

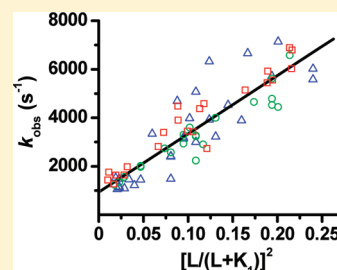
Channel-Opening Kinetic Mechanism of Wild-Type GluK1 Kainate Receptors and a C-Terminal Mutant

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Supporting Information

ABSTRACT: GluK1 is a kainate receptor subunit in the ionotropic glutamate receptor family and can form functional channels when expressed, for instance, in HEK-293 cells. However, the channel-opening mechanism of GluK1 is poorly understood. One major challenge to studying the GluK1 channel is its apparent low level of surface expression, which results in a low whole-cell current response even to a saturating concentration of agonist. A low level of surface expression is thought to be contributed by an endoplasmic reticulum (ER) retention signal sequence. When this sequence motif is present as in the C-terminus of wild-type GluK1-2b, the receptor is significantly retained in the ER. Conversely, when this sequence is either lacking, as in wild-type GluK1-2a (i.e., a different alternatively spliced isoform at the C-terminus), or disrupted, as in a GluK1-2b mutant (i.e., R896A, R897A, R900A, and K901A), there is a higher level of surface expression and a greater whole-cell current response. Here we characterize the channel-opening kinetic mechanism for these three GluK1 receptors expressed in HEK-293 cells by using a laser-pulse photolysis technique. Our results show that wild-type GluK1-2a, wild-type GluK1-2b, and the GluK1-2b mutant have identical channel opening and channel closing rate constants. These results indicate that the amino acid sequence near or within the C-terminal ER retention signal sequence, which affects receptor trafficking and/or expression, does not affect channel gating properties. Furthermore, as compared with the GluK2 kainate receptor, the GluK1 channel is faster to open, close, and desensitize by at least 2-fold, yet the EC₅₀ value of GluK1 is similar to that of GluK2.



Kainate receptor channels are involved in regulating both excitatory and inhibitory neurotransmission.^{1–3} Abnormal function of kainate receptors, however, has been implicated in some neurological disorders, such as epilepsy.^{4–6} Kainate receptors have five subunits, named GluK1–GluK5.⁷ GluK1 (previously known as GluR5),⁸ the focus of this study, is alternatively spliced at the C-terminus, producing various receptor isoforms.^{8,9} Among them, GluK1-2b is 49 amino acids longer than GluK1-2a, the shortest, whereas GluK1-2c, the longest, is generated by an in-frame insertion of nucleotides encoding an additional 29 amino acids into the GluK1-2b sequence (Figure S1 of the Supporting Information).⁹

To date, our understanding of the biophysical properties of kainate receptors at a molecular level had emerged largely from studies of GluK2.² The kinetic properties of GluK1 receptors are not well understood, in part because of their intrinsic low level of surface expression. GluK1, like the other kainate receptor subunits GluK2 and GluK3, can form homomeric channels in a heterologous expression system.^{8,9} However, GluK1, when expressed in human embryonic kidney (HEK-293) cells, for instance, produces a low current response to even a saturating concentration of agonist.^{9–12} In a study of GluK1, Ren et al.¹⁰ identified an endoplasmic reticulum (ER) retention signal from the intracellular C-terminus of GluK1-2b. The motif consists of a series of positively charged residues or mostly arginines. Among them, Arg896 is critical for its ER retention and low level of surface expression.¹⁰ If this ER retention motif in wild-type GluK1-2b is disrupted by mutation,

the ER exit of the receptor is promoted and the surface delivery is enhanced, resulting in a larger whole-cell current.¹⁰ A sizable whole-cell current response is essential for studying the biophysical properties of a receptor channel. For instance, properties of the closed channel conformation are studied at a low agonist concentration, which evokes a low current response.¹³ A low current response becomes even lower in the presence of an inhibitor. Furthermore, a sizable whole-cell current response can serve as a functional output at the cell surface for studying trafficking of kainate receptors, which is involved in affecting neurotransmission during synaptic plasticity and neuronal development.^{1–3}

Here we focus on the wild-type GluK1-2b receptor subunit, wild-type GluK1-2a (which does not contain the ER retention signal, unlike wild-type GluK1-2b), and a mutant GluK1-2b(R896A/R897A/R900A/K901A). GluK1-2a is a commonly used wild-type isoform representative of GluK1 in various studies.^{9,11,12,14,15} Mutations of mostly arginines within the ER retention signal located in the C-terminus of GluK1-2b are known to abolish ER retention.¹⁰ Therefore, the mutant GluK1-2b receptor lacks the ER retention signal and is expected to be expressed better than wild-type GluK1-2b in HEK-293 cells for electrophysiological experiments. In this

Received: September 16, 2011

Revised: December 19, 2011

Published: December 21, 2011



study we investigated (a) the kinetic and equilibrium constants for the opening of the GluK1 channels, (b) whether a mutation in the ER retention signal or the trafficking motif affects these constants, and (c) whether a GluK1 isoform that lacks the entire ER retention signal (i.e., GluK1-2a) has different channel opening kinetic constants. To measure these channel opening constants, we used a laser-pulse photolysis technique with a photolabile precursor of glutamate or caged glutamate,¹⁶ which provides ~60 μ s time resolution.^{17–19}

