Kinetic Mechanism of Channel Opening of the $GluRD_{flip}$ AMPA Receptor†

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ABSTRACT: AMPA-type ionotropic glutamate receptors mediate the majority of fast excitatory neurotransmission in the mammalian central nervous system and are essential for brain functions, such as memory and learning. Dysfunction of these receptors has been implicated in a variety of neurological diseases. Using a laser-pulse photolysis technique, we investigated the channel opening mechanism for $GluRD_{flip}$ or $GluR4_{flip}$ (i.e., the flip isoform of GluRD), an AMPA receptor subunit. The minimal kinetic mechanism for channel opening is consistent with binding of two glutamate molecules per receptor complex. The $GluRD_{flip}$ channel opens with a rate constant of $(6.83 \pm 0.74) \times 10^4$ s^{-1} and closes with a rate constant of $(3.35 \pm 0.17) \times 10^3$ s^{-1}. On the basis of these rate constants, the channel opening probability is calculated to be 0.95 ± 0.12. Furthermore, the shortest rise time (20−80% of the receptor current response to glutamate) is predicted to be 20 μs, which is ∼8 times shorter than the previous estimate. These findings suggest that the kinetic property of $GluRD_{flip}$ is similar to that of $GluR2Q_{flip}$, another fast-activating AMPA receptor subunit.

Individual AMPA[1] receptor subunits, i.e., GluR1−4 or GluRA−D, can form homomeric functional channels in heterologous expression systems, such as human embryonic kidney (HEK) 293 cells (*1*). Recent studies using individual receptor subunits have yielded much information about the structure and function of this important subtype of ionotropic glutamate receptor (*1*, *2*). However, the kinetic mechanism of receptor channel opening is not well understood. In the microsecond time region, an AMPA receptor can open its channel upon binding of glutamate to transmit a nerve impulse, whereas within 1−10 ms, the same receptor desensitizes to an inactive, channel-closed form with glutamate still bound (*3*). The mechanism of desensitization is relatively well understood (*3*−*7*), largely because the time resolution of various fast solution exchange techniques is generally in the range of a few hundred microseconds, and is therefore sufficient for the assessment of the desensitization reaction. However, the solution exchange techniques have not been successful in measuring the channel opening process of AMPA receptors, which occurs in the microsecond time domain.

Determining the kinetic mechanism of receptor channel opening is useful. Knowing the rate constant will enable a more quantitative prediction of the time course of the open channel as a function of neurotransmitter concentration, which determines the change in transmembrane voltage and in turn controls synaptic neurotransmission. Furthermore, knowing the kinetic mechanism will provide clues for mechanism-based design of therapeutic compounds for treating neurological diseases involving AMPA receptors, such as post-stroke cellular lesion and amyotrophic lateral sclerosis (*8*). In addition, a systematic characterization of all AMPA receptors will be necessary to determine their functional differences. Knowledge of these functional traits will aid both the understanding of the properties of possible subunit combinations forming heteromeric AMPA receptors and the development of subunit-specific inhibitors and drugs.

$$A + L \underset{}{\overset{K_1}{\rightleftharpoons}} AL \overset{K_1}{\rightleftharpoons} AL_2 \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \overline{AL_2}$$

FIGURE 1: Minimal kinetic mechanism of channel opening. A represents the active, unliganded form of the receptor and L the ligand (glutamate). $\underline{AL}$ and AL_2 represent the ligand-bound closed channel forms, and $\overline{AL_2}$ represents the open channel form of the receptor. For simplicity, it is assumed that glutamate binds at both steps with equal affinity, designated by K_1. Other symbols are defined in the text, along with a more full discussion of the mechanism. This mechanism is used to describe the channel opening kinetics for $GluRD_{flip}$.

We recently characterized the kinetic mechanism of glutamate-induced channel opening (Figure 1) for GluR1 (*9*) and GluR2 (*10*), two of the four homomeric AMPA receptor channels. In the study presented here, we investigated the channel opening mechanism for the GluRD or GluR4 receptor. As in our previous studies, we used a laser-pulse photolysis technique, which permits free glutamate to be photolytically liberated from caged glutamate [i.e., γ-*O*-(α-carboxy-2-nitrobenzyl)glutamate] with a $t_{1/2}$ of ∼30 μs (*11*). The laser-pulse photolysis approach enabled us to measure the channel opening rate process, which occurs on the microsecond time scale, without the complication of channel desensitization.

