Opening of a ligand-gated ion channel is the step at which the binding of a neurotransmitter is transduced into the electrical signal by allowing ions to flow through the transmembrane channel, thereby altering the postsynaptic membrane potential. We report the kinetics for the opening of the GluR1Qflip channel, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit of the ionotropic glutamate receptors. Using a laser-pulse photolysis technique that permits glutamate to be liberated photolytically from γ-O-(α-carboxy-2-nitrobenzyl)glutamate (caged glutamate) with a time constant of ~30 μs, we show that, after the binding of glutamate, the channel opened with a rate constant of \((2.9 \pm 0.2) \times 10^4 \) s\(^{-1}\) and closed with a rate constant of \((2.1 \pm 0.1) \times 10^3 \) s\(^{-1}\). The observed shortest rise time (20–80% of the receptor current response), i.e. the fastest time by which the GluR1Qflip channel can open, was predicted to be 35 μs. This value is three times shorter than those previously reported. The minimal kinetic mechanism for channel opening consists of binding of two glutamate molecules, with the channel-opening probability being 0.93 ± 0.10. These findings identify GluR1Qflip as one of the temporally efficient receptors that transduce the binding of chemical signals (i.e. glutamate) into an electrical impulse.

The rate at which a ligand-gated ion channel opens is important to know because it has major implications in signal transmission and regulation. First, knowing the constants for the channel-opening rate will allow one to predict more quantitatively the time course of the open channel form of the receptor as a function of neurotransmitter or ligand concentration, which determines the transmembrane voltage change and in turn controls synaptic neurotransmission. Second, that knowledge will provide clues for mechanism-based design of compounds to regulate receptor function more effectively. Third, characterizing the effect of structural variations on the rate constants for channel opening will offer a test of the function, which is relevant to the time scale on which the receptor is in the open channel form, rather than in the desensitized form, i.e. ligand-bound, but closed channel form. Examples of structural variations include those due to RNA editing, RNA splicing, and site-specific mutations for investigating the structure-function relationship. Finally, knowing the channel-opening rate constants will be required to understand quantitatively the integration of nerve impulses that arrive at a chemical synapse or that originate from the same synapse, but from different receptors responding to the same chemical signals (neurotransmitters) such as glutamate.

We here report the kinetics for the opening of the GluR1Qflip receptor channel. GluR1 is one of the four subunits of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (1, 2). As a subtype of ionotropic glutamate receptors, AMPA receptors mediate fast synaptic neurotransmission in the mammalian central nervous system (1, 2). The GluR1 subunit plays a specific role in a wide range of biological functions such as the expression of synaptic plasticity (3–6), the formation of memory (7–9), the development of the dendritic architecture of motor neurons (10), the excitotoxic necrosis induced via acid sphingomyelinase-mediated and NF-κB-mediated signal transduction pathways (11), and the generation of sensitization by drugs of abuse (12–14).

The kinetic properties of the GluR1 receptor related to desensitization have been well characterized. For example, GluR1 desensitizes rapidly with a maximal time constant of 4 ms, achieved at saturating glutamate concentration (15–18). Amino acid residue 750 (i.e. serine at GluR1Qflip) has been identified as sensitive to allosteric modulators of AMPA receptors such as cyclothiazide (16). Furthermore, the single channel recording of GluR1 revealed that the major component (73%) has a lifetime of 0.24 ms (6). However, the kinetic mechanism of channel opening is not known. The rate of channel opening appears to be too fast to be resolved by commonly used kinetic approaches such as solution exchange techniques. Consequently, the kinetic constants pertaining to the channel-opening process have been estimated only by fitting the rate parameters associated with the deactivation and desensitization processes that occur relatively slowly compared with channel opening (17, 19). By no means, however, is this “slow” time scale actually slow: even early studies of the native AMPA receptors show that, within a few milliseconds, the receptors desensitize (20, 21). By inference, the channel must open faster.

In this study, we used a laser-pulse photolysis technique to release biologically active glutamate from biologically inert caged glutamate or γ-O-(α-carboxy-2-nitrobenzyl)glutamate with a time constant of ~30 μs (Fig. 1) (22) and characterized the kinetic mechanism of glutamate-induced channel opening prior to channel desensitization. We found that the GluR1Qflip channel opens in response to the binding of the natural neurotransmitter glutamate with a rate constant of 29,000 s\(^{-1}\) and closes with a constant of 2100 s\(^{-1}\). Once bound to glutamate,
the receptor has a 0.93 probability to open. We offer possible interpretations for the biological significance of these results in the context of other glutamate receptors.

MATERIALS AND METHODS

Expression of cDNA and Cell Culture—The original cDNA encoding GluR1Qflip in a pBluescript vector was provided by Prof. Steve Heinemann (Salk Institute) and cloned into the pcDNA3.1 vector (Invitrogen). The plasmid was propagated in an Escherichia coli host (DH5α) and purified using a kit from QIAGEN Inc. (Valencia, CA). HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a 5% CO2 humidified incubator at 37 °C. GluRIQflip was transiently expressed in these cells using a calcium phosphate method (23). Unless otherwise noted, HEK-293 cells were also cotransfected with a plasmid encoding green fluorescent protein (GFP; a generous gift from Prof. Ben Szaro, State University of New York at Albany). GFP was used as an intracellular marker, and green cells were selected for recordings. The weight ratio of the plasmid for GFP to that for GluR1 was 1:10, and the GluR1 plasmid was used for transfection at ~3–5 μg/35-mm dish. Transfected cells were allowed to grow for ~48 h before use.

Whole-cell Current Recording—Recordings were performed using Axopatch 200B amplifier at a cutoff frequency of ~2-20 kHz with a built-in, 4-pole Bessel filter and digitized at sampling frequencies of ~5-50 kHz using a Digidata 1322A apparatus (Axon Instruments, Inc., Union City, CA). The data acquisition software used was pCLAMP 8 (Axon Instruments, Inc.).

