

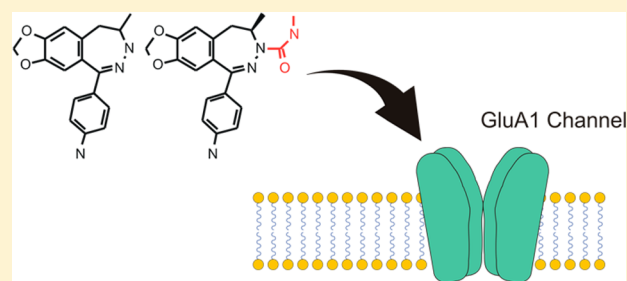
Mechanism of Inhibition of the GluA1 AMPA Receptor Channel Opening by the 2,3-Benzodiazepine Compound GYKI 52466 and a *N*-Methyl-Carbamoyl Derivative

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Supporting Information

ABSTRACT: 2,3-Benzodiazepine derivatives, also known as GYKI compounds, represent a group of the most promising synthetic inhibitors of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Here we investigate the mechanism of inhibition of the GluA1 channel opening and the site of inhibition by GYKI 52466 and its *N*-3 methyl-carbamoyl derivative, which we term as BDZ-*f*. GluA1 is a key AMPA receptor subunit involved in the brain function. Excessive activity and elevated expression of GluA1, however, has been implicated in a number of neurological disorders. Using a laser-pulse photolysis technique, which provides $\sim 60 \mu\text{s}$ resolution, we measured the effect of these inhibitors on the rate of GluA1 channel opening and the amplitude of the glutamate-induced whole-cell current. We found that both compounds inhibit GluA1 channel noncompetitively. Addition of an *N*-3 methyl-carbamoyl group to the diazepine ring with the azomethine feature (i.e., GYKI 52466) improves the potency of the resulting compound or BDZ-*f* without changing the site of binding. This site, which we previously termed as the “M” site on the GluA2 AMPA receptor subunit, therefore favorably accommodates an *N*-3 acylating group. On the basis of the magnitude of the inhibition constants for the same inhibitors but different receptors, the “M” sites on GluA1 and GluA2 are different. Overall, the “M” site or the binding environment on GluA2 accommodates the same compounds better, or the same inhibitors show stronger potency on GluA2, as we have reported previously [Wang et al. *Biochemistry* (2011) 50, 7284–7293]. However, acylating the *N*-3 position to occupy the *N*-3 side pocket of the “M” site can significantly narrow the difference and improve the potency of a resulting compound on GluA1.



The α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptor is one of the three receptor subtypes of the glutamate ion channel receptor family with the other two subtypes being the *N*-methyl-D-aspartate (NMDA) receptor and kainate receptor.^{1,2} AMPA receptors are the principal excitatory neurotransmitter receptors in the brain and are indispensable for brain activities, such as memory and learning.^{1,2} AMPA receptors have four subunits, i.e., GluA1–4. GluA2 is subject to RNA editing at the glutamine/arginine (Q/R) site. GluA1, 2Q (the unedited isoform of GluA2), 3 and 4 can all form functional, homomeric channels. However, GluA2R (the edited isoform of GluA2) must coassemble with other subunits to form functional channels.^{1,2}

The GluA1 AMPA receptor subunit plays important roles in the brain. GluA1 is predominantly expressed in the forebrain, including the hippocampus, a region that has been implicated in memory formation.³ In hippocampus, homomeric GluA1 channels are thought to constitute $\sim 8\%$ of the total AMPA receptor complexes.⁴ In pyramidal neurons of the adult hippocampus, the GluA1/GluA2R AMPA receptor is one of the two major complex AMPA receptor channels.⁵ Furthermore, GluA1 is critical for hippocampal synaptic transmission and plasticity.^{6,7} For example, phosphorylation of S831 of

GluA1 mediates such plasticity, as phosphorylation of this site increases during long-term potentiation (LTP).^{8,9} Studies with the use of GluA1 knockout mice have revealed that this subunit is essential for LTP in the CA1 region of the adult hippocampus,⁶ at least in the rapidly decaying, GluA1-dependent phase of LTP at hippocampal CA3–CA1 synapses.¹⁰

Excessive activity and elevated expression of GluA1 may be linked to neurological diseases.^{11–14} For example, the GluA1 expression level at the dentate gyrus of Alzheimer's patients is thought to be elevated.¹⁵ After conditioning administration of a drug of abuse, the GluA1 expression in central nucleus of the amygdala is significantly increased, but only acutely.¹⁶ In response to acute pain, GluA1 is upregulated.¹⁷ The increase of GluA1 and the concurrent decrease of GluA2 expression occur at the synapses between peripheral nociceptive and dorsal horn neurons.^{18,19} Therefore, it is useful to develop selective AMPA receptor antagonists, particularly those that target the GluA1 subunit. These inhibitors can be used as potential drugs and/or

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selective tools in the investigation of the specific function of the GluA1 subunit *in vivo*.