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[1] Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GFP, green fluorescence protein; HEK-293 cells, human embryonic kidney (293) cells.

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MATERIALS AND METHODS

Expression of cDNA and Cell Culture. The plasmid encoding the full-length $GluRD_{flip}$ subunit was propagated in *Escherichia coli* DH5α cells and purified using a QIAGEN (Valencia, CA) kit. $GluRD_{flip}$ was transiently expressed in HEK-293S cells using a procedure previously described (*9*). Unless otherwise noted, HEK-293S cells were also cotransfected with a plasmid encoding green fluorescence protein (GFP), and the green fluorescent cells were selected for patch clamping 48 h after transfection. The weight ratio of the plasmid for GFP to that for $GluRD_{flip}$ used in transfection was 1:10, and the amount of $GluRD_{flip}$ plasmid was ~3−5 μg/35 mm Petri dish.

Whole-Cell Current Recording. The procedure for whole-cell current recording was previously described (*9*). Briefly, the electrode had a resistance of ~3 MΩ after it was filled with the electrode solution: 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM $CaCl_2$, 5 mM EGTA, and 10 mM HEPES (pH 7.4 adjusted by CsOH). The external bath solution contained 150 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). All chemicals were from commercial sources. A U-tube flow device was used to apply glutamate to a cell, and the resulting rise time of the glutamate-induced whole-cell current response (10−90%) observed was ~2 ms (*9*). The current traces were sampled at 5−50 kHz and filtered at 2−20 kHz by an eight-pole Bessel filter. The data were acquired using pCLAMP 8 (Axon Instruments, Union City, CA). All whole-cell recordings were at −60 mV and 22 °C.

Dose-Response Relationship. On the basis of the channel opening mechanism in Figure 1, eq 1 was derived to describe the dose-response relationship

$$I_A = I_M R_M \frac{L^2}{L^2 + \Phi(L + K_1)^2} \quad (1)$$

where I_A represents the current amplitude, L the molar concentration of the ligand, I_M the current per mole of receptor, and R_M the number of moles of receptor in the cell. Φ^{-1} is the channel opening equilibrium constant, and K_1 is the intrinsic dissociation constant for the ligand. For simplicity and with no experimental evidence to the contrary, the affinity of glutamate, K_1, was assumed to be the same for both the first and second glutamate binding steps (see further discussion of this mechanism in the text). All current traces were corrected for desensitization by a method previously described (*12*). The corrected current amplitude was used to construct the dose-response relationship.

Laser-Pulse Photolysis. The procedure for the laser-pulse photolysis experiment has been described previously (*9*). In brief, caged glutamate (Molecular Probes, Eugene, OR) was dissolved in the external bath buffer and applied to a cell in the whole-cell mode using the U-tube flow device. A laser pulse from the third harmonic output (355 nm, 8 ns pulse length) of a Minilite II pulsed Q-switched Nd:YAG laser (Continuum, Santa Clara, CA) was used to photolyze the caged glutamate after it was equilibrated with a cell for 250 ms. The laser energy was adjusted to 200−800 μJ at the end face of the fiber through which the laser light was introduced to that cell. To determine the concentration of photolytically released glutamate, at least two free glutamate solutions with known concentrations were used to calibrate the current amplitudes from the same cell. The current amplitudes obtained from known glutamate concentrations were compared with the amplitude evoked by glutamate photolytically released from a photolysis measurement, with reference to the dose-response relationship.

Using the laser-pulse photolysis technique, we measured the rate constant for the rising phase of the whole-cell current generated by glutamate that was released photolytically. The rising phase was found to be monoexponential at all concentrations of glutamate, and the observed first-order rate constant (k_{obs}) was thus determined using eq 2. This observation was consistent with the assumption that the channel opening rate was slow compared with the ligand binding rate (see further discussion in the text). Accordingly, the channel opening (k_{op}) and channel closing (k_{cl}) rate constants were calculated by fitting the k_{obs} versus the ligand concentration according to eq 3, together with the use of K_1 obtained from the dose-response relationship described above. In eq 2, I_t represents the current amplitude at time t and I_A the maximum current amplitude.