Laser-pulse Photolysis—The setup for the laser-pulse photolysis experiment has been described previously (24, 25). γ-O-(α-carboxy-2-nitrobenzyl)glutamate (Molecular Probes, Inc., Eugene, OR) (22) was dissolved in the external buffer solution and applied to a cell using a cell-flow device (see below). Once a HEK-293 cell was in the whole-cell mode, it was lifted from the bottom of the dish and suspended in the external bath solution. After the cell was equilibrated with caged glutamate for 250 ms, the laser was fired to liberate the free glutamate. A single laser pulse at 355 nm with a pulse length of 8 ns was generated from a Minilite II pulsed Q-switched Nd:YAG laser (Continuum, Santa Clara, CA) tuned by a third harmonic generator. The laser light was coupled to a fiber optic (FiberGuide Industries, Stirling, NJ), and the power was adjusted to 200–800 μW, as detected by a joulemeter (Gentec, Quebec, Canada).

To vary the concentration of the photolytically released glutamate in kinetic measurements, the power of the laser was adjusted, and/or the concentration of the caged glutamate was varied. To determine the concentration of the photolytically released glutamate, at least two glutamate solutions with known concentrations were used to measure the current amplitudes from the same cell before and after a laser pulse. The free glutamate solution was applied to the cell using the cell-flow device (see below). The current amplitudes obtained from the cell-flow measurements were compared with the amplitude from the laser measurement, with reference to the dose-response relationship. These measurements also permitted us to monitor any damage to the receptors and/or the cell for successive laser experiments with the same cell.

Cell-flow Measurements—The cell-flow device (26) was used to deliver either caged glutamate for laser-pulse photolysis or free glutamate to monitor the damage and to calibrate the concentration of photolytically released glutamate. The cell-flow device consisted of a U-tube with an aperture of ~150 μm. The linear flow rate, controlled by two peristaltic pumps, was 4 cm/s. A HEK-293 cell was placed 50–100 μm away from the U-tube aperture. The rise time of the glutamate-induced whole-cell current response (10–90%) was 2.3 ± 0.1 ms, an average of the measurement from >100 cells expressing the receptor. When free glutamate was used, the amplitude of the whole-cell current was corrected for receptor desensitization during the rise time by a method described previously (26). This correction is necessary because the observed current amplitude depends on the rise time of the amplitude, the fraction of the open channel (i.e. the percentage of the channel that is open), and the desensitization rate constant. The reduction in the current amplitude due to desensitization is particularly significant when the receptor of interest desensitizes rapidly, as does the GluR1 receptor (6, 16, 17). The method used to correct the receptor desensitization during the rise time is based on hydrodynamic theories that describe fluid flowing over a spherical object, such as, by approximating the HEK-293 cell suspended in the external bath solution. When a solution flows over a cell, the time that the ligand molecules in the solution mix with receptors on the cell surface varies because of uneven flow rates of the ligands over the spherical surface of a cell. This asynchroniztion in mixing distorts both the rate and amplitude of the current rise. By the correction method, the time course of the current is first divided into a constant time interval, and then the observed current (I_{obs}) is corrected for the desensitization that occurs during each time interval (∆t). After the current is determined for each of n constant time intervals (∆t = t_i, where t_i is equal to or greater than the current rise time), the corrected total current (I_A) is given by Equation 1,

\[ I_A = \sum_{i=1}^{n} I_{obs} \Delta t_i + (I_{inf}) \Delta t_i \]

where s represents the rate constant for receptor desensitization, and \( I_{inf} \) is the observed current during the ith time interval. \( I_{inf} \), obtained from this correction method is independent of the flow speed of the solution. The validity of this method was demonstrated using several independent approaches (26). (The current correction program was kindly provided by Prof. George P. Hess, Cornell University.)

All recordings were made with cells that were voltage-clamped to −60 mV, pH 7.4, and 22 °C. Each data point is an average of at least three measurements collected from at least three cells unless otherwise noted. Linear regression and nonlinear fitting (Levenberg-Marquardt and simplex algorithms) were performed using Origin Version 7 software (Origin Lab, Northampton, MA). Uncertainties are reported as S.E. of the fits unless noted otherwise.

RESULTS

Glutamate-induced GluRIQflip Response—A typical glutamate-induced whole-cell response is illustrated in Fig. 2A. The current through the GluRIQflip homeric channel expressed in HEK-293 cells increased rapidly, indicating channel opening, and then returned toward the baseline, indicating desensitization. As a control, both non-transfected cells and cells expressing only GFP gave no response even at 10 μM glutamate, a concentration that otherwise would have evoked a maximal current response for the transfected cells expressing GluRIQflip (see the dose-response curve in Fig. 3A). The receptor desensitization was rapid (Fig. 2B) and essentially complete (Fig. 2A), which is consistent with previous observations (6, 15–17). A first-order rate was adequate to describe >95% of the progression of the desensitization reaction at all concentrations of glutamate. This analysis agreed with those previously reported (6, 15, 16), although Robert et al. (17) described an additional but minor (0.5–2%) desensitization process with a much slower rate. The desensitization rate constant increased with increasing glutamate concentration, but eventually became invariant (Fig. 2B). The mean value of the maximal rate constant, independent of ligand concentration, was 230 s^-1 or a time constant of ~4 ms.

Based on the magnitude and profile of the desensitization rate, we evaluated whether the presence of GFP affected the kinetic properties of GluRIQflip expressed in the green fluorescent HEK-293 cells (the green color was due to the expression of GFP) because the green cells were selected for measurements. We found that the desensitization rate constant obtained from green cells expressing both GFP and GluRIQflip at
a given glutamate concentration was statistically no different from the rate constant obtained from non-green cells expressing only GluR1Qflip (Fig. 2B). Furthermore, the relative current amplitude determined under the same conditions was comparable (Fig. 3A). Therefore, we concluded that the presence of GFP in the same cell did not affect the kinetic property of GluR1Qflip and GFP was thus used for convenient identification of cells expressing GluR1Qflip in HEK-293 cells expressing both GluR1Qflip and GFP. Each data point is an average of at least three measurements from three cells.