In the study, we investigate the mechanism of inhibition of the GluA1 channel opening and the site of inhibition by two 2,3-benzodiazepine (2,3-BDZ) compounds, i.e., GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine) and its N-3 methyl-carbamoyl derivative (i.e., (R)-5-(4-aminophenyl)-8-methyl-7-(N-methyl-carbamoyl)-8,9-dihydro-7H-1,3-dioxolo[4,5-h][2,3]benzodiazepine) (for convenience, we term this compound as BDZ-*f*; see chemical structures of both compounds in Figure 1). 2,3-BDZ

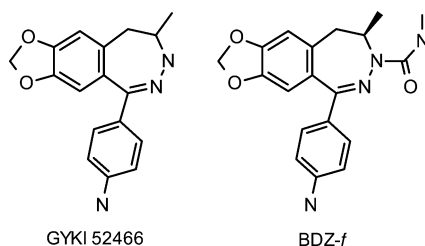


Figure 1. Chemical structures of the 2,3-benzodiazepine derivatives GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine) and BDZ-*f* (GYKI 53784, LY 303070, (R)-5-(4-aminophenyl)-8-methyl-7-(N-methyl-carbamoyl)-8,9-dihydro-7H-1,3-dioxolo[4,5-h][2,3]benzodiazepine).

derivatives, also known as GYKI compounds, represent one of the most promising group of compounds synthesized as selective inhibitors of AMPA receptors and drug candidates for treatment of a number of neurological diseases involving excessive activity of AMPA receptors.^{20,21} To date, hundreds of 2,3-BDZ derivatives have been synthesized, based on the structure of the prototypic compound GYKI 52466.^{22–24} In a series of studies of the structure–activity relationship for 2,3-BDZ compounds that we published earlier, we described that the compounds like these two (Figure 1) bind to the same site, which we have termed as the “M” site, on the GluA2Q receptors.^{25–28} This is because both GYKI 52466 and BDZ-*f* contain a C-4 methyl group and the 7,8-methylenedioxy ring (Figure 1). Furthermore, BDZ-*f* is more potent than GYKI 52466 on GluA2Q, because addition of an acylating group to the N-3 position of the 2,3-benzodiazepine ring is favorable for compounds that bind to the “M” site.²⁷ However, how these compounds act on GluA1 and whether they bind to the same site on GluA1 are not known.

To investigate the mechanism of inhibition of the GluA1 channel opening, we use a laser-pulse photolysis technique, together with a photolabile precursor of glutamate or caged glutamate. This technique provides a time resolution of $\sim 60 \mu\text{s}$, sufficient for measuring the rate of channel opening and thus the effect of an inhibitor on the channel-opening rate without the complication of channel desensitization on the millisecond (ms) time scale. Therefore, our kinetic study permits us to characterize these 2,3-BDZ compounds with the GluA1 receptor channels while the receptors are still in the functional state.

MATERIALS AND METHODS

Cell Culture and GluA1 Expression. In this study, we chose the flip variant of rat GluA1 or GluA1_{flip}.^{29,30} GluA1_{flip} was transiently expressed in human embryonic kidney (HEK)-293S cells. The cells were grown in modified Eagle medium

(Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units of penicillin/mL, and 0.1 mg streptomycin/mL (Sigma-Aldrich, St. Louis, MO). The cells were grown in a humidified incubator at 37 °C and 5% CO₂. The cells were transfected with the cDNA encoding the GluA1_{flip} receptor subunit by a standard calcium phosphate protocol.^{29,30} For transfection, 4–6 μg of the GluA1_{flip} plasmid was used, together with green fluorescent protein and simian virus large T-antigen at a ratio of 10:2:1.³⁰ The cells were used for recording from 24 h after transfection.

Whole-Cell Current Recording. Glutamate was used as the agonist. All whole-cell current recordings were collected with transfected HEK-293S cells that were voltage-clamped at -60 mV and at ambient temperature. Specifically, an Axopatch 200B at a cutoff frequency of 2–20 kHz by a built-in, 4-pole low-pass Bessel filter was used. The current traces were digitized at a 5–50 kHz sampling frequency using a Digidata 1322A (Molecular Devices, Sunnyvale, CA). The data was acquired with the use of pClamp 8 (Molecular Devices). The electrode solution contained (in mM) 110 CsF, 30 CsCl, 4 NaCl, 0.5 CaCl₂, 5 EGTA, and 10 HEPES (pH 7.4 adjusted by CsOH). The extracellular bath buffer contained (in mM) 150 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.4 adjusted by NaOH). All chemicals used for making buffers were from commercial sources.

Laser-Pulse Photolysis Measurement. The laser-pulse photolysis technique has been previously described.^{25,27,29–31} Briefly, we used 4-methoxy-7-nitroindolyl-caged-L-glutamate (Tocris Bioscience, Ellisville, MS).³² An HEK-293 cell that expressed GluA1_{flip} was equilibrated with the caged glutamate for at least 250 ms before photolysis. A single laser pulse at 355 nm generated from a pulsed Q-switched Nd:YAG laser (Continuum, Santa Clara, CA), with a pulse length of 8 ns and energy output in the range of 200–1000 μJ (measured at the end of the optical fiber), was applied to an HEK-293S cell via optical fiber. Free glutamate solutions were used to calibrate the whole-cell current responses from the same cell before and after a laser flash to estimate the concentration of photolytically released glutamate. Free glutamate and/or caged glutamate solutions in the absence and presence of inhibitor were delivered by the use of a U-shaped flow device.²⁵ The time resolution of this flow device, determined by the rise time of the whole-cell current response (10–90%) to saturating glutamate concentrations, was $1.0 \pm 0.2 \text{ ms}$, an average of the measurement from >100 cells expressing the same receptor.³² We also found that full inhibition by either GYKI 52466 or BDZ-*f* was achieved only by preincubating the inhibitor with the GluA1_{flip} receptor for at least 6 s, similar to what we reported for other 2,3-BDZs with GluA2Q_{flip} receptor.^{25,27} It should be noted that neither GYKI 52466 nor BDZ-*f* activated the GluA1 receptor. This was based on the observation that a recorded trace did not deviate from the baseline during preincubation of only an inhibitor in the flow measurement or when the inhibitor mixed with the caged glutamate was exposed to the receptor prior to a laser flash in the laser-pulse photolysis measurement. It should be also noted that the amplitude of the whole-cell current measured by using the flow device was corrected for receptor desensitization for data analysis, as previously described.^{25,27}