$$I_t = I_A(1 - e^{-k_{obs}t}) \quad (2)$$

$$k_{obs} = k_{cl} + k_{op}\left(\frac{L}{L + K_1}\right)^2 \quad (3)$$

Channel Opening Probability (P_{op}). The P_{op} or the probability by which the $GluRD_{flip}$ channel opens once it is bound with ligand(s) was calculated using eq 4 (*9*) in which k_{op} and k_{cl} were determined experimentally.

$$P_{op} = \frac{k_{op}}{k_{op} + k_{cl}} \quad (4)$$

Time Course of the Opening of the $GluRD_{flip}$ Channel. The time course of the opening of a receptor channel reflects the observed rate by which the open channel is formed at a given concentration of ligand. The rate at which a channel is open at a given concentration of ligand is characteristic of k_{op} and k_{cl}. Specifically, to represent the time course, we used the rise time of the current response, defined by the increase in receptor current from 20 to 80% (*9*). The rise time was obtained first by using eq 3 to obtain k_{obs} based on the experimentally determined k_{cl} and k_{op} for $GluRD_{flip}$ as a function of glutamate concentration and then by using eq 2 to obtain the time corresponding to the current rise from 20 to 80%.

Unless noted otherwise, each data point was an average of at least three measurements collected from at least three cells. Origin 7 (Origin Lab, Northampton, MA) was used for both linear and nonlinear regression. Uncertainties are reported as the standard error of the mean.

RESULTS

Dose-Response Relationship and Minimal Kinetic Mechanism of Channel Opening. Glutamate-induced whole-cell current was observed from the HEK-293 cells that expressed $GluRD_{flip}$ (Figure 2A, inset). To demonstrate that the current response was from the opening of the $GluRD_{flip}$ channel, we tested the cells that had current response to glutamate yet found no current response to the external buffer. We had

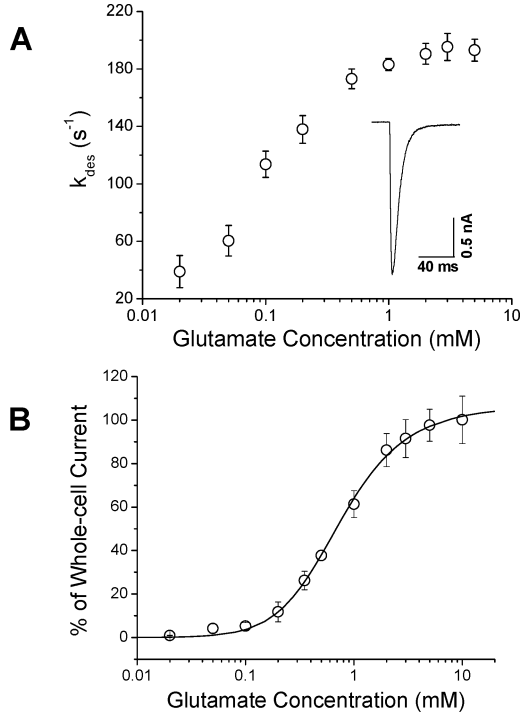

FIGURE 2: (A) Dependence of the desensitization rate constant, k_{des}, of $GluRD_{flip}$ on glutamate concentration. The inset is a representative whole-cell current response of $GluRD_{flip}$ expressed in an HEK-293S cell to 2 mM glutamate, by using flow measurement. The desensitization rate was characterized by a first-order rate constant and is shown with the standard error of the mean. Each data point is an average of at least three measurements from three cells. (B) Dose-response relationship. The whole-cell currents from different cells were normalized to the current obtained at 0.5 mM glutamate, and the current amplitude at 5 mM was set to 100%. Best-fit parameters using eq 1 (—) were as follows: $K_1 = 1.11 \pm 0.40$ mM, $\Phi = 0.24 \pm 0.16$, and $I_M R_M = 131 \pm 20$.

previously observed that both nontransfected cells and transfected cells expressing only GFP, a cell marker, gave no response even at 10 mM glutamate (*10*), a concentration that evoked a maximum current response for the transfected cells expressing $GluRD_{flip}$ (see the dose-response curve in Figure 2B). Furthermore, coexpression of GFP in the same cell that expressed $GluRD_{flip}$ did not affect the kinetic properties of the receptor as evidenced by the desensitization rate constant being identical to those of cells transfected with only $GluRD_{flip}$ (data not shown). In Figure 2A, the desensitization rate constant is shown as a function of glutamate concentration. A first-order rate law was sufficient to characterize more than 95% of the current response at all concentrations of glutamate, consistent with previous reports (*3*, *13*). The average value of the maximum desensitization rate constant is $\sim$190 s^{-1}, which is seen at a glutamate concentration of greater than 3 mM (Figure 2A).