EXPERIMENTAL PROCEDURES

Expression of cDNAs and Cell Culture. The DNA plasmids encoding wild-type GluK1-2a(Q), wild-type GluK1-2b(Q), and the mutant GluK1-2b(R896A/R897A/R900A/K901A) were from P. Seeburg and G. Swanson. The 3132 bp fragment harboring the open reading frame that encodes GluK1-2a was cloned into pcDNA3.1(+). The cDNA plasmids were propagated through an *Escherichia coli* host (DH5 α) and purified using a QIAGEN kit. A receptor was transiently expressed in HEK-293 cells by a calcium phosphate method, as previously described.¹⁷ The HEK-293 cells were also cotransfected with a DNA plasmid encoding green fluorescent protein and another plasmid encoding the SV40 large T-antigen.¹⁷ The weight ratio of the DNA plasmid for receptor:green fluorescent protein:large T-antigen was 20:1:2, with the receptor DNA plasmid being 20 μ g per 35 mm Petri dish. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin in a 37 °C, 5% CO₂, humidified incubator. Transfected cells were allowed to grow for at least 48 h before they were used for recording.

Whole-Cell Current Recording. Electrodes for whole-cell recording were made from glass capillaries from World Precision Instruments (Sarasota, FL) and fire polished. The electrode had a resistance of ~3 M Ω when filled with the pipet solution. The pipet solution contained 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES (pH 7.4, adjusted with NaOH). The external cellular solution contained 150 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH 7.4, adjusted with NaOH). Green fluorescence in the transfected cells was visualized using a Carl Zeiss (Thornwood, NY) Axiovert S100 microscope equipped with a fluorescent detection system. The glutamate-induced whole-cell current was recorded using an Axopatch200B amplifier at a cutoff frequency of 2–20 kHz by a built-in, four-pole low-pass Bessel filter and was digitized at a 5–50 kHz sampling frequency using a Digidata 1322A digitizer from Molecular Devices (Sunnyvale, CA). Data were acquired by using pClamp 8 (also from Molecular Devices). All data were collected from the transfected HEK-293 cells voltage-clamped at –60 mV and 22 °C.

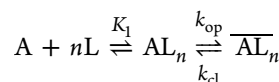
Laser-Pulse Photolysis Measurement. In the laser-pulse photolysis measurement, γ -O-(α -carboxy-2-nitrobenzyl)-glutamate or caged glutamate (Invitrogen)^{16–18} was photolyzed to liberate glutamate for measuring the rate of GluK1 receptor channel opening. We also used another caged glutamate, i.e., 4-methoxy-7-nitroindolyl-glutamate,²⁰ for some of our measurements. We tested the two caged glutamate compounds extensively with several glutamate receptor channels and found no difference in the rate constant measurement. Caged glutamate (or free glutamate) was applied to an HEK-293 cell suspended in the extracellular solution by using a U-tube flow device.^{17,18} The cell was equilibrated with caged glutamate

for 250 ms before being irradiated with a laser pulse. A Minilite II pulsed Q-switched Nd:YAG laser (Continuum, Santa Clara, CA), tuned by a third harmonic generator, produced single pulses of 8 ns at 355 nm. The laser light was introduced into a cell via a fiber optic, and the power was adjusted to 200–800 μ J. For a laser-pulse photolysis measurement of a channel opening kinetic rate constant, we also used at least two known concentrations of free glutamate with the same cell before and after a laser flash to calibrate the concentration of photolytically released glutamate.^{17,18,21} The current amplitudes obtained from flow measurements were compared with that of the laser measurement with reference to the dose–response relationship. The flow measurement also allowed us to monitor any potential damage to the receptors and/or the cell for successive laser flashes with the same cell.

Data Analysis. In the laser-pulse photolysis measurement of the channel opening reaction, we observed that the whole-cell current rise followed a single-exponential rate process (>95%) over the entire range of ligand (glutamate) concentrations that we were able to measure [i.e., 60–400 μ M glutamate or ~4–52% of the fraction of the open channel population (see the dose–response curve in Results)]. Therefore, the observed rate constant of channel opening, k_{obs} , was empirically determined by eq 1

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

where I_t and I_A represent the whole-cell current amplitude at time t and the maximal current amplitude, respectively. To describe the relationship between k_{obs} and agonist concentration, we introduced a general mechanism of channel opening.^{17,18,21}



where A stands for the active, unliganded form of the receptor, L the ligand or glutamate, AL_n the closed channel state with n ligand molecules bound, and $\text{AL}_n^{\text{open}}$ the open channel state. The number of glutamate molecule(s) to bind to the receptor and to open its channel, n , can be from 1 to 4, assuming that a receptor is a tetrameric complex and each subunit has one glutamate binding site.^{22–26} It is further assumed that a ligand does not dissociate from the open channel state. k_{op} and k_{cl} are the channel opening and channel closing rate constants, respectively. For simplicity and without contrary evidence, it is assumed that glutamate binds at all binding steps with an equal affinity or K_1 , the intrinsic equilibrium dissociation constant. By this mechanism, eq 2 was derived.