Experimental Design and Data Analysis. We first characterized the effect of GYKI 52466 and BDZ-*f* on both the channel-opening (k_{op}) and the channel-closing (k_{cl}) rate processes of the GluA1_{flip} receptor. Specifically, the effect on k_{op}

and k_{cl} was determined as a function of inhibitor concentration at two glutamate concentrations. An observed rate constant (k_{obs}) of GluA1_{flip} channel opening followed a first-order rate process with or without an inhibitor (see eq 1 and all other equations in the Supporting Information). As shown in eq 2, k_{obs} is a function of ligand concentration (L) and includes the rate terms of k_{op} and k_{cl} . If the ligand concentration is low (i.e., $L \ll K_1$; K_1 is the intrinsic equilibrium constant, in Figure 4), eq 2 is reduced to $k_{obs} \approx k_{cl}$ and k_{cl} is correlated to the lifetime of an open channel (or $\tau = 1/k_{cl}$, where τ is the lifetime).³⁰ The effect of an inhibitor on, and its inhibition constant for, the open-channel state are thus determined (eq 4). At a higher ligand concentration, where $k_{obs} > k_{cl}$, the k_{op} value is determined by the difference between k_{obs} and k_{cl} or by rearranging eq 2 such that $k_{obs} - k_{cl} = k_{op} [L/(L+K_1)]$. Similarly, the effect of an inhibitor on k_{op} and the inhibition constant for the closed-channel state are determined (eq 5). We have previously established the criteria by which k_{cl} can be determined from the measurement of k_{obs} .^{25–28} For GluA1_{flip}, k_{cl} is numerically equal to the k_{obs} value obtained at a glutamate concentration of 40 μM , which corresponds to $\sim 4\%$ of the fraction of the open-channel form.³⁰ In this study, the effect of GYKI 52466 and BDZ-*f* on k_{cl} was determined at 40 μM glutamate concentration. The effect of these compounds on k_{op} was determined at 300 μM glutamate concentration.

The whole-cell current amplitude in the absence and presence of an inhibitor was also measured for independently determining an inhibition constant.²⁵ Similarly, we used 40 μM glutamate concentration (i.e., $L \ll K_1$), at which most receptors were in the closed-channel state (see Figure 4; defined as the unliganded, singly, and doubly liganded forms), to determine the inhibition constant for the closed-channel state (see eqs 6a and 6b). Conversely, we used a saturating ligand concentration ($L \gg K_1$), where most of the receptors were in the open-channel state, to determine the inhibition constant for the open-channel state.²⁵ For GluA1 receptors, we chose 2 mM glutamate concentration as a saturating concentration, which corresponded to $\sim 93\%$ of the receptors being in the open-channel form.³⁰

Whether GYKI 52466 and BDZ-*f* bound to the same site or two different sites on GluA1_{flip} was investigated using a double-inhibitor experiment (see detail in Supporting Information). In this experiment, the concentration of one inhibitor was kept constant while the concentration of the other was varied. The current amplitude in the absence and presence of two inhibitors was measured. An apparent inhibition constant obtained from the two-inhibitor experiment (or the slope of the $A/A_{I,P}$ plot; see eqs 7 and 8) was compared to that obtained from one-inhibitor experiment (or the slope of the A/A_1 plot; see eq 6). All other conditions were the same as described before.

For data analysis and plotting, we used Origin 7 (Origin Lab, Northampton, MA). Unless otherwise noted, each data point shown in a plot was an average of at least three measurements collected from at least three cells. The error reported refers to the standard deviation from the mean.

RESULTS

GYKI 52466 and BDZ-*f* Inhibited the Channel-Opening Process of GluA1_{flip}. Using the laser-pulse photolysis technique, we first characterized the effect of GYKI 52466 and BDZ-*f* on the channel-opening rate process of GluA1_{flip}. As shown in a pair of whole-cell recording traces (Figure 2A) initiated by laser-pulse photolysis of the caged glutamate, the

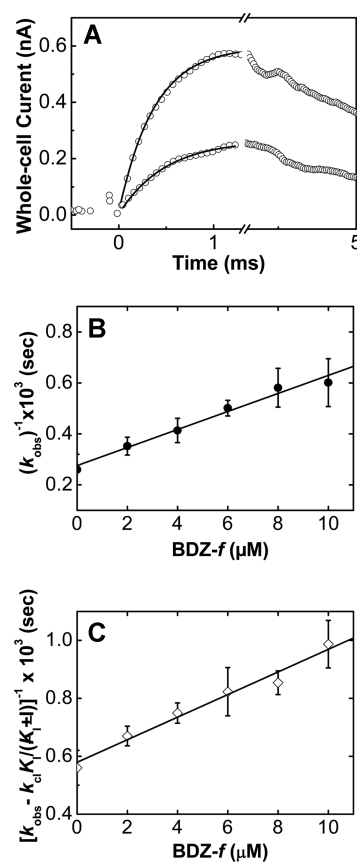


Figure 2. (A) Representative whole-cell current traces from the laser-pulse photolysis experiment with BDZ-*f* as an example. As shown, BDZ-*f* inhibited both the rate and amplitude of the opening of the GluA1_{flip} channels (lower trace with 10 μM BDZ-*f*; $k_{obs} = 2,299 \text{ s}^{-1}$; $A = 0.24 \text{ nA}$) as compared to the control (upper trace; $k_{obs} = 3376 \text{ s}^{-1}$; $A = 0.56 \text{ nA}$). The solid line superimposed in each trace was a single exponential fit using eq 1 (Supporting Information). For data plotting, we used every fourth point (or the point at every 100 μs); for plotting the desensitization phase, we used the data points at every 500 μs . (B) Effect of BDZ-*f* on k_{cl} was obtained at 40 μM of photolytically released glutamate and as a function of BDZ-*f* concentration. From this plot, a K_i^* of $30 \pm 3 \mu\text{M}$ was determined. (C) Effect of BDZ-*f* on k_{op} obtained at 300 μM of photolytically released glutamate and as a function of BDZ-*f* concentration. From this plot, a K_i^* of $15 \pm 1 \mu\text{M}$ was determined. Each data point shown in this plot was an average of at least three measurements collected from at least three cells.