The desensitization rate constant obtained using whole cells in this study is smaller than the value previously reported using outside-out patches at a given glutamate concentration (*3*). An example can be seen in Figure 2A, in which the desensitization rate constant at 3 mM glutamate was 196 s^{-1}, but was 260 s^{-1} when outside-out patches were used (*3*). This phenomenon has been thoroughly investigated for the $GluR1Q_{flip}$ receptor expressed in HEK-293 cells (*14*, *15*), and the largest reported difference is 2.9-fold; i.e., the rate constant obtained from whole cells is 2.9-fold smaller than the value obtained from outside-out patches (*15*). The discrepancy in rate constant at an identical glutamate concentration but seemingly different membrane configurations could be attributed to a slower solution exchange rate with a whole cell, because of its geometry (*14*−*16*). Alternatively, this discrepancy could arise from a faster desensitization rate constant observed with outside-out patches because of altered kinetic properties of the receptor in such a membrane configuration (*14*−*16*). Nevertheless, the mean conductance of the $GluRD_{flip}$ channel observed using whole-cell recording was similar to that using outside-out patches (*17*). These results indicate that the whole-cell current recording was suitable for studying the $GluRD_{flip}$ channel.

Next, we determined the dose-response relationship for the $GluRD_{flip}$ channel, which describes the dependence of the current amplitude as a function of glutamate concentration. The best fit by eq 1 yielded an intrinsic dissociation constant of glutamate, K_1, of 1.11 ± 0.40 mM (Figure 2B). The K_1 value is qualitatively comparable with the reported values of EC_{50} (the ligand concentration that corresponds to 50% of the maximum response), which range from 0.5 to 1.8 mM (*1*, *13*).

Characterization of the Channel Opening Kinetics of $GluRD_{flip}$. Before using the laser-pulse photolysis technique to characterize the channel opening rate constant, we tested the biological properties of caged glutamate with the $GluRD_{flip}$ receptor. We found that the whole-cell current was identical (data not shown) in the absence and presence of 2 mM caged glutamate, the highest concentration of caged glutamate used in this study, suggesting that caged glutamate is biologically inert. This finding is consistent with the conclusion from both the initial report (*11*) and our recent studies of other AMPA receptor channels using the caged glutamate (*9*, *10*).

Using the laser-pulse photolysis technique and the caged glutamate, we measured the channel opening rate constant for $GluRD_{flip}$. Figure 3A shows a representative whole-cell response to photolytically released glutamate. The current increased initially as a result of the channel opening and then decreased because of channel desensitization. In analyzing the rising phase of the whole-cell current induced by photolysis, we observed that $\sim$95% of the current rise was adequately ascribed to a single-exponential rate process (see the solid line in Figure 3A). Equation 2 was thus used to calculate the first-order rate constant, k_{obs}, which corresponded to a particular glutamate concentration. From the plot of k_{obs} versus glutamate concentration according to eq 3 (in Figure 3B), a k_{cl} of $(3.35 \pm 0.17) \times 10^3$ s^{-1} and a k_{op} of $(6.83 \pm 0.74) \times 10^4$ s^{-1} were thus obtained.

As seen in Figure 3A, the rising phase of the current followed a single-exponential rate process for the entire glutamate concentration range from 80 to 310 μM (the latter was the highest concentration of glutamate collected in photolysis). This kinetic feature is consistent with the assumption that the rate of channel opening is slow relative to the rate of ligand binding. On the basis of this assumption, the rising phase of the receptor response is expected not only to follow a single-exponential rate process, reflecting the channel opening rate step, but also to remain single-exponential even when the concentration of ligand is varied (*9*). Accordingly, eq 3 was derived and used for calculating