It has been documented that the desensitization rate observed using whole cells is slower than that using outside-out patches for the same receptor (6, 16, 17). The largest difference reported is 2.9-fold (16). The reason for this discrepancy in rate is not known, although several explanations have been proposed (6, 16, 17), including the slower solution exchange rate with a whole cell because of its geometry and/or the faster desensitization rate constant observed with outside-out patches because of the altered kinetic properties of the receptor in such a membrane configuration (27). Nevertheless, our purpose for measuring desensitization (shown in Fig. 2B) was to use the desensitization rate constant as a relative measure to test whether the presence of GFP in the same cell affected the desensitization rate of the receptor. Furthermore, the magnitude of the desensitization rate we observed using the whole-cell recording was consistent with values reported by others. For instance, at 1 mM glutamate, the desensitization time constant we obtained was 6 ms (or a rate constant of 160 s⁻¹). This value was identical, within experimental error, to the value observed in the whole-cell recording of the same receptor by both Partin et al. (16) and Derkach et al. (6).

FIG. 2. Coexpression of GFP does not affect the kinetic properties of GluR1Qflip in HEK-293 cells. A, whole-cell current response to glutamate at concentrations of 2000 (lower trace), 500 (middle trace), and 200 (upper trace) μM. B, comparison of the desensitization rate constants obtained from cells with and without coexpression of GFP. The desensitization rate was characterized by a first-order rate constant and is shown with the S.E. □, measurements for HEK-293 cells expressing only GluR1Qflip. □, measurements for HEK-293 cells expressing both GluR1Qflip and GFP. Each data point is an average of at least three measurements from three cells.

FIG. 3. Whole-cell current amplitude as a function of glutamate concentration or dose-response relationship. A, the whole-cell currents from different cells were normalized to the current obtained at 1 mM glutamate, and the current amplitude at 10 mM was set to 100%. The best fit parameters using Equation 2 (solid line) were as follows: K₁ = 0.53 ± 0.06 mM, φ = 0.19 ± 0.04, and IₐRₐ = 123 ± 5. □, measurements for HEK-293 cells expressing only GluR1Qflip. □, measurements for HEK-293 cells expressing both GluR1Qflip and GFP. The observed whole-cell current was corrected for desensitization (see “Materials and Methods”). B, shows a minimal kinetic mechanism for the channel opening of GluR1Qflip. The mechanism involves two ligand-binding steps. A, the active unliganded form of the receptor; L, the ligand (glutamate); AL₁ and AL₂, ligand-bound closed channel forms; AL₂, the open channel form of the receptor. For simplicity, it is assumed that glutamate binds to the two sites with equal affinity (designated by K₁).

Minimal Kinetic Mechanism for Channel Opening—Fig. 3A shows the dose-response relationship, established with the current amplitude corrected for receptor desensitization (see “Materials and Methods”), as a function of glutamate concentration. The relationship is described by Equation 2,

\[ I_A = I_M R_M \frac{L^2}{L^2 + \Phi(L + K_1)^3} \]  

(Eq. 2)

where \( I_A \) represents the current amplitude, \( L \) is the molar concentration of the ligand, \( I_M \) is the current/mole of receptor, and \( R_M \) is the moles of receptor in the cell. \( \Phi \) is the reciprocal of the channel-opening equilibrium constant, and \( K_1 \) is the intrinsic dissociation constant for the ligand. The derivation of Equation 2 was based on a minimal kinetic mechanism for channel opening, shown in Fig. 3B. This mechanism is a general one for ligand-gated ion channels (28), including glutamate receptors (20, 29–33), in which the binding of two glutamate molecules is sufficient to open the channel (29, 34, 35). For simplicity, it was assumed that the intrinsic equilibrium dissociation constant (\( K_1 \)) for both ligand-binding steps was the same (see below for additional discussion of this mechanism).

Accordingly, the best fit of the dose-response curve yielded \( K_1 = 0.53 \pm 0.06 \) mM using Equation 2. The \( K_1 \) value from this study is comparable with the reported values of EC₅₀ (the ligand concentration that corresponds to 50% of the maximal response), ranging from ~0.5 to 0.7 mM (6, 16, 36).

Characterization of Caged Glutamate with GluR1Qflip in HEK-293 Cells—In this study, we used a lazer-pulse photolysis technique to characterize the channel-opening kinetics for the GluR1Qflip homomeric receptor. This technique permits rapid photolytic release of free glutamate, with \( t_{\frac{1}{2}} \approx 30 \) μs, from its photolabile precursor or caged glutamate (Fig. 1) (22). To use this technique, the caged glutamate must be biologically inert with respect to the GluR1Qflip receptor expressed in HEK-293 cells. As shown in Fig. 4, the glutamate-elicited receptor re-
Fig. 4. Caged glutamate is biologically inert with respect to GluR1Qflip expressed in HEK-293 cells. Superimposed are the whole-cell currents induced by 200 μM glutamate in the absence (solid line) and presence (closed circles) of 2 mM caged glutamate. The sampling frequency was 5 kHz. For clarity, however, the number of points shown in the closed circle trace was reduced in various regions of the current trace.

responses in the presence and absence of caged glutamate had identical current amplitudes and desensitization rates. In this test, the concentration of caged glutamate was 2 mM, the highest used in the laser experiments. Therefore, this result (Fig. 4) demonstrated that the caged glutamate did not activate the GluR1Qflip channel, nor did it inhibit or potentiate the glutamate response. This conclusion is consistent with the earlier characterization of the caged glutamate using endogenous glutamate receptors in rat hippocampal neurons (22).