time course of the whole-cell current rise was slowed, and the current amplitude was reduced in the presence of BDZ-*f* (here BDZ-*f* was used as an example). We ascribed the reduction in both the rate and the amplitude to the inhibition of the channel-opening process of GluA1_{flip} by BDZ-*f*. This interpretation was based on the fact that the rate of the current rise in the laser-pulse photolysis measurement was pertinent to the channel opening.³⁰ Furthermore, the macroscopic current amplitude was correlated to the number of channels on a single cell surface (see eq 6b in the Supporting Information). Therefore, the reduction of the amplitude in the presence of an inhibitor would be counted for by the notion that a certain number of the channels on the cell surface was blocked from ionic conductance by the inhibitor (eq 6a). It should be further noted that the observed effects of BDZ-*f* on both the rate of current rise and the amplitude were measured prior to the

channel desensitization, reflected by the falling phase of the current on a longer time scale (Figure 2A).

That the time course of the whole-cell current rise reflected the rate of channel opening (see the minimal mechanism of channel opening in the Supporting Information and eq 2) was based on the assumption that ligand binding rate(s) of all steps were fast, relative to the rate of channel opening. On the basis of this assumption, the k_{op} of the GluA4 receptor we obtained,³³ for instance, is indeed 3 orders of magnitude smaller than the glutamate binding rate constant estimated by Madden and co-workers using an extracellular portion of the same receptor.³⁴ It should be noted, however, that the rate constant of glutamate binding to any intact AMPA receptor (e.g., either unliganded or singly or doubly liganded forms) is not known. On the basis of the same assumption, the k_{cl} we obtained for an AMPA receptor is well corroborated with the lifetime obtained from single-channel recording of the same receptor.^{30,31,35} In addition, if an observed rate constant represents the rate of the transition from the closed- to the open-channel state and is slow as compared to the ligand binding, the rising phase of the receptor response is expected to be a single exponential rate process and to remain so even when the concentration of the ligand varies. Indeed, an observed rate constant of the current rise followed a single exponential rate law for over 93% of the rising phase (i.e., the solid lines in Figure 2A). This observation was without exception in all inhibitor and glutamate concentrations used not only in this but also in our previous studies of AMPA receptors in the absence and presence of other inhibitors.^{25,27}

The Effect of GYKI 52466 and BDZ-f on k_{op} and k_{cl} . Next we characterized the effect of GYKI 52466 and BDZ-f on both k_{op} and k_{cl} of GluA1_{flip}. At the 40 μ M glutamate concentration where k_{cl} was measured (see Materials and Methods), \bar{K}_1^* or the inhibition constant for the open-channel state was found to be $30 \pm 3 \mu$ M for BDZ-f (Figure 2B) by the use of eq 4. At a higher concentration [i.e., 300 μ M of glutamate in this study, where $k_{obs} > k_{cl}$ ³⁰], K_1^* or the inhibition constant for the closed-channel state was characterized, from the effect of BDZ-f on k_{op} , to be $15 \pm 1 \mu$ M (Figure 2C) by the use of eq 5. All of these values for BDZ-f are also summarized in Table 1, along with the inhibition constants for GYKI 52466 (i.e., the effect of GYKI 52466 on the k_{op} and k_{cl} of GluA1 was characterized similarly, and the original data as well as the plots of GYKI 52466 are shown in Figure S1 in the Supporting Information). The fact that GYKI 52466 and BDZ-f inhibited both k_{cl} and k_{op} of GluA1_{flip} was consistent with a non-competitive mechanism of inhibition.^{25–28} In contrast, an uncompetitive inhibitor would be expected to inhibit only k_{cl} but not k_{op} , whereas a competitive inhibitor would be expected to inhibit k_{op} but not k_{cl} .^{25–28}

Effect of GYKI 52466 and BDZ-f on the Whole-Cell Current Amplitude. In the laser-pulse photolysis measurement of both GYKI 52466 and BDZ-f, we also used the amplitude of the whole-cell current response (an example is shown in Figure 2A) in the absence and presence of an inhibitor to determine the inhibition constant. From the ratio of the current amplitude in the absence and presence of an inhibitor, such as BDZ-f (Figure 3A), we found a K_1 of $6 \pm 1 \mu$ M for the closed-channel state (i.e., at 40 μ M glutamate as in Figure 3A, solid circles; see also Materials and Methods and eqs 6 and 7). A K_1 of $5 \pm 1 \mu$ M was estimated at 300 μ M glutamate for BDZ-f (Figure 3A, open circles). Similarly, the inhibition constant of GYKI 52466 for the closed-channel and the open-

