, the inhibition constants for GYKI 52466, evaluated from the amplitudes in the presence of both GYKI 52466 and BDZ-2 (Figure 6), would be identical to those evaluated from GYKI 52466 alone (Figure 4). By the use of eq 8, together with a 10 μM concentration of BDZ-2 with $\overline{K_I}$ = 7 μM (Table 1), a $\overline{K_I}$ of 25 μM was determined for GYKI 52466 for the open-channel state (legend of Figure 6A). Similarly, by the use of 30 μM BDZ-2 with K_I = 25 μM for BDZ-2 (Table 1), a K_I of 17 μM for GYKI 52466 for the closed-channel state was obtained. These values were comparable with $\overline{K_I}$ of 30 μM and K_I of 14 μM, as determined from GYKI 52466 alone (Figure 4 and Table 1), suggesting that the two sites to which GYKI 52466 and BDZ-2 bound did not have a significant interaction, if any.

The results from this study establish that GYKI 52466 and BDZ-2 act as noncompetitive inhibitors on GluA2Q_{flip} receptor. The minimal mechanism of inhibition consists of a two-step process involving the formation of loosely bound, partially conducting intermediates. BDZ-2 preferentially inhibits the open-channel state, whereas GYKI 52466 is more selective for the closed-channel state. Although the two inhibitors are

structurally similar, each inhibitor binds to its own noncompetitive site, and the two sites do not interact allosterically. These results provide not only specific implications on the functional consequence of the substitution of the azomethine moiety on the diazepine ring with ε-lactam moiety but also broad implications in the structure−activity relationship for the 2,3-benzodiazepine compound series.

If we use the two inhibition constants assigned for the initial step, that is, K_I^* associated with the closed-channel state and $\overline{K_I}^*$ associated with the open-channel state (see columns 1 and 2 in Table 1), as a rough measure of how tight the complexes are (i.e., the tighter the complex, the more potent the inhibitor), the comparison of K_I^* and $\overline{K_I}^*$ allows us to draw the following implications. Both compounds have a tighter affinity for the closed-channel rather than the open-channel conformation (GYKI 52466 shows a 2-fold higher affinity or 61 vs 128 μM, whereas BDZ-2 shows 4-fold difference or 48 vs 194 μM). This comparison suggests that the open-channel conformation is more rigid whereas the closed-channel is more flexible, consistent with the notion we have proposed previously.[14] Upon isomerization, GYKI 52466 improves its overall potency on both the closed- and open-channel conformations. Specifically, the inhibition constant is reduced from 61 to 15 μM and from 128 μM to 30 μM for the closed- and open-channel conformations, respectively, representing a 4-fold improvement on both the closed-channel and the open-channel conformations. Although the isomerization reaction tightens the complex, it does not reverse the conformation selectivity of GYKI 52466: overall GYKI 52466 inhibits the closed-channel conformation with a 2-fold higher selectivity. For BDZ-2, however, the isomerization reaction reverses its selectivity in that BDZ-2 is selective for the open-channel over the closed-channel conformation by more than 3-fold (i.e., from 25 μM to 7 μM after isomerization). This means that the isomerization reaction doubles the potency of BDZ-2 for the closed-channel conformation (i.e., from 48 μM to 25 μM). However, the same reaction brings about a 28-fold improvement of potency for the open-channel conformation (i.e., from 194 to 7 μM; see Table 1). As compared with the original GYKI 52466,[5] we conclude that (i) BDZ-2 is a better compound by 4-fold, but the improvement is only on its selectivity toward the open-channel conformation ($\overline{K_I}$ = 7 μM for BDZ-2 vs $\overline{K_I}$ = 30 μM for GYKI 52466); (ii) the loose intermediates formed in the first step do not have much influence on the overall selectivity of these compounds.