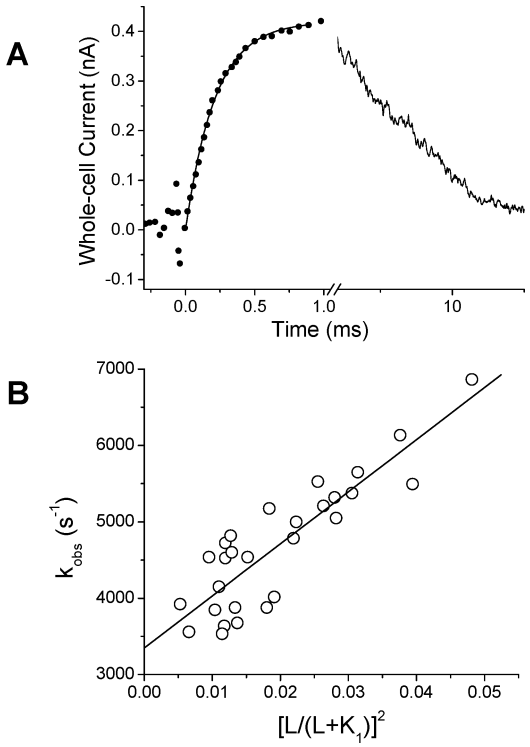

FIGURE 3: Laser-pulse photolysis measurement of the channel opening rate for $GluRD_{flip}$. A representative whole-cell current is shown in panel A for the opening of the $GluRD_{flip}$ channel initiated by the laser-pulse photolysis of caged glutamate at time zero. The fitting of the rising phase by eq 2, shown as the solid line, yielded a k_{obs} of 4800 ± 250 s^{-1}, corresponding to 190 μM photolytically released glutamate. Note that the direction of the current response was plotted opposite to that recorded. For clarity, the number of the data points was reduced for plotting. From the plot of k_{obs} vs glutamate concentration by eq 3 in panel B, k_{cl} and k_{op} were determined to be (3.35 ± 0.17) × 10^3 and (6.83 ± 0.74) × 10^4 s^{-1}, respectively. Each point represents a k_{obs} obtained at a particular concentration of photolytically released glutamate.

k_{op} and k_{cl}. It should be pointed out that deviation would be observed from this monophasic kinetic feature in the rising phase of the current if the bimolecular ligand binding step were either rate-determining or comparable to the rate of channel opening (*9*).

Lower Limit of Ligand Concentration for Determining the Channel Opening Rate Constants. As shown in Figure 1, ligand binding is a bimolecular process, and it precedes channel opening. The ligand concentration thus affects the kinetic observation. When the ligand concentration is sufficiently high, it is always valid to assume that the rate of channel opening is slow compared to the rate of ligand binding. Consequently, the rising phase reflects the channel opening, and eq 3 is valid. When the ligand concentration is low enough, however, the ligand binding will become rate-limiting. As a result, eq 3 will no longer be valid. Therefore, we established the following criterion to set the lower limit of ligand concentration to ensure that the channel opening reaction was measurable.

Specifically, the lowest concentration of glutamate which we used for the calculation of k_{op} and k_{cl} by eq 3 (in Figure 3B) was chosen to be 80 μM. The glutamate concentration of 80 μM corresponded to the fraction of the channel in the open form being ~4% (the fraction of the open channel can be determined by eq 1; see Figure 2B). At the 4% fraction of the open channel form, the k_{obs} obtained by the laser-pulse photolysis technique was found to be comparable to the numerical value of k_{cl} (this is true when $L \ll K_1$, then $k_{obs} \approx k_{cl}$; see eq 3). Moreover, the k_{cl} can be compared to the lifetime of the open channel, which may be measured independently using single-channel recording (*18*−*21*). Indeed, we found that the k_{cl} value we determined using the laser-pulse photolysis technique was well corroborated with the value obtained from single-channel studies (*9*, *10*, *22*). For instance, the k_{cl} of 2600 s^{-1} obtained from the laser-pulse photolysis measurement of the $GluR2Q_{flip}$ AMPA receptor (*10*) is comparable to the lifetime of the open channel (~0.32 ms in time constant or 3100 s^{-1} in rate constant) obtained from single-channel recording (*23*). We therefore concluded that the ligand concentration that correlates to the 4% fraction of the open channel form is sufficiently high not to limit the rate of ligand binding. Consequently, ligand concentrations that correspond to 4% or more of the fraction of the open channel form can be used to calculate the rate constants of channel opening.