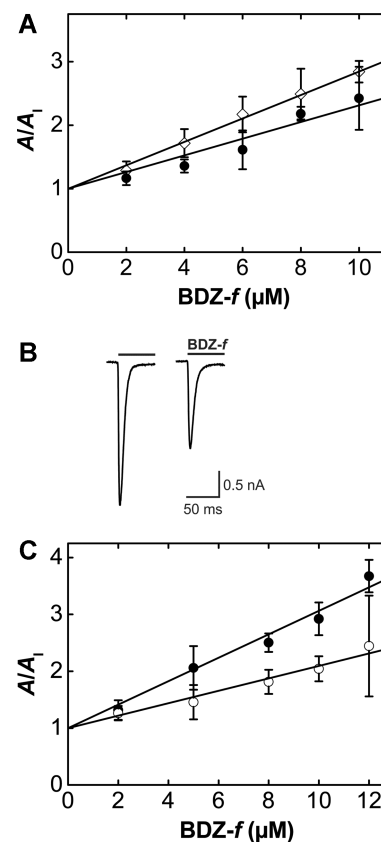


Figure 3. (A) Effect of BDZ-f on the amplitude of the whole-cell current obtained from laser-pulse photolysis measurements. A K_1 of $6 \pm 1 \mu$ M was obtained from the A/A_1 value as a function of BDZ-f concentration (\bullet) for the closed-channel state at 40 μ M of photolytically released glutamate. At 300 μ M photolytically released glutamate concentration (\diamond), the K_1 was determined to be $5 \pm 1 \mu$ M. (B) Representative whole-cell current traces of GluA1_{flip} channels in the absence (left) and presence (right) of BDZ-f obtained by the flow measurement. The concentrations of glutamate and the inhibitor were 2 mM and 6 μ M, respectively. (C) Effect of BDZ-f on the whole-cell current amplitude of GluA1_{flip} receptors obtained from the flow measurement. The inhibition constant of K_1 of $5 \pm 1 \mu$ M was determined for the closed-channel state (40 μ M glutamate, \bullet), whereas a \bar{K}_1 of $9 \pm 1 \mu$ M was obtained for the open-channel state (2 mM glutamate, \circ).

channel state of the GluA1_{flip} were determined, respectively (see Figure S2 in Supporting Information). All the inhibition constants obtained from the laser photolysis measurement are summarized in Table 1.

We then compared the magnitude of the inhibition constants obtained from the rate of the current rise (Figure 2B,C) with those obtained from the current amplitude (Figure 3A) observed in the same experiment (i.e., the laser-pulse photolysis experiment). We found, however, that the inhibition constant of BDZ-f calculated from the amplitude was \sim 2-fold smaller than the one calculated from the rate for the closed-channel state (i.e., 40 μ M glutamate concentration; see Table 1). For the inhibition constant of BDZ-f collected at 300 μ M glutamate concentration, the inhibition constant calculated from the amplitude (Figure 3A) was almost 6-fold smaller than the one obtained from the rate (Figure 2C, and Table 1). The same trend was observed for GYKI 52466 (see Table 1). Because these inhibition constants were obtained at the same glutamate concentrations and in the same experiment, we suspected that

Table 1. Summary of the Inhibition Constants of GYKI 52466 and BDZ-*f*, Obtained from Rate and Amplitude Measurements, for the Closed-Channel and Open-Channel States of GluA1_{flip}

	rate measurement		amplitude measurement			
	K_I^* (μM) ^{a,c}	$\bar{K}_I^{*,a,d}$	K_I (μM) ^{a,c}	K_I (μM) ^{a,d}	K_I (μM) ^{b,c}	\bar{K}_I (μM) ^{b,e}
BDZ- <i>f</i>	15 ± 1	30 ± 3	6 ± 1	5 ± 1	5 ± 1	9 ± 1
GYKI 52466	145 ± 30	281 ± 70	59 ± 9	52 ± 5	53 ± 2	140 ± 5

^aLaser-pulse photolysis measurement. ^bFlow measurement. ^cMeasurements at 40 μM glutamate for the closed-channel state. ^dMeasurements at ~ 300 μM glutamate. ^eMeasurements at 2 mM glutamate.

the discrepancy between the corresponding inhibition constants determined from the amplitude and rate measurements was due to the nature of the inhibition, rather than the difference in the measurements. We therefore decided to use a different technique, i.e., a solution flow measurement (see Materials and Methods), to independently evaluate an inhibition constant.

As shown in glutamate-evoked whole-cell current traces in the absence (left trace of Figure 3B) and presence (right trace) of BDZ-*f* in the solution flow measurement, BDZ-*f* inhibited the current amplitude from the GluA1_{flip} receptor channels. From the data of the current amplitude collected at glutamate concentrations of 40 μM and 2 mM³⁰ (see Materials and Methods), we calculated a K_I of 5 ± 1 μM for the closed-channel and a \bar{K}_I of 9 ± 1 μM for the open-channel state, respectively. Likewise the inhibition constants of GYKI 52466 were determined to be 53 ± 2 μM and 140 ± 5 μM , respectively (all these values are also summarized in Table 1).

On the basis of these results, we drew the following conclusions. First, the magnitude of the inhibition constant of BDZ-*f* or GYKI 52466 obtained at the 40 μM glutamate concentration from the amplitude data of the laser-pulse photolysis measurement was in good agreement with that of the solution flow measurement (columns 3 and 5 in Table 1). Second, because the laser-pulse photolysis experiments permitted the estimate of inhibition constants at 300 μM glutamate concentration, we further conducted the flow measurement at the same concentration. The inhibition constant at 300 μM glutamate concentration, calculated from the amplitude of the flow measurement, was found to be 5 ± 1 μM for BDZ-*f* and 55 ± 6 μM for GYKI 52466, respectively (see Figure S3 in Supporting Information). Like the 40 μM data, these values are in good agreement with the corresponding data collected from the rate measurement (i.e., the values in column 4 in Table 1). Third, the inhibition constant of BDZ-*f* or GYKI 52466 obtained at 300 μM glutamate concentration was similar to its respective value at 40 μM (compare values in column 4 with those in column 3), but different from the value obtained at 2 mM glutamate concentration (compare values in column 4 with those in column 6). These results could be explained by the fact that at glutamate concentrations of 40 μM , 300 μM , and 2 mM, the fraction of the open channel population from the ensemble current was approximately 6%, 42%, and 93%, respectively [note that the K_I value for GluA1_{flip} is 530 μM ,³⁰ and the EC₅₀ value ranges from 500 to 700 μM ^{36–38}]. Furthermore, the channel-opening probability of GluA1_{flip} is ~ 0.93 .³⁰ Consequently, the magnitudes of inhibition constants at 300 μM glutamate concentration would be expected to be more similar to those at lower glutamate concentration.