What makes BDZ-2 a more potent and more open-channel conformation-selective inhibitor than GYKI 52466? The most obvious explanation is the replacement of the methylimine function of GYKI 52466 with a carboxyamide group. If this replacement is responsible for this selectivity reversal, it suggests that the electron character of a functional group at the C4 position is a structural feature of the inhibitor. In particular, a carbonyl group at the C4 position makes the inhibitor selective toward the open-channel conformation whereas the presence of a methyl group changes the inhibitor into a closed-channel preferred compound. This can be explained on the basis of the ability of the 4-carbonyl group to act as a hydrogen bond acceptor with respect to the 4-methyl group in GYKI 52466. The ability to form a hydrogen bond involving this carbonyl group on the benzodiapine ring with the receptor may reflect the intimate interaction of the amino acids in the receptor site surrounding this group. More studies with different compounds are obviously needed in order to test if this hypothesis is generalizable. If so, 2,3-benzodiazepine inhibitors with a predictable conformation selectivity on $GluA2Q_{flip}$ can be tailor-made by the substitution of the azomethine moiety in GYKI 52466 with an ε-lactam moiety.

The functional consequence of a carbonyl group at the C4 position of BDZ-2 with respect to the 4-methyl group in GYKI 52466 is further manifested by the fact that this structural substitution changes not only the conformation selectivity for the same receptor but also the site of binding. In other words, BDZ-2 no longer binds to the same site as GYKI 52466 does. This is totally different from the derivatization on the N3 position of the benzodiazepine ring we reported earlier where acetylating the N3 position by adding a methylcarbamoyl group changes potency but does not change the site of binding for the new compound.[14] Therefore, our results suggest that a functional group at the C4 position on the diazepine ring is less accommodated by the receptor site surrounding the group and thus is more critical in defining a site of noncompetitive inhibition. Currently, however, neither the location of the site(s) of these noncompetitive inhibitors nor the identity of the surrounding amino acids on the receptor inferred from this study is known.

CONCLUSION

In the present study, we establish the mechanism of inhibition and the site of interaction for two structurally related inhibitors, GYKI 52466, the prototypic 2,3-benzodiazepine compound, and BDZ-2, by measuring the effects of these inhibitors on both the rate and the current amplitude of channel-opening process of $GluA2Q_{flip}$ receptors. Our results are consistent with a noncompetitive mechanism of inhibition for both compounds, and the double-inhibitor experiments further show that GYKI 52466 and BDZ-2 bind to two regulatory sites that appear to be independent. In the context of the structure−activity relationship, our findings reveal that the substitution of the azomethine group on the diazepine ring of GYKI 52466 with a ε-lactam group imparts conformation selectivity and site of binding on $GluA2Q_{flip}$ for BDZ-2. Future studies of more 2,3-benzodiazepine compounds are needed to better understand these noncompetitive sites and to investigate if the site selection is influenced by the presence of a hydrogen bond acceptor at the C4 position on the diazepine ring. The results from this study illustrate a potential utility of applying the same approach to the study of the mechanism of inhibition of other ligand-gated ion channel receptors, including other subtypes of glutamate ion channel receptors. The mechanistic clues learned from a detailed kinetic study can be used in synthesizing new molecules with predictable functionality for a more quantitative control of the activity of target receptors.

METHODS

Expression of cDNA and Cell Culture. The DNA plasmid encoding $GluA2Q_{flip}$ was prepared as described.[14,15] HEK-293S cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and in a 37 °C, 5% CO_2, humidified incubator. $GluA2Q_{flip}$ was transiently expressed in these cells using a calcium phosphate method. HEK-293S cells were also cotransfected with a plasmid encoding for green fluorescent protein (GFP) as a transfection marker and a separate plasmid encoding large T-antigen to enhance the receptor expression at the single cell level.[27] The weight ratio of the plasmid for GFP and the large T-antigen to that for GluA2

was 1:10, respectively, and the $GluA2Q_{flip}$ plasmid used for transfection was ~5−10 μg/35 mm dish. The cells were used 48 h after transfection.