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

In the derivation of eq 2, the rate of ligand binding was assumed to be fast relative to the rate of channel opening. This assumption is consistent with kinetic evidence that the whole-cell current rise (described in detail in Results) follows a first-order rate law (eq 1) not only in this study but also in all of our previous studies of the channel-opening mechanism for other kainate receptors.¹⁸ From eq 2, a set of k_{cl} and k_{op} values as well as K_1 were found to be correlated with a particular number of ligands (n) that bound to and subsequently opened the receptor channel.

Furthermore, K_1 was independently estimated from the dose–response relationship, as in eq 3, which was also derived on the basis of the general mechanism of channel opening described above.

$$I_A = I_M R_M \frac{L^n}{L^n + \Phi(L + K_1)^n} \quad (3)$$

I_M is the current per mole of receptor, R_M is the number of moles of receptors on the cell surface, and Φ^{-1} is the channel opening equilibrium constant. It should be noted that n as in both eqs 2 and 3 is the number of glutamate molecules to bind to and open the channel and is not the Hill coefficient. Like the Hill equation,²⁷ eq 3 accounts for macroscopic current response to the entire range of agonist concentration. As such, eq 3, together with eq 2, does not take into account the contribution of different conductance levels and the change in conductance with an increasing agonist occupation. Nonetheless, we found that the best fit value of n is 2 from the use of eqs 2 and 3, as described in Results.

Unless otherwise noted, each data point shown in the plots of this study was an average of at least three measurements collected from at least three cells. Linear regression and nonlinear fitting were performed using Origin 7 (Origin Lab, Northampton, MA). Unless otherwise noted, the standard error of the mean was reported.

RESULTS

Expression and Detection of Wild-Type GluK1-2a, Wild-Type GluK1-2b, and the Mutant GluK1-2b Channels by Whole-Cell Recording. We expected that the mutant GluK1-2b has a higher expression level and thus a higher current amplitude than wild-type GluK1-2b for the following reason. The mutant GluK1-2b was constructed to change the amino acid sequence of the ER retention signal so that its ER exit and surface expression would be enhanced compared with that of wild-type GluK1-2b.¹⁰ On the other hand, GluK1-2a does not contain the alternatively spliced C-terminal domain or the ER retention signal that exists in the wild-type GluK1-2b sequence. If the ER retention sequence motif regulates only the receptor trafficking and cell surface expression, we expect that wild-type GluK1-2a, wild-type GluK1-2b, and the mutant GluK1-2b all have similar, if not identical, gating properties.

To test this hypothesis, we first measured the whole-cell current response of each of the receptors individually expressed in HEK-293 cells. As shown (Figure 1A), whole-cell current responses to glutamate at various concentrations initially increased as a result of channel activation and then decreased toward baseline due to channel desensitization. Using the amplitude of whole-cell current (Figure 1B), we compared the relative surface expression level for the three receptors in HEK-293 cells. For this comparison, a total of 28 cells expressing wild-type GluK1-2a, 11 cells expressing wild-type GluK1-2b, and 15 cells expressing the mutant GluK1-2b were used, and the amount of plasmid we used for transient transfection was the same (24 μ g per 35 mm dish).

The average whole-cell current amplitude at each of the three glutamate concentrations (0.05, 0.2, and 3 mM) for the mutant GluK1-2b was >5-fold larger than that of wild-type GluK1-2b (Figure 1B). The highest current response, as we observed, of the GluK1-2b mutant to 3 mM glutamate is \sim 2 nA. These observations are comparable with those reported by Ren et al.¹⁰ previously. In the study by Ren et al.,¹⁰ the GluK1-2b mutants

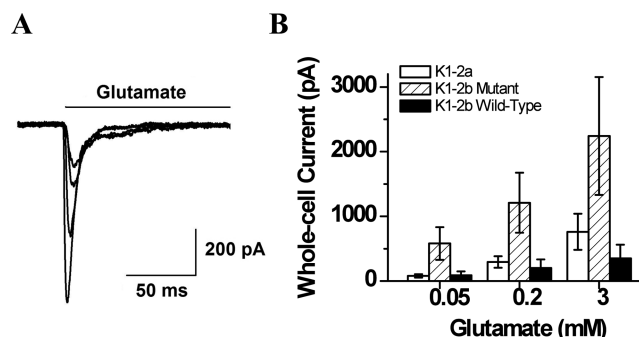


Figure 1. (A) Representative traces of the whole-cell current measured from a single HEK-293 cell expressing the mutant GluK1. Glutamate was applied at time zero (upper bar), and the concentrations of glutamate were, from bottom up, 10, 1, 0.2, and 0.1 mM, respectively. (B) Average of the whole-cell current amplitudes of wild-type GluK1-2a (white column), wild-type GluK1-2b (black column), and the mutant GluK1-2b (cross-hatched column). The number of cells used for comparison for each receptor was described in the text.

tested contain at most three mutations (i.e., R896A, R900A, and K901A). The mutant we tested contains an additional mutation or the R897A mutation besides R896A, R900A, and K901A. Furthermore, the average of the whole-cell current amplitude for wild-type GluK1-2a was only slightly higher than that of wild-type GluK1-2b (Figure 1B). The average whole-cell current response of GluK1-2a to 3 mM glutamate in our experiments (Figure 1B) was only \sim 800 pA. This value is comparable with those documented in the literature, i.e., 300–800 pA.^{6,9,12,14,28} This result may suggest a different regulatory process for surface expression of GluK1-2a.¹⁰