Mechanism of Inhibition of GluA1_{flip} by GYKI 52466 and BDZ-*f*. On the basis of the results we described above, we conclude that both compounds inhibited the GluA1_{flip} channel

noncompetitively. This conclusion is supported by the fact that GYKI 52466 and BDZ-*f* inhibited the whole-cell current response from both the closed-channel and the open-channel states of GluA1_{flip}, and the two compounds inhibited both k_{cl} and k_{op} . The k_{cl} is correlated to the open-channel state, whereas the k_{op} is correlated to the closed-channel state.³⁰

For both GYKI 52466 or BDZ-*f*, we observed that the inhibition constants determined using the amplitude ratio from both the laser and flow measurements were in good agreement (K_I values of columns 3–5 in Table 1); yet those constants were ~ 2 – 3 -fold smaller than the inhibition constants determined from the measurement of the channel-opening rate (K_I^* values of columns 1 and 2 in Table 1). To explain this difference, we proposed a two-step mechanism of inhibition (Figure 4). By this mechanism, the initial binding of GYKI

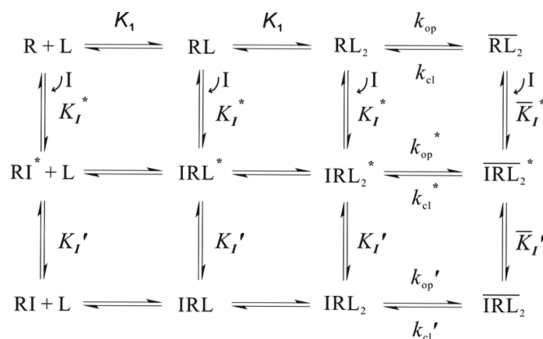


Figure 4. A minimal mechanism of the inhibition of GluA1 by 2,3-benzodiazepine compounds. *L* represents ligand (glutamate) and the number of ligands that bind to and open the channel is assumed to be two. *R* represents the active, unliganded form of the receptor; *I* represents an inhibitor. For simplicity and without contrary evidence, it is assumed that glutamate binds with equal affinity or K_I , the intrinsic equilibrium dissociation constant, at all binding steps. An asterisk indicates those species in the intermediate state, i.e., loose receptor:inhibitor complexes, whereas those species bound with inhibitor but without asterisk represent those in the final state of the receptor complexes through a rapid isomerization reaction. All species related to *R*, *RL*, and *RL₂*, including those bound with inhibitors, are in the closed-channel state, whereas those related to $\bar{R}L_2$ refer to the open-channel state.

52466 or BDZ-*f* to GluA1_{flip} forms a loosely bound intermediate (e.g., $\bar{I}AL_2^*$) in both the closed-channel and open-channel states of the receptor. A receptor:inhibitor intermediate is partially conducting, which yields partial inhibition of channel activity. In the second step, the receptor:inhibitor intermediate rapidly isomerizes into a more tightly bound complex ($\bar{I}AL_2$), and such a complex is no longer capable of conducting ions.

This two-step mechanism of inhibition (Figure 4) can account for the results we obtained. On the basis of the K_I value calculated from the amplitude, a larger inhibition constant

obtained from the rate (Figure 2A) reflects that the inhibitory effect we observed from the rate only correlates to a partial inhibition. Additional step, therefore, must be involved in producing the full inhibition. The ensuing step following the initial formation of the receptor:inhibitor intermediate must be also faster. In other words, the observable step in the rate measurement can only be the slow step, which correlates to the formation of the receptor:inhibitor intermediate in the first step. The first step only generates partial inhibition; the second step or the isomerization reaction that turns the initial, partially conducting complex into a totally inhibitory complex produces additional inhibition. On the other hand, unlike the rate of channel opening, the amplitude is an equilibrium measure, which reflects the overall inhibition.

If the rate of the second step were slow or comparable to that of the first step, we would expect complete or nearly complete inhibition or the inhibition constants calculated from the rate data would agree fully or nearly fully with those from the amplitude. The fact that $(1/k_{\text{obs}})$ increased linearly with increasing inhibitor concentration as predicted by eqs 4 and 5 (Figure 2B,C) in both the closed-channel and open-channel states of the receptor further supports the assumption that the rate of the isomerization should be faster than the initial step. As such, the K_i value (Table 1) obtained from the rate measurement at a high glutamate concentration should be pertinent to the inhibition of the closed-channel by either GYKI 52466 or BDZ-*f* through the initial inhibitor:receptor intermediate, whereas the K_i^* value (Table 1) should be assigned to the inhibition of the open-channel state by the corresponding initial inhibitor:receptor intermediate.