Whole-Cell Current Recording. A recording electrode was made from glass capillary (World Precision Instruments, Sarasota, FL) and had a resistance of ~3 MΩ when filled with the electrode solution.[14] The electrode solution was composed of (in mM) 110 CsF, 30 CsCl, 4 NaCl, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES (pH 7.4 adjusted by CsOH). The external solution contained (in mM) 150 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH 7.4 adjusted by NaOH). All chemicals were from commercial sources. The whole-cell current was recorded using an Axopatch-200B amplifier at a cutoff frequency of 2−20 kHz by a four-pole, low-pass Bessel filter, and digitized at 5−50 kHz sampling frequency using a Digidata 1322A apparatus from Axon Instruments. The data were acquired using pCLAMP 8 (Molecular Devices, Sunnyvale, CA). All whole-cell recordings were performed at −60 mV, pH 7.4, and 22 °C.

Laser-Pulse Photolysis Measurements. The use of the laser-pulse photolysis technique to measure the channel-opening kinetics has been described previously.[14] Briefly, the caged glutamate[13] (Invitrogen, Carlsbad, CA) was dissolved in the external buffer and applied to a cell using a flow device. In the photolysis measurement, a single laser pulse at 355 nm was generated from a Minilite II pulsed Q-switched Nd:YAG laser from Continuum (Santa Clara, CA). The pulse energy was adjusted to be 200−800 μJ, measured at the end of a fiber optic into which the laser was coupled. To determine the concentration of glutamate generated photolytically by laser photolysis, we calibrated the receptor response in the same cell by applying two solutions of free glutamate with known concentrations before and after laser flash, with reference to the dose−response relation.[14] These measurements also permitted us to monitor any damage to the receptors and/or the cell for successive laser experiments with the same cell. Furthermore, in all experiments reported in this study, we found that both GYKI 52466 and BDZ-2 required at least 3 s preincubation in order to exert full inhibition, a phenomenon we previously observed with other 2,3-benzodiazepine compounds.[14] Preincubation with any of these compounds for as long as 10 s caused no further current reduction. Therefore, a 6 s preincubation protocol was used in both the flow and laser-pulse photolysis measurements for both the rate and amplitude measurements.

Experimental Design and Data Analysis. The determination of the effect of an inhibitor on k_{op} and k_{cl} as a function of inhibitor concentration and at two glutamate concentrations from the laser-pulse photolysis measurement is described in the text, and the equations used for data analysis are located in the Appendix of the Supporting Information. Also located in the Appendix are the description and the equations for measuring the effect of an inhibitor on the current amplitude, including the use of double-inhibitor experiment to assess whether two inhibitors bind to the same site or to two separate sites on the same receptor. In addition, when the free glutamate was used to evoke the receptor response either in the absence or in the presence of an inhibitor, the amplitude of the whole-cell current was corrected for receptor desensitization during the rise time by a method previously described.[14,28] The corrected current amplitude was used for data analysis, as described in the Appendix. Origin 7 (Origin Lab, Northampton, MA) was used for both linear and nonlinear regressions (Levenberg−Marquardt and simplex algorithms). Each data point shown in the plots of this study was an average of at least three measurements collected from at least three cells unless otherwise noted. The error reported refers to the standard error of the fits.

■ ASSOCIATED CONTENT

Supporting Information. Figure showing the double-inhibitor experiment by keeping the concentration of GYKI 52466 constant but varying the concentration of BDZ-2, and Appendix listing all equations for the analysis of the rate and amplitude data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.R. conducted most of the experiments; C.Z.W. did double inhibitor experiments and produced some plots; N.M. and R.E. synthesized and characterized compounds used; L.N. directed the research and wrote the paper based on the draft written by M.R.

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■ ABBREVIATIONS

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDZ, 2,3-benzodiazepine compounds; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; BDZ-2, 1-(4-aminophenyl)-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one; caged glutamate, γ-*O*-(α-carboxy-2-nitrobenzyl)glutamate; HEK-293 cells, human embryonic kidney cells

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