Dependence of the Whole-Cell Current Response on Glutamate Concentration. The dose–response relationship for the two wild-type and mutant GluK1 receptors was characterized to estimate K_1 (i.e., the intrinsic equilibrium dissociation constant of the ligand) and the EC_{50} value. As seen in Figure 2A, the dose–response relationships of wild-type GluK1-2a, wild-type GluK1-2b, and mutant GluK1-2b receptors were statistically indistinguishable. Quantitatively, the best fit of the combined dose–response data by eq 3 yielded a K_1 of $480 \pm 50 \mu$ M (Figure 2A and Table 1) with an n of 2 (where n represents the number of ligand molecules bound to the receptor to open the channel; Table 1 also contains the best fit values of K_1 for n values of 1, 3, and 4). When the dose–response curve was analyzed separately using the same approach, K_1 was $400 \pm 170 \mu$ M for wild-type GluK1-2a, $470 \pm 130 \mu$ M for wild-type GluK1-2b, and $530 \pm 140 \mu$ M for the mutant receptor (data not shown). From the same dose–response data, we also estimated the EC_{50} value by the Hill equation.²⁷ We found that $EC_{50} = 460 \pm 34 \mu$ M, and the Hill coefficient = 1.1 ± 0.1 (Figure S2 of the Supporting Information) for the combined dose–response data. When the dose–response relationships of the two wild-type and mutant GluK1 receptors were analyzed separately, the EC_{50} value and the Hill coefficient were $510 \pm 40 \mu$ M and 1.2 ± 0.1 for wild-type GluK1-2a, $415 \pm 30 \mu$ M and 1.0 ± 0.1 for wild-type GluK1-2b, and $570 \pm 70 \mu$ M and 1.1 ± 0.1 for the mutant GluK1-2b, respectively (data not shown). On the basis of these results, we concluded that the wild-type and mutant GluK1-2b as well as wild-type GluK1-2a had identical dose–response relationships. They also had the same n value from the best fit; namely, the number of glutamate molecules bound to and open

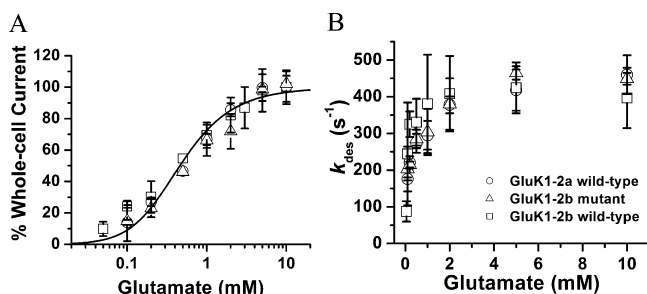


Figure 2. (A) Dose–response relationship of wild-type GluK1-2a (○), wild-type GluK1-2b (□), and the mutant GluK1 receptors (△). Each datum is an average of at least three measurements from three different cells. A total of 53 cells were measured for the two wild-type receptors and the mutant GluK1 receptors. The whole-cell current amplitudes from different cells were normalized to that of the control or 0.5 mM glutamate, and the average of the current amplitude collected at glutamate concentrations of ≥ 5 mM was set to 100%. The solid line is the nonlinear fit of the combined data based on eq 3 (see the detailed results of the fitting in Table 1). From the combined data, when $n = 2$, a K_1 of $480 \pm 50 \mu\text{M}$, a Φ of 0.45 ± 0.15 , and an $I_{\text{M}R_{\text{M}}}$ of 146 ± 20 were obtained. (B) Dependence of desensitization rate constant on glutamate concentration for wild-type GluK1-2a (○), wild-type GluK1-2b (□), and the mutant GluK1 receptors (△). Each datum is an average of at least three data points from three cells.

Table 1. Nonlinear Fitting of the Dose–Response Curve for Wild-Type and Mutant GluK1

n^a	K_1 (mM)	Φ	$I_{\text{M}R_{\text{M}}}$ (nA)	R^2
1	1.63 ± 0.16	0.51 ± 0.05	161 ± 7	0.99
2	0.48 ± 0.05	0.45 ± 0.15	146 ± 20	0.97
3	0.26 ± 0.06	0.38 ± 0.16	137 ± 22	0.95
4	0.18 ± 0.03	0.43 ± 0.21	142 ± 28	0.94

^a n was fixed as an integer.

a channel was 2. This value was corroborated by the fitting of both the dose–response data (Figure 2A and Table 1) and the channel opening rate data, described below. Furthermore, the EC_{50} value of $510 \pm 40 \mu\text{M}$ for GluK1-2a, which we determined, is similar to the EC_{50} of $631 \mu\text{M}$ for the same receptor reported by Sommer et al.⁹ (Table S1 of the Supporting Information). However, no EC_{50} value for GluK1-2b or any of its mutants has been previously reported.