Although the effect of either GYKI 52466 or BDZ-*f* on k_{op} and k_{cl} is partial, that GYKI 52466 or BDZ-*f* inhibits both k_{op} and k_{cl} is consistent with a noncompetitive mechanism but inconsistent with either a competitive or an uncompetitive mechanism of inhibition. By a competitive mechanism, an inhibitor would compete with glutamate for the same binding site. Consequently, only the effect on k_{op} , but not on k_{cl} , would be expected. In other words, there would be no $[\bar{K}_i^*/(\bar{K}_i^* + I)]$ term associated with k_{cl} in eq 3, and thus $1/k_{\text{obs}}$, as in eq 4, would be independent of inhibitor concentration.^{25,27,39} By an uncompetitive mechanism or an open-channel blockade mode of action, GYKI 52466 or BDZ-*f* would inhibit the open-channel state only. In this case, only the effect on k_{cl} , but not on k_{op} , would be expected. Consequently the $[K_i^*/(K_i^* + I)]$ term associated with k_{op} in eq 3 would not exist. Furthermore, the $(k_{\text{obs}} - k'_{\text{cl}})$ term, as in eq 5, would be invariant or independent of inhibitor concentration.

BDZ-*f* and GYKI 52466 Bind to the Same Site on GluA1_{flip}. We have previously hypothesized that the 2,3-benzodiazepine compounds with the azomethine group on the diazepine ring, which contains the 4-methyl group (see Figure 1), bind to the same site, which we termed it as the “M” site.^{28,40} These compounds must also contain the 7,8-methylenedioxy ring feature (Figure 1). However, that hypothesis is based on our work with the GluA2Q_{flip} channel.^{28,40} Here we asked whether GYKI 52466 and BDZ-*f* competed to the binding at the same site on GluA1_{flip}, because both compounds fulfill the same structural characteristic for the “M” site, at least for the GluA2Q_{flip} receptor. To address this question, we did a double-inhibitor experiment (see detail in the Materials and Methods, and Supporting Information) in which the concentration of GYKI 52466 was kept constant while the concentration of BDZ-*f* was varied (Figure 5). From

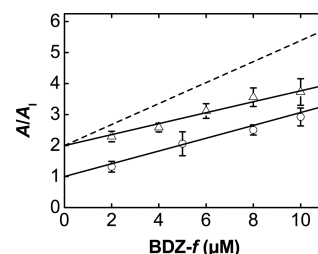


Figure 5. The double-inhibitor experiment for GYKI 52466 and BDZ-*f* on GluA1_{flip} is shown using the ratio of current amplitude (upper solid line), as compared with the ratio of the amplitude in the presence of a single inhibitor (lower solid line). The concentration of GYKI 52466 was fixed at 50 μM , while the concentration of BDZ-*f* varied from 2 to 10 μM . The upper solid line represents the best fit to a one-site, double-inhibitor model, as compared with the lower solid line (i.e., the best fit to the data with BDZ-*f* alone). The dashed line is the simulated data for a two-site, double-inhibitor model.

this experiment, we determined that the apparent K_i' value for the double inhibition (or the upper solid line in Figure 5) was $\sim 5 \mu\text{M}$ (using eq 7 in Supporting Information). This value was comparable with the K_i of 5 μM for BDZ-*f* alone (the lower solid line in Figure 5). This finding was consistent with the conclusion that both inhibitors competed the binding to the same noncompetitive site on GluA1_{flip}. If GYKI 52466 and BDZ-*f* had bound to two different noncompetitive sites on GluA1_{flip}, an additive effect of inhibition or a stronger inhibition than just a single inhibitor would have been observed. This is shown, in Figure 5, by the dashed line, which simulates the two-site model (using eq 8 in the Supporting Information).

DISCUSSION

Using a laser-pulse photolysis and a rapid solution flow technique, we have characterized the mechanism of action and the site of binding for GYKI 52466 and BDZ-*f* by measuring their inhibitory effects on the channel-opening rate process and the whole-cell current amplitude of the GluA1_{flip} receptors. Our findings, as summarized in Table 1, establish that (i) both compounds are noncompetitive inhibitors on GluA1_{flip} and have the same mode of action on GluA1_{flip} that involves a two-step, minimal mechanism of inhibition. Both compounds bind to the same site on GluA1_{flip}. (ii) Both compounds prefer to inhibit the closed-channel over the open-channel state, albeit the preference in the case of BDZ-*f* is much smaller than GYKI 52466 (i.e., values in column 5 vs column 6). (iii) On the basis of the magnitude of the overall inhibition constant, BDZ-*f* is a much stronger inhibitor of GluA1_{flip} than GYKI 52466. Specifically, BDZ-*f* is ~ 10 -fold stronger in inhibiting the closed-channel state (i.e., K_i is 5 μM for BDZ-*f* and 52 μM for GYKI 52466, respectively; see Table 1, column 4). However, BDZ-*f* is ~ 15 -fold stronger in inhibiting the open-channel state than GYKI 52466 (i.e., \bar{K}_i is 9 μM for BDZ-*f* and 140 μM for GYKI 52466, respectively; see Table 1, column 6).

The finding that BDZ-*f* is a stronger inhibitor on GluA1_{flip} than GYKI 52466 can be best accounted for on the basis of the structure–activity relationship. (i) Because BDZ-*f* and GYKI 52466 bind to the same noncompetitive site on GluA1_{flip}, our results suggest that the addition of an N-3 methyl-carbamoyl group on the diazepine ring of GYKI 52466 improves potency without changing the site of binding of the new compound, i.e., BDZ-*f*. This suggests that occupying the N-3 side pocket of the site is favorable.²⁸ (ii) Furthermore, occupying the N-3 side

pocket of this site is already evident even at the first step or the step of the formation of the initial, partially inhibitory receptor:inhibitor intermediate (Figure 4). Specifically, the GluA1_{flip}:BDZ-*f* intermediate formed in the both the closed-channel and the open-channel state is about 10-fold more inhibitory than the GluA1_{flip}:GYKI 52466 complex (i.e., $K_i^* = 15 \mu\text{M}$ for BDZ-*f* and $145 \mu\text{M}$ for GYKI 52466 for the closed-channel state; for the open-channel state, $K_i^* = 30 \mu\text{M}$ for BDZ-*f* and $281 \mu\text{M}$ for GYKI 52466; see Table 1).