DISCUSSION

In this study, we characterized the kinetic mechanism of glutamate-induced opening of the $GluRD_{flip}$ homomeric channel by using a laser-pulse photolysis technique with a time resolution of ~60 μs. Our results provide new insights into the function of this subunit.

Kinetic Mechanism of Channel Opening. The opening of the $GluRD_{flip}$ homomeric channel upon glutamate binding is illustrated by the minimal kinetic mechanism in Figure 1, in which k_{op} and k_{cl} as well as K_1 have been determined. The magnitude of k_{op} (6.83 × 10^4 s^{-1}) defines how fast the conformation of the doubly liganded, closed form of the receptor changes to the open channel conformation. On the basis of the structural events proposed by Armstrong and Gouaux (*24*, *25*), the k_{op} may be correlated to the rate process of the glutamate-induced bilobe closure seen in the S1S2 extracellular binding domain, which presumably leads to the channel opening. Then, the $t_{1/2}$ of the bilobe closure can be calculated to be 10 μs from k_{op}. The magnitude of this value is comparable with the one observed by NMR in a study of the motion of amino acid residues in ligand-binding pockets (*26*). Conversely, the magnitude of k_{cl} (3400 s^{-1}) defines the rate at which an open channel returns to the doubly liganded closed state. Thus, it represents the lifetime of the open channel or a τ of 0.30 ms (i.e., $\tau = 1/k_{cl}$).

By the mechanism in Figure 1 and consistent with our data, the opening of the $GluRD_{flip}$ channel requires a minimal binding of two glutamate molecules per receptor complex. This mechanism is general for other AMPA receptors (*22*, *27*−*30*), including the $GluR1Q_{flip}$ and $GluR2Q_{flip}$ channels we characterized using the same kinetic technique (*9*, *10*). Binding of two glutamate molecules per receptor complex with a tetrameric composition, one for each dimer rather than to each subunit, is a plausible stoichiometry for opening the channel considering that the receptor complex is a dimer-of-dimers assembly (*2*). The stoichiometry issue was also investigated by analyzing the conductance states at the single-channel level. It was found that both native and recombinant AMPA receptors exhibit multiple conductance levels and the receptor channel dwells on larger conductance levels as the agonist concentration is increased (*17*, *31*). Using single-

channel recording, Rosenmund et al. (*32*) carried out an elegant study and concluded that binding of two agonist molecules per tetrameric receptor complex is necessary to open the channel. Binding of up to four agonist molecules increases mean single-channel conductance as compared with binding of two agonist molecules.

To determine k_{op} and k_{cl}, the rate of glutamate binding to the receptor was assumed to be fast compared with the rate of channel opening, consistent with the kinetic behavior of the rising phase of the whole-cell current. At present, however, the association rate constant of glutamate with the whole receptor is unknown. Pioneering studies by Madden and co-workers (*33*) on the kinetics of glutamate binding to the GluRD-derived S1S2 extracellular binding domain provide some clues about how fast glutamate may bind to the whole receptor. The rate constant of association of glutamate with the S1S2 partial receptor was $\sim 10^7 - 10^8$ M^{-1} s^{-1} at 5 °C (*33*). Because the rate of association of glutamate with wild-type S1S2 was too fast, it was not possible to further separate the microscopic rate constants for step 1, the agonist docking step, from step 2, the subsequent locking process (*33*). Nevertheless, the association rate constant determined at 5 °C is expected to become even larger at room temperature, assuming that the association rate constant behaves according to the Arrhenius equation (*34*). The rate of glutamate binding to its site on the S1S2 receptor may be even faster because of a favorable electrostatic interaction between the receptor and glutamate, which steers glutamate to its site when a glutamate molecule approaches by free diffusion (*35*). Such a favorable electrostatic interaction to steer ligand or substrate binding to its target protein, thus accelerating the basal association rate constant, was observed in other association processes, such as the binding of acetylcholine, a neurotransmitter, to acetylcholinesterase (*36*).