Dependence of the Desensitization Rate Constant on Glutamate Concentration. Next we examined the desensitization rate (as shown in Figure 1A). The desensitization proceeded with a single, first-order rate process for >98% of the reaction and for the entire range of glutamate concentrations for all three receptors. The desensitization rate constant, or k_{des} , for the two wild-type receptors and the mutant GluK1 increased with an increasing glutamate concentration but reached a plateau around 5 mM glutamate (Figure 2B). We found that the channel desensitization rate profile and the dependence of the channel desensitization rate constant on glutamate concentration appeared to be identical (Figure 2B). The maximal k_{des} was $464 \pm 50 \text{ s}^{-1}$ for wild-type GluK1-2a, $450 \pm 70 \text{ s}^{-1}$ for wild-type GluK1-2b, and $457 \pm 17 \text{ s}^{-1}$ for the mutant (Figure 2B). These results showed that all three receptors were desensitized with identical rate constants at any given glutamate concentration (Figure 2B). Therefore, our results suggest that the difference in the amino acid sequence among wild-type GluK1-2a, wild-type GluK1-2b, and the

mutant GluK1-2b (Figure S1 of the Supporting Information) did not affect the channel desensitization kinetics.

The maximal k_{des} of 464 s^{-1} for wild-type GluK1-2a we determined is in good agreement with the rate constant for the fast desensitizing process of the same receptor reported previously.¹⁵ We did not, however, observe a slow desensitizing rate as previously documented¹⁵ (note that the total number of cells we recorded was 28). The magnitude of the k_{des} of 457 s^{-1} is also similar to the value reported by Ren et al.¹⁰ for another GluK1-2b mutant with three point mutations (i.e., R896A, R900A, and K901A). We did observe that $\sim 20\%$ of cells (from a total of 20 cells) that expressed wild-type GluK1-2b showed a slower desensitization rate. The rate constant for the slow desensitization rate was 3.8-, 4.0-, and 4.4-fold smaller at 50 μM , 200 μM , and 3 mM glutamate, respectively (data not shown). However, on average, our ratio is ~ 2 -fold larger than that reported previously.¹⁵ In contrast, only one cell (of 25) that expressed the mutant GluK1-2b exhibited a slower rate of desensitization. It should be noted that we did not study any of these slow desensitizing cells that expressed either wild-type GluK1-2b or its mutant.

Channel Opening Rate Constants. Using the laser-pulse photolysis technique,¹⁷ we measured the channel opening (k_{op}) and channel closing (k_{cl}) rate constants of wild-type GluK1-2a, wild-type GluK1-2b, and the mutant GluK1-2b receptors. The magnitude of k_{op} reflects how fast a channel opens after the binding of agonist, whereas the magnitude of k_{cl} reflects how long a channel stays open or the lifetime of the open channel.¹⁹ Therefore, a putative difference between rate constants would be attributed to the difference in the amino acid sequence of the C-termini among the three receptors (Figure S1 of the Supporting Information).

As seen in an example (Figure 3A), laser-pulse photolysis of the caged glutamate triggered a rapid rise in whole-cell current. The time course of the rising phase (Figure 3A), represented by an observed rate constant of channel opening, or k_{obs} , was adequately described by a single-exponential rate process (see the solid line as the fit to this rate process in Figure 3A by eq 1). Various k_{obs} values were obtained as a function of the concentration of photolytically released glutamate. Inspection of these k_{obs} values in the entire range of glutamate concentrations showed that there was not a statistically significant difference among the two wild-type channels and the mutant GluK1 channel (Figure 3B). The best fit, using eq 2, of k_{obs} as a function of the glutamate concentration, based on the combined data from the mutant and the two wild-type receptors, yielded an n of 2, and the k_{op} and k_{cl} values were $(2.6 \pm 0.1) \times 10^4$ and $(1.1 \pm 0.2) \times 10^3 \text{ s}^{-1}$, respectively (Figure 3B and Table 2). If the rate data were analyzed separately, the best fit yielded k_{op} and k_{cl} values of $(2.2 \pm 0.2) \times 10^4$ and $(0.9 \pm 0.2) \times 10^3 \text{ s}^{-1}$ for wild-type GluK1-2a, $(2.4 \pm 0.1) \times 10^4$ and $(1.4 \pm 0.2) \times 10^3 \text{ s}^{-1}$ for wild-type GluK1-2b, and $(2.6 \pm 0.3) \times 10^4$ and $(0.8 \pm 0.3) \times 10^3 \text{ s}^{-1}$ for the mutant GluK1, respectively, at $n = 2$. We therefore conclude that GluK1-2a, GluK1-2b, and the GluK1-2b mutant had identical k_{op} and k_{cl} values. These results suggest that the difference in the amino acid sequence located in the C-terminal tail that defines wild-type GluK1-2a and the ER retention signal motif as in the wild-type sequence of GluK1-2b (Figure S1 of the Supporting Information) does not affect the channel opening rate process (Figure 3B and Table 2).