The inhibitory properties of GYKI 52466 and BDZ-*f* we have observed in this study with the GluA1_{flip} receptor are similar to those we reported for the same compounds but with the GluA2Q_{flip} receptor. Therefore, we believe that the site to which both GYKI 52466 and BDZ-*f* bind is still the “M” site. We have previously identified three different types of noncompetitive sites on GluA2Q_{flip}. The 2,3-benzodiazepine compounds with the azomethine group on the diazepine ring, which contains the 4-methyl group (see Figure 1), bind to the “M” site.^{28,40} Enlarging the 7,8-methylenedioxy ring into the 7,8-ethylenedioxy one makes the resulting compound and its derivatives bind to the “E” site.³⁹ However, 2,3-benzodiazepine compounds with an ϵ -lactam structure, which contains the 4-carbonyl group, bind to a different site, which we termed as the “O” site.^{26,28} Apparently, more studies using a series of 2,3-BDZs with GluA1_{flip} are needed to better define the sites we have previously reported on GluA2Q_{flip}. It should be further pointed that currently, there is no structural information about a noncompetitive inhibitor bound to an AMPA receptor, nor the exact location of any of these noncompetitive sites, including the “M” site.

When we compare the inhibition constants of the same compounds with different receptors, we can further infer some difference in the “M” site between GluA1 and GluA2. This can be illustrated in Figure 6, where the inhibition constants of GYKI 52466 and BDZ-*f* on GluA1_{flip} are compared with those

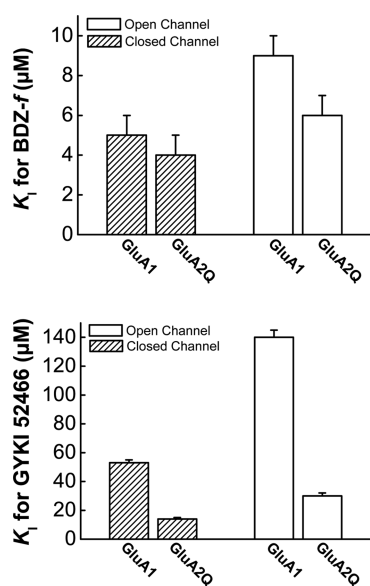


Figure 6. A comparison of the inhibition constant (K_i , in μM) between GluA1_{flip} and GluA2Q_{flip} for BDZ-*f* (upper panel) and GYKI 52466 (lower panel). The data for GluA2Q_{flip} with the same compounds, i.e., GYKI 52466 and BDZ-*f*, were published earlier.²⁷ A dashed column represents the closed-channel state, whereas a hollow column represents the open-channel state.

on GluA2Q_{flip}. For GYKI 52466, there is clear difference between different receptor subunits: its inhibition constant on GluA2Q_{flip} is >3-fold and >4-fold smaller for the closed-channel and the open-channel states, respectively, than that on GluA1_{flip}. However, the inhibition constants of BDZ-*f* for the closed-channel and the open-channel states of GluA1_{flip} are similar to the corresponding values on GluA2Q_{flip}. These results suggest that the “M” sites of the GluA1 and GluA2 receptors are different, although the difference is not that large. Furthermore, the side pocket that accommodates an acylating group at the N-3 position for the “M” site is especially important in defining the difference in the binding environment between the GluA1 and GluA2. Overall, the “M” site or the binding environment on GluA2 accommodates the same compounds better than that on GluA1 or the same inhibitors show stronger potency on GluA2 than on GluA1. However, acylating the N-3 position to occupy the N-3 side pocket of the “M” site on GluA1 can significantly narrow the difference, especially on the closed-channel state of GluA1 (Figure 6). This can be seen by the fact that the second step or the isomerization step generates ~2–3-fold more inhibition as compared to the first step for both GYKI 52466 and BDZ-*f* on GluA1, whereas the same compounds produced ~4-fold additional inhibition in the isomerization step on GluA2Q_{flip} (Figure 6). It should be noted that in drawing this inference in the difference of the properties of the “M” site between GluA1 and GluA2, we have assumed that the structures of the same compounds, i.e., GYKI 52466 and BDZ-*f*, remain the same regardless of whether they bind to different receptors. As such, the difference in their inhibition constants reflects the difference in the binding environment between GluA1 and GluA2.

In summary, we have shown that the addition of an N-3 methyl-carbamoyl group to the diazepine ring with the azomethine feature (i.e., GYKI 52466) is what makes BDZ-*f* a better inhibitor of the GluA1_{flip} receptor subunit than the original template GYKI 52466. In other words, adding an N-3 methyl-carbamoyl group improves its potency but does not change the site of binding. At a mechanistic level, adding an N-3 methyl-carbamoyl group significantly strengthens potency of the resulting compound because occupancy of the N-3 side pocket of the “M” site is structurally favorable to inhibit the GluA1. Therefore, for developing more potent 2,3-BDZs that bind to the “M” site on GluA1, it is desirable to acylate the N-3 position of the 2,3-benzodiazepine ring.

■ ASSOCIATED CONTENT

📄 Supporting Information

Original data for the model and the equations used for data analysis, the effect of GYKI 52466 on k_{op} and k_{cl} , as well as on the current amplitude. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDZ, 2,3-benzodiazepine compounds; BDZ-*f*, GYKI 53784, LY 303070, (–)-3-*N*-methylcarbamyl-1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; HEK-293 cells, human embryonic kidney 293 cells; LTP, long-term potentiation; μ s, microsecond; ms, millisecond

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