Channel-opening Kinetics Characterized by the Laser-pulse Photolysis Technique—Using the laser-pulse photolysis technique with caged glutamate, we determined the rate constants for the opening of the GluR1Qflip channel. A representative whole-cell current response obtained in these experiments is illustrated in Fig. 5A. The current increased as a result of the opening of the receptor channel and then decreased because of channel desensitization. A single exponential rate law (given in Equation 3) accounted for ~95% of the increase in current.

\[ I_t = I_A(1 - e^{-\lambda t}) \]  

(Eq. 3)

\( I_t \) represents the current amplitude at time \( t \), and \( I_A \) represents the maximal current amplitude. (An example of the fit using Equation 3 is indicated by the solid line in Fig. 5A.) Moreover, the rising phase of the current remained monophasic over the entire range of concentrations of photolytically released glutamate (i.e. between 50 and 250 μM). This result was consistent with the assumption that the ligand-binding rate was fast relative to the channel-opening rate (see the mechanism in Fig. 3B and the discussion below). The observed first-order rate process, as shown in Fig. 5A, therefore represented the channel-opening rate step. Accordingly, Equation 4 was derived, which enabled us to determine the channel-opening \( (k_{\text{op}}) \) and channel-closing \( (k_{\text{cl}}) \) rate constants.

\[ k_{\text{obs}} = k_{\text{cl}} + k_{\text{op}} \left( \frac{L}{L + K_L} \right)^2 \]  

(Eq. 4)

Shown in Fig. 5B is the best fit of the observed first-order rate constant \( (k_{\text{obs}}) \) as a function of glutamate concentration by Equation 4, yielding \( k_{\text{cl}} = (2.1 \pm 0.1) \times 10^3 \) s⁻¹ and \( k_{\text{op}} = (2.9 \pm 0.2) \times 10^4 \) s⁻¹.

In kinetic analysis of the channel-opening rate using Equation 4, we assumed that the rate of channel opening is slow relative to the rate of ligand binding in both the first and second steps (Fig. 3B). Consequently, the observed rate process reflects the transition from the doubly liganded, closed channel form to the open channel form. Kinetically, then, 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 ligand is varied. In our experiments, at all concentrations of photolytically liberated glutamate, the rising phase was accounted for adequately by a single first-order rate constant. Thus, this result is consistent with the assumption that the channel-opening rate is slower than the ligand-binding rate. Conversely, if the ligand-binding rate were similar to the channel-opening rate, there would be a biphasic rate process in the rising phase as the concentration of ligand is varied. In that case, one rate would represent ligand binding, whereas the other would reflect channel opening. If the ligand-binding rate were slow compared with the rate of channel opening, the concentration of ligand would not be adequately described by Equation 4. For instance, the rate would be linearly dependent on the concentration of glutamate if the binding rate for the first and second steps is assumed to be the same (37).

Because ligand binding is a bimolecular process, at sufficiently low concentrations of ligand, the rate of ligand binding will eventually become rate-limiting for the kinetic mechanism shown in Fig. 3B. To ensure that ligand binding was always fast so that the relatively slow channel-opening rate process could be observed, the lowest concentration of glutamate at
which we measured \( k_{\text{obs}} \) was chosen as 40 \( \mu M \) (Fig. 5B). The 40 \( \mu M \) ligand concentration corresponded to the fraction of the channel in the open form being \(-4\%\) (the fraction of the open channel is defined by Equation 2 and is shown in Fig. 2B). This was the same fraction at which the \( k_{\text{obs}} \) was comparable with the \( k_d \) for the nicotinic acetylcholine receptor (38). (Using the fraction of the open channel, rather than the absolute concentration, takes into account the different \( K_i \) values for different receptors.) Furthermore, the \( k_d \) for the nicotinic acetylcholine receptor was in agreement with the lifetime of the major component, rather than the absolute concentration.

Likewise, the \( k_d \) for the channel opening, the desensitization process, takes into account the different \( K_i \) values for different receptors. Thus, \( k_d \) represents the rate constant estimated for channel opening, the desensitization rate constant for the channel opening is 3800 s\(^{-1}\), obtained in this study for the GluR1Qflip receptor, is close to the value of the lifetime (of the major component), \(-0.3\) ms or a rate constant of 3100 s\(^{-1}\), for the same receptor, but obtained from single channel recording (see details under “Discussion”) (6). Therefore, the fraction of the open channel at \(-4\%\), which corresponded to the 40 \( \mu M \) glutamate concentration for the GluR1Qflip receptor, should be high enough such that the rate constant we measured should pertain to the channel-opening process rather than to ligand binding.

Presently, the rate of glutamate binding to the receptor, which leads to the opening of the channel, is not known. However, Madden and co-workers (39) reported that the rate constant for glutamate binding to the extracellular portion of the GluR4 receptor, known as S1S2, is indeed large. The association rate constant at 5 °C is \( 1.6 \times 10^7 M^{-1} s^{-1} \) (39). At room temperature, this rate constant is expected to become even larger provided that ligand binding behaves linearly according to the Arrhenius equation (40). However, that rate constant, as the authors pointed out, should be taken in the context that S1S2 is only a partial protein and lacks the ability to form the channel.

The Channel-opening Rate Can Be Separated from the Channel Desensitization Rate in the Laser-pulse Photolysis Measurements—As shown in Fig. 5B, the observed-channel opening rate became faster as the glutamate concentration increased (by the relationship given in Equation 4). Concurrently, the observed desensitization rate also became faster (Fig. 2B). However, the rate of channel opening, seen as the rise in the whole-cell current, was always faster than the rate of desensitization, seen as the fall in current (Fig. 5A). This was observed in all the current recordings of the laser-pulse photolysis measurements. Consequently, simultaneous fitting of both the rising and falling phases by two first-order rate equations yielded a \( k_{\text{obs}} \) value that was identical (±5% error range) to the \( k_{\text{obs}} \) value obtained in the single exponential fit using Equation 3. Thus, \( k_{\text{obs}} \) was treated as an elementary rate process, using Equation 4, without the complication of the desensitization reaction.