Estimation of (a) the Minimal Number of Glutamate Molecules Bound to a Receptor To Open Its Channel

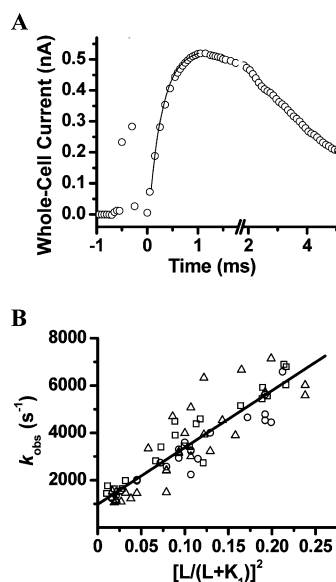


Figure 3. (A) Representative whole-cell current trace generated by the laser-pulse photolysis measurement with an HEK-293 cell expressing wild-type GluK1-2a. A laser pulse was fired at time zero. The concentration of glutamate released was estimated to be 180 μM . A k_{obs} was calculated from the rising phase of the current to be $3300 \pm 76 \text{ s}^{-1}$ by using eq 1 (solid line). Note that the current is plotted opposite the direction that was recorded. (B) Linear plot of the k_{obs} data combined from the two wild-type and mutant GluK1 receptors as a function of glutamate concentration by eq 2. Each data point represents a single k_{obs} value obtained at a particular concentration of photolytically released glutamate. Overall, a total of 20, 22, and 30 cells were measured for wild-type GluK1-2a (\circ), wild-type GluK1-2b (\square), and the mutant GluK1-2b (\triangle), respectively. The best linear fit yielded, at $n = 2$, k_{op} and k_{cl} values of $(2.6 \pm 0.1) \times 10^4$ and $(1.1 \pm 0.2) \times 10^3 \text{ s}^{-1}$, respectively.

Table 2. Fitted k_{op} and k_{cl} Values with Different n Values for Wild-Type and Mutant GluK1

n^a	$k_{\text{op}} (\times 10^4 \text{ s}^{-1})$	$k_{\text{cl}} (\times 10^3 \text{ s}^{-1})$	R^2
1	1.5 ± 0.1	-0.7 ± 0.2	0.91
2	2.6 ± 0.1	1.1 ± 0.2	0.91
3	5.5 ± 0.3	1.7 ± 0.1	0.89
4	11.7 ± 0.8	2.1 ± 0.1	0.85

^a n was fixed as an integer, and K_1 was fixed at 480 μM .

and (b) the K_1 Value Using the Rate Data. In the study described above (Figure 3), we measured k_{obs} for the two wild-type receptors and the mutant GluK1 using up to 400 μM glutamate generated by laser photolysis, which correlated with up to 52% of the fraction of the open channel population. The wide range of k_{obs} values versus glutamate concentration permitted us to simultaneously evaluate, by eq 2, k_{op} and K_1 as well as n , the number of glutamate molecules bound to the receptor to open the channel. To achieve a better estimate of n , K_1 , and k_{op} by nonlinear regression using eq 2, we decided to first fix the value of k_{cl} . This is reasonable because when $L \ll K_1$, eq 2 can be reduced to $k_{\text{obs}} \approx k_{\text{cl}}$, suggesting that (a) k_{obs} at a low glutamate concentration would reflect k_{cl} and (b) the value of k_{cl} is independent of the n value, as in eq 2.^{19,29} On the basis of this rationale, we identified a k_{obs} of 1000 s^{-1} , determined at $\sim 60 \mu\text{M}$ glutamate or an $\sim 4\%$ fraction of the open channel,¹⁹ to be k_{cl} for the wild-type and mutant GluK1 receptors. By nonlinear regression with a k_{cl} of 1000 s^{-1} , the n values turned

out to be consistently close to 2 (Table S2 of the Supporting Information). Furthermore, choosing k_{cl} values different from 1000 s^{-1} did not significantly affect the output of n , K_1 , or k_{op} values (Table 3). These results are therefore consistent with an

Table 3. Nonlinear Fitting of the Wild-Type and Mutant GluK1 Data for Estimating n , k_{op} , and K_1

$k_{\text{cl}}^a (\text{s}^{-1})$	n	$k_{\text{op}} (\times 10^3 \text{ s}^{-1})$	$K_1 (\text{mM})$	R^2
400	1.8 ± 0.4	2.7 ± 0.4	0.44 ± 0.17	0.80
600	1.9 ± 0.5	2.6 ± 0.3	0.44 ± 0.16	0.81
800	2.0 ± 0.6	2.4 ± 0.3	0.43 ± 0.15	0.83
1000	2.1 ± 0.5	2.3 ± 0.3	0.43 ± 0.15	0.83
1200	2.0 ± 0.5	2.5 ± 0.4	0.48 ± 0.15	0.84
1400	2.0 ± 0.6	2.5 ± 0.4	0.51 ± 0.17	0.82

^a k_{cl} is fixed as specified.

n of 2 being the best fit (Table 3). This conclusion is also consistent with results from our study of other kainate receptor channels.^{17,19,21,30} Furthermore, as is known for kainate receptors, channel activation via a subset of subunits instead of all four in a tetrameric complex can indeed occur.³¹ Thus, we favor an interpretation of k_{op} and k_{cl} at an n of 2 being the representative values of the channel opening and channel closing rate constants, respectively, for wild-type GluK1-2a, wild-type GluK1-2b, and the mutant GluK1-2b receptors.