The Channel Opening Rate Can Be Uniquely Measured Using the Laser-Pulse Photolysis Technique. The use of the laser-pulse photolysis technique made it possible to resolve the channel opening process in the microsecond time domain from the ensuing desensitization reaction in the millisecond time region. The separation of the two rate processes is apparent via examination of the following experimental evidence. As seen in Figure 3A, the k_{obs} for the rising phase is 4800 s^{-1}. However, at the same glutamate concentration, 190 μM, the observed desensitization rate constant is 130 s^{-1} as seen in Figure 2B. Using these rate constants, we can estimate the percentage of desensitization that occurred within the rising phase of the current from which the channel opening rate constant was measured. If glutamate binding had initiated the desensitization at time zero, as it initiated the channel opening, in 0.6 ms the desensitization reaction would have proceeded ~8%. However, the channel opening reaction would progress to 95% of completion, the extent to which the k_{obs} was measured in our experiment as shown in Figure 3A. Therefore, the desensitization did not interfere appreciably with the measurement of the channel opening rate of $GluRD_{flip}$. Furthermore, because the k_{obs} and the time interval for the current rise used for this estimation were based on a virtually synchronized release of free glutamate triggered by the laser-pulse photolysis, this estimate was thus reasonable. Consistent with this conclusion, we found that simultaneous fitting of the current rise and fall yielded a k_{obs} value similar to the value obtained by a single-exponential fit of the current rise using eq 2. Together, these results show that the channel opening rate constant can be measured from the rising phase of the macroscopic receptor current, and this measurement is free from the complication of the desensitization reaction. Hence, the formulation of the kinetic scheme for channel opening in Figure 1 does not involve the reaction pathway(s) for channel desensitization and in turn gives rise to a simple kinetic equation, i.e., eq 3, for determining k_{op} and k_{cl}.

Table 1: Channel Opening and Channel Closing Rate Constants for Glutamate Receptors[a]

glutamate receptor type	k_{op} (s^{-1})	k_{cl} (s^{-1})	technique	ref
$GluR1Q_{flip}$	2.9×10^4	2.1×10^3	laser-pulse photolysis	*9*
	–	$\sim 3.3 \times 10^3$	single-channel recording[b]	*14*
$GluR2Q_{flip}$	8.0×10^4	2.6×10^3	laser-pulse photolysis	*10*
	–	3.1×10^3	single-channel recording[b]	*23*
$GluR4_{flip}$	6.8×10^4	3.4×10^3	laser-pulse photolysis	this work
	4.0×10^4	8.0×10^3	fitting	*13*
	–	5.9×10^3	single-channel recording	*17*
GluR6Q	1.1×10^4	4.2×10^2	laser-pulse photolysis	*22*
	1.0×10^4	4.4×10^2	fitting	*41*
	1.0×10^4	–	flow measurement	*40*

[a] In all data cited, glutamate is the agonist. [b] The lifetime refers to the major component.

Earlier studies suggested that when the glutamate concentration is very low, glutamate binding may desensitize AMPA receptors without ever opening the channel (*4*, *37*–*39*). Furthermore, binding of even one glutamate molecule per receptor complex is thought to be sufficient to desensitize the channel (*13*). Under any of these circumstances, the receptor would be pre-desensitized through the closed states and would be thus electrically silent. Such a process would not be detected, at least not directly, by any electrophysiological methods. These receptor states were thus not included in the minimal mechanism in Figure 1.

Comparison of the k_{op} and k_{cl} Values with Reported Ones for $GluRD_{flip}$ and Other Glutamate Receptors. Previously, k_{op} has not been experimentally determined for *$GluRD_{flip}$*. On the basis of a limited number of kinetic parameters, Robert and Howe (*13*) estimated the rate constant of k_{op} to be 4.0×10^4 s^{-1} (assuming a 2β value or the channel opening is initiated after binding of two glutamate molecules from their fitting). That estimated value is 1.7-fold smaller than our value of k_{op} (6.83×10^4 s^{-1}). However, the lifetime of the open channel for $GluRD_{flip}$ was determined using single-channel recording (*17*). A single time constant of 0.17 ms or an equivalent rate constant of ~6000 s^{-1} was calculated from the histogram of the open times, which was compiled from all conductance states (*17*). The mean lifetime of the open channel in this case was close to the resolution of openings in the single-channel recording, which was 0.15–0.2 ms (*17*). Although not large, we do not yet know the reason for this 1.7-fold discrepancy in the value of k_{cl} between our study (i.e., $k_{cl} = 3400$ s^{-1}) and the single-channel approach. Qualitatively, however, the two values do suggest that the channel closing process is rapid.