 Unlike ours, earlier mechanisms proposed for the channel opening of various glutamate receptors, including GluR1, all involved the desensitization reaction, and such reaction was assumed to occur once glutamate was bound (33, 35, 41). The omission of desensitization in our kinetic analysis of channel opening was based on our experimental evidence that the desensitization reaction did not proceed appreciably during the current rise, had the desensitization reaction occurred. This evidence is apparent in Figs. 2B and 5A. The observed first-order rate constant for the channel opening is 3800 s\(^{-1}\) (Fig. 5A), whereas the rate of channel desensitization is 120 s\(^{-1}\) at the same glutamate concentration, i.e. 160 \( \mu M \) (Fig. 2B). Therefore, when the current increased to 95%, where the first-order rate constant was estimated for channel opening, the desensitization reaction proceeded to only \(-7\%\). This estimate is plausible because it is based on a virtually synchronized activation of all channels on the cell surface triggered by the laser-pulse photolysis of caged glutamate. We therefore conclude that the channel-opening rate process can be measured effectively as a rate process that is kinetically distinct and separable from the slower desensitization process, which becomes appreciable only on a longer time scale.

The comparison of the rate of glutamate-induced channel opening with the rate of channel desensitization, as described above, further demonstrates that the rate of channel opening for GluR1Qflip far exceeds the rate of desensitization. This should be especially the case at high concentrations of glutamate. Physiologically, the synaptic concentration of glutamate can be as high as 1 mM (42, 43). However, when its concentration is very low, glutamate might desensitize AMPA receptors without ever opening the channel, as previously suggested (20, 30, 42, 44).

In our experiments, we were not able to determine whether there were receptors that never opened the channel because (a) these receptors were pre-desensitized through closed states, and/or (b) they were trapped in the ligand-bound closed states following the binding of glutamate. All these receptor states would be electrically “silent.” As in any other electrophysiological recording methods, these electrically silent states are not observable (at least not directly). Thus, it was implicit that these receptor states were not included in the minimal mechanism in Fig. 3B.

The activation of the glutamate channel as a kinetic process separate from desensitization was proposed (33, 35) and demonstrated explicitly in several early studies. For example, binding of cyclothiazide to glutamate receptors has been shown to prevent desensitization (45). A single leucine-to-tyrosine substitution (L497Y in GluR1Qflip or L507Y in GluR3) also abolishes desensitization for the corresponding homomeric channels (46). By measuring the rate of GluR2 channel closure after ligand removal (\( k_{\text{off}} \) versus \( k_{\text{des}} \)), the \( k_{\text{off}}/k_{\text{des}} \) ratio was 15 for glutamate, compared with 2.2 for quisqualate, another agonist (47). Therefore, the high ratio indicates that the GluR2Q channel, once opened after glutamate binding, preferentially returns to the closed states without entering the desensitization state (47). Here we have demonstrated that, using the laser-pulse photolysis technique, the rate of channel opening can be indeed measured uniquely and prior to channel desensitization.

**DISCUSSION**

The kinetic process of GluR1Qflip receptor activation to form the transmembrane ion channel is thought to proceed rapidly after the binding of the natural neurotransmitter glutamate. However, little is known about the kinetic mechanism of channel opening. Some critical kinetic information was obtained previously by fitting the slower deactivation/desensitization rate constants. In this study, we applied the laser-pulse photolysis technique with caged glutamate, which provided \(-60-\mu s\) time resolution. This technique enabled us to measure directly the kinetic constants that govern the transition between the doubly liganded, closed state and the open state.

Channel-opening \( (k_{\text{op}}) \) and Channel-closing \( (k_{\text{cl}}) \) Rate Constants—The channel-opening rate constant \( (k_{\text{op}} = 29,000 s^{-1}) \) defines the time scale by which the GluR1Qflip channel opens after the binding of glutamate. It therefore reflects the rate of the conformational change from the doubly liganded, closed form of the receptor to the open channel form. Armstrong and Gouaux (48) and Armstrong et al. (49) suggested that the opening of the channel is triggered by the closure of receptor domains or lobes 1 and 2 after the binding of agonist. The “trapping” of agonists such as glutamate by domain closure causes a conformational strain in the extracellular portion of the receptor. Such strain is translated into the gate, presum-
ably in the transmembrane region, thereby opening the channel (48, 50). By this notion, \( k_{op} \) likely represents the rate of this domain closure induced by the binding of glutamate. Furthermore, the magnitude of \( k_{op} \) indicates that the \( t_{1/2} \) of channel opening is 24 \( \mu s \). This value is consistent with the microsecond time scale by which the amino acid residues in the ligand-binding pockets can undergo motions, as observed by NMR spectroscopy (51).

The channel-closing rate constant (\( k_{cl} \approx 2100 \text{ s}^{-1} \)) is a measure of how fast an open channel returns to the doubly liganded, closed state (Fig. 3B). Thus, it reflects the lifetime of the open channel for the GluR1Qflip receptor (\( k_{cl} = 1/r \), where \( r \) is the lifetime expressed as a time constant). The lifetime of the GluR1 channel has been determined using single channel recording (6). The distribution of the open times was described by two time constants of \(-0.3 \text{ ms} \) (73%) and \( 2 \text{ ms} \) (27%) (6). The major component has an equivalent rate constant of \(-3000 \text{ s}^{-1} \), which is slightly higher than the \( k_{cl} \) of \( 2100 \text{ s}^{-1} \) obtained in this study. One possible reason for the disparity in the rate constant is that our value reflects the rate constant for the ensemble rate process originating from the macroscopic receptor response, rather than individual channel events observed at the single channel level. Nevertheless, the two values may be roughly comparable, reflecting in general a short duration of the open channel for the majority of the receptors.