The evaluation of n as described above was based on the channel opening rate data, independent of the analysis of the dose–response relationship (as in Figure 2A). Using the same rate data, we also independently evaluated K_1 , the intrinsic equilibrium dissociation constant (see the scheme in Experimental Procedures). Specifically, nonlinear regression of k_{obs} versus glutamate concentration using eq 2 yielded an average K_1 of $400 \pm 150 \mu\text{M}$ for wild-type GluK1-2a, wild-type GluK1-2b, and the mutant GluK1-2b receptors (Table S1 of the Supporting Information). This value was in good agreement with the K_1 value of $480 \pm 50 \mu\text{M}$ obtained from the dose–response measurement (Figure 2A and Table 1). When K_1 was further constrained to be 480 μM , in addition to a fixed k_{cl} , the fit values of n were, again, close to 2, and the fit values of k_{op} were relatively invariant, the average of which was $2.6 \times 10^4 \text{ s}^{-1}$ (Table S3 of the Supporting Information). Finally, if k_{cl} and n were kept at 1000 s^{-1} and 2, respectively, we obtained a k_{op} of $2.1 \times 10^4 \text{ s}^{-1}$ on average. Similarly, K_1 was found to be 380 μM (Table S4 of the Supporting Information). It should be noted that the K_1 value, which was obtained from the channel opening rate data, was also in agreement with the K_1 value obtained independently from the dose–response relationship.

Measurement of the Channel Opening Rate Constant.

To characterize the mechanism of channel opening for the GluK1 receptors, we took into account several experimental observations in our kinetic data analysis to obtain k_{op} and k_{cl} . For example, the rising phase of the whole-cell current (as in Figure 3A) proceeded with a single-exponential rate law over the entire concentration range of glutamate (i.e., 60–400 μM). This observation was consistent with the assumption that the channel opening rate or the rate of transition from the closed channel form, AL_n , to the open channel form, AL_n^* , was slow compared with the rates of all preceding steps involving glutamate binding. On the basis of this assumption, eq 2 was derived. If the channel opening rate was either comparable to or faster than the ligand binding rate, a single-exponential rate

law would fail. For instance, when the ligand concentration is too low such that the ligand association rate dominates the rate process, the rising phase of the current will represent a bimolecular association rate process instead.^{19,30} To ensure that ligand binding was fast even at low glutamate concentrations, we did not use any k_{obs} values at any glutamate concentrations lower than ~4% of the fraction of the open channel form.¹⁹ For GluK1 receptors, 4% of the fraction of the open channel form correlates with ~60 μM glutamate (see the dose–response relation). Under this condition, k_{obs} reflected mainly k_{cl} .¹⁹ To demonstrate that the method of data analysis used here is valid, we note that the k_{cl} values,^{19,30} calculated using eq 2 and by using ~4% of the fraction of the open channel form, are generally in agreement with the lifetimes determined by single-channel recording of the same α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) channels with glutamate as the agonist (i.e., $k_{\text{cl}} = 1/\tau$, and τ is the lifetime expressed as a time constant).^{17,19,24,32}

Swanson et al.³³ previously studied the homomeric GluK1 receptors using single-channel recording. In the presence of domoate as the agonist, GluK1 channels exhibited three conductance states, and two time constants, i.e., 0.3 and 0.7 ms, from the best fit of the open-time distribution. These time constants are equivalent to rate constants of ~3300 and 1400 s^{-1} , respectively. However, no single-channel study of any GluK1 receptors using glutamate as the agonist has been reported thus far. Furthermore, the k_{op} and k_{cl} values of GluK1 receptor channels we determined in this study, together with K_1 , EC_{50} , and n values, were based on the macroscopic current response to glutamate. Consequently, the k_{op} and k_{cl} values reflect only the average rate constants of an ensemble of single channels, or they do not correlate with kinetic constants of individual channels of the ensemble.

DISCUSSION

In this study, we characterized the channel-opening mechanism for three kainate receptors, namely, wild-type GluK1-2a, wild-type GluK1-2b, and a GluK1-2b receptor mutated at the C-terminal tail such that the ER retention signal is disrupted. As expected, the GluK1-2b mutant receptor that lacks the ER retention signal motif is expressed much better in HEK-293 cells than wild-type GluK1-2b. Furthermore, wild-type GluK1-2a, wild-type GluK1-2b, and the mutant GluK1-2b share identical k_{des} , k_{op} , k_{cl} , K_1 , and EC_{50} values. The minimal number of glutamate molecules that bind to any one of the three receptors to open the channel is 2. Because all three GluK1 receptors have different amino acid sequences in the C-terminal region but identical sequences elsewhere, our results suggest that the C-terminal region, precisely the sequence surrounding the ER retention and trafficking signal, does not affect the channel gating properties of GluK1. Our results therefore demonstrate that the GluK1-2b mutant can be used to represent the GluK1-2a and GluK1-2b wild-type receptors in studying their structure–function relationships. Having a better expressed mutant and consequently larger whole-cell current response would be further advantageous for studying the function and regulation of the GluK1 receptors. The fact that the mutant GluK1-2b receptor produces a larger current response but has kinetic properties identical to those of wild-type GluK1-2a and GluK1-2b further suggests a possibility of finding additional mutants, by changing the amino acid sequences within or near the ER retention and trafficking

signal, that are expressed even better in HEK-293 cells and produce an even larger whole-cell current response.