Comparison of the k_{op} and k_{cl} values of $GluRD_{flip}$ with those of other AMPA and kainate homomeric channels (Table 1) shows two notable features. First, all AMPA channels have rate constants for channel opening and closing larger than the corresponding values of the GluR6Q kainate

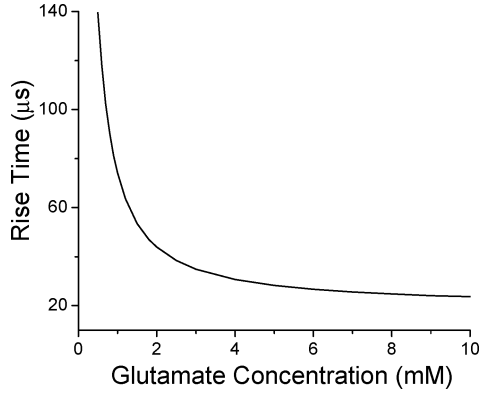

FIGURE 4: Dependence of the rise time on glutamate concentration for the opening of the $GluRD_{flip}$ channel. The rise time is defined as the time for the receptor current to rise from the 20% to the 80% level. The rise time is calculated as described in Materials and Methods. The fastest rise time is predicted to be ~20 μs.

receptor (*22*, *40*, *41*). Second, comparison of $GluRD_{flip}$ with other AMPA receptor subunits (Table 1) shows that the kinetic properties of $GluRD_{flip}$ are more similar to those of $GluR2Q_{flip}$ than to those of $GluR1Q_{flip}$. However, the $GluRD_{flip}$ channel closes slightly faster than does the $GluR2Q_{flip}$ channel, whereas the channel opening rate constant is roughly identical. Interestingly, the intrinsic dissociation constant for glutamate or the K_1 value for $GluRD_{flip}$ (1.1 mM) is also similar to that of $GluR2Q_{flip}$ (1.3 mM) (*10*), compared with that of $GluR1Q_{flip}$ (0.53 mM) (*9*).

Channel Opening Probability (P_{op}). On the basis of the experimentally determined k_{op} and k_{cl} values, the P_{op} for the $GluRD_{flip}$ channel was calculated to be 0.95 ± 0.12 by eq 4. This value is comparable to those previously reported for two other homomeric AMPA receptor channels, all of which are above 0.90 (*9*, *10*). Quantitatively, the P_{op} of 0.95 indicates that the rate of the forward reaction (i.e., the channel opening) is ~20 times faster than the rate of the backward reaction (i.e., channel closing). Thus, the presumed conformational change from the doubly liganded, closed channel state to the open channel state is relatively favorable. A large P_{op} value, like the one obtained here, further suggests that the open channel state of the receptor is relatively stable, because $k_{cl} \ll k_{op}$.

Time Course of the Opening of the $GluRD_{flip}$ Channel. The time course of the opening of the $GluRD_{flip}$ channel was determined as described in Materials and Methods. As seen in Figure 4, the rise time can be predicted at any given concentration of glutamate. The rise time decreases with increasing concentrations of ligand and becomes the shortest, ~20 μs, when glutamate binding is saturated. Thus, 20 μs sets the fastest observed time scale for this channel to open. It should be pointed out the contribution of desensitization to the time course of channel opening or to the rise time is negligible and thus not included, for reasons described in the Results.

Solution exchange techniques were previously used to estimate the fastest rise time of the macroscopic current for several AMPA receptor channels (*5*, *42*, *43*). In particular, a recent study by Grosskreutz et al. (*43*) reported a 120 μs time constant as the fastest current rise for the opening of AMPA receptor homomeric channels, including GluRD. In contrast, the time constant of the channel-opening process for the saturation condition in this study is 14 μs [see eq 3; when $L \gg K_1$, $\tau = 1/(k_{op} + k_{cl})$]. Thus, the previous estimate by Grosskreutz et al. (*43*) is 8 times slower than our value. In fact, the time constant measured by Grosskreutz et al. for the channel opening at the ligand saturation condition, 120 μs, is not much larger than the lifetime of the open channel or the equivalent k_{cl} from either the value reported in this study or the value previously measured by Swanson et al. using single-channel recording (*17*). The slower channel opening rate or an equivalent longer rise time, estimated by using various solution exchange techniques, may be attributed to a slower solution exchange time of ~200 μs in general (*1*, *13*, *14*, *42*).

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