The channel-opening probability (\( P_{op} \)) reflects the probability that a channel will open once it is bound with ligand(s) (38). Based on the experimentally determined \( k_{op} \) and \( k_{cl} \) values, \( P_{op} \) for the GluR1Qflip channel was estimated to be \( 0.93 \pm 0.10 \), given by the ratio \( k_{op}/(k_{op} + k_{cl}) \) (52). This value is comparable with those previously reported (ranging from 0.8 to 0.9) by non-stationary variance analysis of glutamate-induced macroscopic currents (6, 17, 19). Quantitatively, the \( P_{op} \) of 0.93 indicates that the rate of the forward reaction (i.e. the reaction of channel opening) is \(-14 \) times faster than the rate of the backward reaction (i.e. the reaction of channel closing). Thus, the presumed conformational change from the doubly liganded, closed channel form to the open channel form is relatively favorable. A high value of \( P_{op} \) like the one obtained here, further implies that the open channel form of the receptor is relatively stable because \( k_{cl} \ll k_{op} \).

**Time Course of Channel Opening**—The time course of the opening of a channel describes the duration of the open channel for the ensemble rate process and takes into account how fast the channel opens, defined by \( k_{op} \), and how long the channel remains open (i.e. the lifetime of the channel), defined by \( k_{cl} \). The time course of channel opening is influenced by the synaptic concentration of ligand because the rate of channel opening is ligand-dependent (see Equation 4). With the \( k_{cl} \) and \( k_{op} \) values known, the time course for the opening of the GluR1Qflip channel at any given concentration of glutamate can be established quantitatively using Equation 4 (Fig. 6). To represent the time course, we used the rise time of the current response, defined by an increase in receptor current of 20–80% in response to glutamate. As shown in Fig. 6, the rise time became shorter with increasing concentrations of ligand. When the ligand concentration reached \( 5 \text{ mM} \), the rise time became virtually invariant. Under this condition, the shortest rise time was calculated to be \(-35 \mu s \). This value sets the lower limit for the duration of the open channel.

Several attempts (all of which used fast solution exchange techniques) have been made previously to estimate the shortest current rise time. These values range from 180 to \( >400 \mu s \), reported as a 10–90% rise time (17, 19). These rise times are at least 3-fold longer than the value obtained in this study (35 \( \mu s \)). In a recent report by Grosskreutz et al. (53), a piezoelectrically driven solution exchange technique was used to measure the activation kinetics for human GluR1Qflip, for which a minimal solution exchange time of \(-50 \mu s \) was claimed. However, at the saturating concentration of glutamate, a time constant of 120 \( \mu s \) was obtained by directly fitting the rising phase of the current. In comparison, we found that the time constant for the channel-opening process under the saturation condition was 32 \( \mu s \) (i.e. \( 1/(k_{op} + k_{cl}) \)). Our value is more than three times shorter than the value reported by Grosskreutz et al. (53). The comparison, as such, should be valid because those authors further reported that the human AMPA receptors have kinetic properties that are similar to those of the rodent AMPA receptors (53), like the one used in our study. Conceivably, the longer rise time observed in all previous measurements for the same channel could be attributed to a slower solution exchange time or the time resolution, which limited the measurement of the faster receptor kinetics. Generally, the time resolution of these solution exchange techniques is \(-200 \mu s \) (1, 6, 17, 19).

When the glutamate concentration decreases to the extent that ligand binding becomes rate-limiting, the rise time as a function of glutamate concentration (Fig. 6) will be no longer tenable. When that happens, the \( t_{1/2} \) value will reflect the rate of ligand binding, the slowest step, rather than channel opening. The rise time may be less sensitive, accordingly, to the change in ligand concentration rather than as predicted by Equation 4. This phenomenon has been observed experimentally in the muscle nicotinic acetylcholine receptor (54). Furthermore, neither the relationship as predicted in Fig. 6 nor the minimal mechanism as presented in Fig. 3B takes into account the possible difference in the properties of the receptors that exhibit different subconductance levels, as observed in single channel recording (6, 19). Rather, the prediction of the rise time (Fig. 6) represents the ensemble kinetic properties of this channel based on the measurement of macroscopic current.

**Comparison of the Channel-opening Rate Constants for GluR1Qflip and Other Receptor Channels**—The \( k_{op} \) of 29,000 \( \text{s}^{-1} \) for the GluR1Qflip homomeric receptor channel suggests that this is a fast activating channel compared with other ligand-gated cation-conducting channels. For instance, the muscle-type nicotinic acetylcholine receptor has a \( k_{op} \) of 9400 \( \text{s}^{-1} \) (38), and the GluR6Q kainate receptor channel has a \( k_{op} \) of 11,000 \( \text{s}^{-1} \) (55). The \( k_{op} \) of 2100 \( \text{s}^{-1} \) for the GluR1Qflip homomeric channel suggests that it also closes more rapidly than most channels. For instance, the channel-closing rate constant for both the muscle-type nicotinic acetylcholine receptor (38) and the GluR6Q kainate receptor (55) is nearly 4-fold smaller than the channel-closing rate constant for GluR1. Compared with native heteromeric AMPA receptors in hippocampal neu-
to glutamate is a result of a concentration and the ratio of the rise time between the two difference of the receptor response as a function of glutamate concentration and the ratio of the rise time between the two receptors were in their functional forms before receptor desensitization, because the desensitization that eventually leads to the closure of the channel does not contribute appreciably during the rise time.