The results from this study using the rat GluK1 receptor now enable us to compare with the results obtained for rat GluK2, the most studied and the best understood kainate receptor channel.^{12,18,21,34–37} The K_1 of $300 \pm 210 \mu\text{M}$ for rat GluK2 determined previously²¹ is similar to the K_1 of $400 \pm 150 \mu\text{M}$ (from the analysis of the combined rate constant data) and the K_1 of $480 \pm 50 \mu\text{M}$ (from the analysis of the combined dose–response relation data) for rat GluK1, suggesting that there is no significant difference between the dose–response relationships of GluK1 and GluK2. Because the peak concentration of glutamate in the synapse cleft is thought to be ~1 mM,^{38,39} both GluK1 and GluK2 kainate receptors can open up to 80% of the receptors under this condition. In other words, both receptors are equally sensitive to glutamate.²¹ Conversely, the channel opening and channel closing rate constants of GluK2, i.e., $(6.4 \pm 0.3) \times 10^3$ and $(3.9 \pm 0.3) \times 10^2 \text{ s}^{-1}$, respectively,²¹ are several-fold smaller than those of the GluK1 receptors reported here. This comparison suggests that the GluK1 channels open, in response to glutamate, and close several-fold faster than the GluK2 receptor channel. Furthermore, the maximal desensitization rate constant of the GluK1 channels we report here (~460 s^{-1}) is almost 2-fold larger than that of the rat GluK2 channels²¹ (~230 s^{-1}). Taken together, our results show that the GluK1 homomeric receptor is a faster kainate channel than GluK2, in that the channel opens followed by the binding of glutamate, closes, and desensitizes faster than GluK2 does. The apparent difference in the channel gating properties between GluK1 and GluK2 may be surprising, because the two subunits are ~80% identical in sequence, and such extensive sequence homology generally suggests common properties.

The rapid kinetic characterization of the GluK1 kainate receptors that we have described here represents our continuing effort to investigate the similarities and differences in the channel opening kinetic properties between the kainate and AMPA receptor subtypes. In a more significant way, the results from this study demonstrate that GluK1 receptors exhibit a much slower rate of channel opening in response to glutamate binding and a slower rate of closing the open channels than do AMPA receptors.^{17,19,30,40,41} The desensitization rate constant of GluK1 is, however, comparable to those of the majority of the AMPA receptors.^{17,19,30,40,41} Specifically, the maximal k_{des} is much faster than that of either the flip or the flop isoform of the GluA1 AMPA receptor channel but is comparable to that of the GluA2Q_{flip} AMPA receptor channel, which is much faster than GluA2Q_{dip}. The K_1 or EC_{50} values for both GluK1 and GluK2^{18,21} are comparable to those of the GluA1 AMPA receptor but are ~2-fold smaller than the corresponding values for the rest of the AMPA receptor homomeric channels.^{17,19,30,40,41} Therefore, the results from this study and the studies we reported previously^{18,21} support the idea that kainate receptors (i.e., GluK1 and GluK2) have different channel properties compared with those of AMPA receptors.

The results for GluK1, together with the results for GluK2,²¹ show that kainate receptors are kinetically slower than AMPA receptors in opening the channels. This conclusion may be useful in understanding the role of kainate receptors, which is much less understood than the role of AMPA receptors, in mediating synaptic plasticity.^{3,5,42} For example, our results may account for the slower time course of the rise and decay of the kainate-mediated excitatory postsynaptic currents (EPSCs)

than the AMPA receptor-mediated EPSCs, as observed in hippocampal CA3 pyramidal cells.^{43–45} The time course of this mixed AMPA and kainate EPSCs is similar to those reported for synapses on CA1 hippocampal interneurons^{46,47} and in layer IV neurons of the primary somatosensory cortex.⁴⁸ The difference in the channel opening kinetic properties, defined by those rate constants, between kainate and AMPA receptors could be an explanation for the different EPSC profiles.¹⁸ Therefore, our results support the hypothesis that kainate receptors encode different ranges of afferent fiber frequency as compared with AMPA receptors.^{49–51} Relatively slow kainate receptor-mediated EPSCs can be also ascribed to kinetic behaviors of heteromeric kainate receptors.⁵² It is known, for instance, that GluK1/GluK5 has gating properties different from those of GluK1 homomeric channels.³³ Association of auxiliary proteins with kainate receptors is also known to slow the decay of EPSCs.^{53,54} Therefore, similar studies of the channel opening kinetic properties of both heteromeric kainate channels and protein–kainate receptor complexes will be also helpful in improving our understanding of kainate receptor-mediated synaptic neurotransmission.

■ ASSOCIATED CONTENT

■ Supporting Information

A schematic figure showing the C-termini of the GluK1 receptor isoforms, a Hill equation fit to the dose–response relationship, and several tables showing the results of the nonlinear regression data for the best fit of n , K_1 , and k_{op} to the observed rate constant of channel opening as a function of glutamate concentration. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Funding

This work was supported by grants from the National Institute of Neurological Disorders and Stroke (R01 NS060812), the U.S. Department of Defense (W81XWH-04-1-0106), and the Muscular Dystrophy Association (to L.N.).

■ ACKNOWLEDGMENTS

We thank Peter Seeburg for the wild-type GluK1-2a DNA plasmid and Geoffrey Swanson for the GluK1-2b DNA plasmids.

■ ABBREVIATIONS

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EPSCs, excitatory postsynaptic currents; ER, endoplasmic reticulum; HEK-293, human embryonic kidney 293.

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