It is especially useful to compare the channel-opening rate constants for GluR1Qflip and GluR2Qflip (56) because both are AMPA receptor subunits, and both share considerable sequence homology, including identical RNA splicing status (i.e., flip variant). Yet, the magnitude of \( k_{op} \) for GluR1Qflip is nearly 3-fold smaller, suggesting that GluR1Qflip opens its channel in response to the binding of the same neurotransmitter, i.e., glutamate, three times slower than GluR2Qflip. Conversely, both channels close roughly on the same time scale based on the \( k_{cl} \) values for GluR1Qflip and GluR2Qflip of 2100 and 2600 s\(^{-1}\), respectively (56).

The comparison of the channel-opening rate constants for these two closely related AMPA receptor subunits suggests a unique pattern of neuronal integration if it is assumed that both GluR1 and GluR2 co-localize at the same postsynapse and can be activated simultaneously in response to the same chemical signal, i.e., glutamate. In fact, it is known that GluR1 and GluR2 are the predominant subunits in the composition of AMPA receptors in the CA1 region of the hippocampus (57). Furthermore, individual AMPA receptor subunits such as GluR1 may function independently (17). To highlight the implications of neuronal integration, Fig. 7 (A and B) shows the difference of the receptor response as a function of glutamate concentration and the ratio of the rise time between the two receptor types. The difference between the receptor responses to glutamate is a result of a \( >2 \)-fold difference in the \( K_i \) value (or roughly the EC\(_{50}\) value). At a glutamate concentration of 0.6 mM and below, a higher fraction of the GluR1Qflip receptor channel could open compared with the GluR2Qflip channel. When the glutamate concentration was lowered (see the left-hand side of Fig. 7B), the rise time became increasingly monophasic due to the opening of the GluR1Qflip channel. If these results are indicative of how the GluR1Qflip and GluR2Qflip receptors function at the same postsynapse, then at lower glutamate concentrations, it is expected that the integrated neuronal signal will be slow and mostly GluR1Qflip-like. However, as the glutamate concentration increases, a biphasic rate process will appear in the overall rise, with the fast component contributed by the GluR2Qflip channel opening.

What is the origin of this large disparity in the \( k_{op} \) value for these two closely related receptor subunits? The answer is presently unknown. Armstrong and Gouaux (48) proposed that the closure of the two extracellular domains or lobes that comprise the glutamate-binding site induces channel opening. Furthermore, the degree of lobe closure is ligand-dependent in that the largest degree of lobe closure can be induced by full agonists such as glutamate and AMPA, an intermediate degree induced by partial agonist, and the smallest degree induced by antagonists (48, 58). Therefore, assuming that \( k_{op} \) is linked to the dynamic movement of the lobe closure, the disparity in \( k_{op} \) between the two receptor channels could be due to the difference in the degree of glutamate-induced bilobe closure in these two receptors. Then, by analogy, the bilobe closure induced by binding of glutamate to GluR2Qflip is more complete than that induced by binding of glutamate to GluR1Qflip. This implies that some structural differences exist in the glutamate-binding pocket between the two receptors because the ligand that binds and opens the two channels in this case is the same. The structural difference may arise from several possible sources such as a difference in lobe 1, to which glutamate binds first, or lobe 2, whose closure is translated into the dynamic movement of the receptor channel (48). Evidently, to test this hypothesis, more study is needed.

**Charaterization of the Channel-opening Kinetics by Macroscopic Receptor Current**—The laser-pulse photolysis technique enabled us to measure the GluR1Qflip channel-opening kinetics based on the glutamate-induced macroscopic current. Photolytic release of free glutamate has a time resolution of \( \sim 60 \mu s \). Such high resolution is required to resolve the kinetic process of channel opening from the ensuing rapid desensitization reaction. Piezoelectric perfusion devices, which have been considered the most rapid, direct ligand application method, have time resolutions in the range of 200–400 \( \mu s \) for solution exchange (1, 6, 17, 19). The laser-pulse photolysis technique therefore offers a better time resolution. This technique is particularly useful for determining the channel-opening kinetic constants for both kainate and AMPA receptors because these receptors have been more difficult to study using the single channel recording technique with glutamate compared with the muscle nicotinic acetylcholine receptor, which has been classically characterized by single channel recording (59–62). Specifically, a much briefer opening duration (i.e., a faster channel-closing rate) of a kainate or an AMPA channel compared with the muscle nicotinic acetylcholine receptor is often observed. Moreover, the brief opening duration of such a channel makes it even more difficult to analyze the “flickering” in open channel bursts to investigate the mechanism of inhibition (63, 64).

Since the first demonstration of its use in the kinetic investigation of the channel-opening mechanism for the muscle-type nicotinic acetylcholine receptor (38), the laser-pulse photolysis technique has been applied to several other receptor types (31, 32, 65), including the GluR6Q kainate receptor (55) and the GluR2Qflip AMPA receptor (56). It has further enabled kinetic investigations of the mechanism of drug-receptor interactions in the microsecond-to-millisecond time domain where the receptors were in their functional forms before receptor desensitization (66–69). It is now possible to explore, with a time resolution of \( \sim 60 \mu s \), the structural and functional relationships of receptor subunits, the role of an individual subunit in heteromorphic receptor complexes, and the mechanism by which this homomeric receptor channel is regulated.
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REFERENCES
1. Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999) Pharmacol. Rev. 51, 7–61
2. Hollmam, M., and Heinemann, S. (1994) Annu. Rev. Neurosci. 17, 31–108
3. Petralia, R. S., Esteban, J. A., Wang, Y. X., Partridge, J. G., Zhao, H. M., Wenthold, R. J., and Malinow, R. (1999) Nat. Neurosci. 2, 31–36
4. Lao, D., Zhang, X., O’Brien, E. H., and Huganir, R. L. (1999) Nat. Neurosci. 2, 37–43
5. Takahashi, T., Sloboda, K., and Malinow, R. (2003) Science 299, 1585–1588
6. Derkach, V., Barria, A., and Soderling, T. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3269–3274
7. Reisel, D., Bannerman, D. M., Schmitt, W. B., Deacon, R. M., Flint, J., Borchardt, T., Seeburg, P. H., and Rawlins, J. N. (2002) Nat. Neurosci. 5, 868–873
8. Bliss, T. V., and Collingridge, G. L. (1993) Nature 361, 31–39
9. Lee, H. K., Takamiya, R., Han, J. S., Man, H., Kim, C. H., Rumbough, G., Yu, S., Ding, L., He, C., Petralia, R. S., Wenthold, R. J., Gallagher, M., and Huganir, R. L. (2003) Cell 112, 631–643
10. Inglis, F. M., Crockett, E., Kanada, S., Abraham, W. C., Hollmam, M., and Kalb, R. G. (2002) J. Neurosci. 22, 8042–8051
11. Yu, Z., Cheng, G., Wen, X., Wu, G. D., Lee, W. T., and Pleasure, D. (2002) Neuron 34, 199–213
12. Fitzgerald, I. W., Ortiz, J., Hamedani, A. G., and Nestler, E. J. (1996) J. Neurosci. 16, 274–282
13. Sutton, M. A., Schmidt, E. F., Choi, K. H., Schad, C. A., Whisler, K., Simmons, D., Karanjan, D. A., Monteggia, L. M., Neve, R. L., and Sel, D. W. (2003) Nature 421, 70–75
14. Carlezen, W. A., Jr., Todtenkopf, M. S., McPhie, D. L., Pimentel, P., Pilakas, A. M., Stellar, J. R., and Trzcinska, M. (2001) Neuropsychopharmacology 25, 234–241
15. Mosbaher, J., Schoepfer, R., Monyer, H., Burnashev, N., Seeburg, P. H., and Ruppersberg, J. P. (1994) Science 266, 1059–1062
16. Partiz, E. M., Fleck, M. W., and Mayer, M. L. (1996) J. Neurosci. 16, 6634–6647
17. Robert, A., Irizarry, S. N., Hughes, T. E., and Howe, J. R. (2001) J. Neurosci. 21, 5374–5386
18. Banke, T. G., Bowie, D., Lee, H., Huganir, R. L., Schousboe, A., and Traynelis, S. F. (1997) J. Physiol. Lond. 282, 383–395
19. Banke, T. G., Bowie, D., Lee, H., Huganir, R. L., Schousboe, A., and Traynelis, S. F. (2000) J. Neurosci. 20, 89–102
20. Raman, I. M., and Truscott, L. O. (1992) Neuron 9, 173–186
21. Fleck, M. W., Bahring, R., Patneau, D. K., and Mayer, M. L. (1996) J. Neurophysiol. 75, 2322–2333
22. Wiedbeldt, R., Gee, K. R., Niu, L., Ramesh, D., Carpenter, B. K., and Hess, G. P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8752–8756
23. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
24. Hess, G. P., and Grewer, C. (1998) Methods Enzymol. 291, 443–473
25. Niu, L., Grewer, C., and Hess, G. P. (1996) J. Neurosci. 16, 543–553
26. Ueda, N., Ito, S., and Hess, G. P. (1998) J. Neurosci. 18, 119–127
27. Raman, I. M., and Truscott, L. O. (1995) Biochem. J. 303, 137–146
28. Wahl, P., Anker, C., Traynelis, S. F., Egebjerg, J., Rasmussen, J. S., Krosgaard-Larsen, P., and Madsen, U. (1996) Mol. Pharmacol. 53, 590–596
29. Udgaonkar, J. B., and Hess, G. P. (1986) J. Membr. Biol. 93, 93–109
30. Matsubara, N., Billingam, A. P., and Hess, G. P. (1992) Biochemistry 31, 5507–5514
31. Abele, R., Keinanen, K., and Madden, D. R. (2000) J. Biol. Chem. 275, 21355–21363
32. Consens, K. A. (1990) Chemical Kinetics: The Study of Reaction Rates in Solution, pp. 245–253, VCH Publishers, Inc., New York
33. Krogsgaard-Madsen, U. (1995) Biochem. J. 310, 465–474
34. Krogsgaard, P., and Hess, G. P. (1995) Biochem. J. 310, 465–474
35. Raman, I. M., and Trussell, L. O. (1999) J. Physiol. (Lond.) 503, 153–152
36. Armstrong, N., and Gouaux, E. (2000) Neuron 28, 165–181
37. Armstrong, N., Mayer, M., and Gouaux, E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5736–5741
38. Sun, Y., Olson, R., Horning, M., Armstrong, N., Mayer, M., and Gouaux, E. (2002) Nature 417, 245–253
39. McFeeters, R. L., and Oswald, R. E. (2002) Biochemistry 41, 10472–10481
40. Raman, I. M., and Hess, G. P. (1997) J. Physiol. (Lond.) 503, 153–152
41. Armstrong, N., Sun, Y., Chen, G. Q., and Gouaux, E. (1998) Nature 395, 913–917
42. Neher, E., and Sakmann, B. (1976) Nature 266, 799–802
43. Colquhoun, D., and Sakmann, B. (1985) J. Physiol. (Lond.) 369, 501–557
44. Auerbach, A., and Sachs, P. (1984) Biophys. J. 45, 187–198
45. Krogsgaard-Madsen, U. (1995) J. Physiol. (Lond.) 339, 663–678
46. Javaraikum, V., Thiran, S., and Hess, G. P. (1999) Biochemistry 38, 11752–11758
47. Niu, L., and Hess, G. P. (1993) Biochemistry 32, 3831–3835
48. Niu, L., Aboud, L. G., and Hess, G. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12008–12013
49. Grewer, C., and Hess, G. P. (1999) Biochemistry 38, 7857–7864
50. Javaraikum, V., Usherwood, P. N., and Hess, G. P. (1999) Biochemistry 38, 4140–4144

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