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Articles

Mechanism of Glutamate Receptor-Channel Function in Rat Hippocampal Neurons Investigated Using the Laser-Pulse Photolysis (LaPP) Technique†

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ABSTRACT: Ionotropic glutamate receptors are members of a large family of plasma membrane proteins expressed by cells of the nervous system. Upon binding glutamate, the receptors transiently open transmembrane channels that allow the entry of sodium ions. The resulting changes in the transmembrane potential of the cell initiates a process that is involved in signal transmission to another cell. The binding of glutamic acid triggers the channel opening in the microsecond time domain and the reversible inactivation (desensitization) of the receptors in the millisecond time region. The channel-opening mechanism of glutamate receptors was investigated in rat hippocampal neurons voltage-clamped to −60 mV at room temperature and pH 7.4. Two rapid chemical reaction techniques were used: (1) a cell-flow method with a 4−10 ms time resolution to apply L-glutamate and (2) a laser-pulse photolysis technique to release glutamate from γ-*O*-(α-carboxy-2-nitrobenzyl)glutamate (αCNB-caged L-glutamate) with a time constant of 30 μs. The rate and equilibrium constants for channel opening were determined. The results are consistent with the receptor binding two molecules of glutamic acid before the channel opens, with an apparent dissociation constant of 600 μM. Channel opening and closing rate constants, k_{op} and k_{cl}, were determined to be $(9.5 \pm 1) \times 10^3$ s^{-1} and $(1.1 \pm 0.1) \times 10^3$ s^{-1}, respectively. The value of the channel-opening equilibrium constant, Φ ($=k_{op}/k_{cl}$), was 8.6 when determined by laser-pulse photolysis and 6.6 in cell-flow experiments. The results suggest that there are at least two forms of glutamate receptors in rat hippocampal neurons that desensitize with different rates. At a concentration of 500 μM glutamate, 80% of the receptors desensitized with a rate of ∼200 s^{-1} and 20% with a rate of ∼50 s^{-1}.

Glutamate is the major excitatory neurotransmitter in the central nervous system where it activates metabotropic and, more importantly for this study, three subfamilies of ionotropic glutamate receptors. The subfamilies are distinguished, on the basis of their selective agonists, as *N*-methyl-D-aspartate (NMDA),[1] α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate selective receptors (*1*). NMDA receptor channels have comparatively slow activation and desensitization kinetics when the channels are opened by glutamate in the presence of the co-agonist glycine (reviewed in refs *2*−*4*). In contrast, the majority of non-NMDA glutamate receptor channels expressed in central nervous system neurons open rapidly upon binding glutamate and then desensitize in the continuous presence of glutamate

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[1] Abbreviations: αCNB-caged L-glutamate, γ-*O*-(α-carboxy-2-nitrobenzyl)glutamate; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole; LaPP, laser-pulse photolysis.

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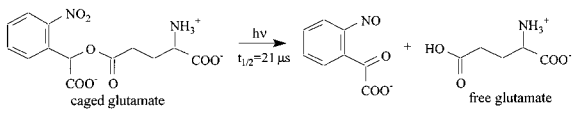
(*5*−*7*). At the high glutamate concentrations that are likely to be released by active presynaptic neurons, the rapid desensitization of non-NMDA glutamate receptor channels has been postulated to limit the duration of postsynaptic responses (*7*; but see also ref *8*). Detailed knowledge of the opening, closing, and desensitization rates of the non-NMDA glutamate receptor channels expressed in individual neurons was the objective of this study. The desired information is considered essential for understanding rapid signal transmission between cells in different parts of the mammalian brain.

In the present study, we investigated the transient kinetics of the opening and closing of glutamate receptor channels expressed in primary cultured rat hippocampal neurons. Two rapid reaction techniques were used to apply glutamate to the surface of isolated single cells. One is a modified U-tube/cell-flow method (*9*, *10*) with a 4−10 ms time resolution, and the other is the laser-pulse photolysis (LaPP) technique previously developed in our laboratory (reviewed in ref *11*). The time resolution of the photolysis method is ∼60 μs, which is ∼50 times faster than that in the cell-flow experiments. Frequently, the primary problem in measuring the chemical reaction kinetics for the opening and closing of a receptor channel is that diffusional access of neurotransmitters to their receptor binding sites on cell membrane surfaces is slow compared to the rate of receptor desensitization (*10*) and channel opening (*12*, *13*; reviewed in ref *10*). This is particularly true for ionotropic glutamate receptors where desensitization rates of 200 s^{-1} are typical but where the desensitization rates can vary between 50 s^{-1} and 1600 s^{-1}, depending upon the cell type or recombinant receptor subtype under investigation (reviewed in refs *3*, *4*, and *10*).

In this study, the problem of rate-limiting diffusional access of glutamate to its binding sites on the receptors was avoided by first equilibrating the receptors with αCNB-caged L-glutamate [γ-*O*-(α-carboxy-2-nitrobenzyl)glutamate]. We chose this caged derivative of glutamic acid because we ascertained that it and its photolysis side products were biologically inactive, that the compound was sufficiently stable in aqueous solution, and that it is photolyzed by a single laser pulse to release free glutamate with a 30 μs time constant (*14*). Thermal hydrolysis of the caged compound in a 10 mM Hepes buffer (pH 7.4 and 27 °C) is negligible in transient chemical kinetic measurements in which the caged glutamate needs to be exposed to the solution for only a few minutes (*14*). The absence of free glutamate in the solution of caged glutamate was ascertained in each experiment, as described in the Materials and Methods section. In locating glutamate receptors in brain slices, considerably longer exposure of the tissue to the caged compounds may be required (*15*−*17*). The synthesis of more thermostable caged glutamate derivatives than the one we are using for kinetic measurements has been described [*N*-(*o*-nitromandeylyl)oxycarbonyl-L-glutamatic acid (Nmoc-caged glutamate) (*18*) and 4-methoxy-7-nitroindolynyl-caged-L-glutamate (MNI-caged L-glutamate) (*19*)].

The chemical reaction for photolysis of αCNB-caged L-glutamate (*14*) is given below.



The LaPP technique using αCNB-caged neurotransmitters (reviewed in refs *11* and *20*) has allowed for the determination of the rate constant for channel opening, the channel-opening equilibrium constant, and the receptor−neurotransmitter dissociation constant of the muscle nicotinic acetylcholine, glycine, γ-aminobutyric acid, and kainate receptors (reviewed in refs *11* and *20*). It has also allowed for the determination of the effects of inhibitors on the rate constants for channel opening and closing (*21*−*25*).

MATERIALS AND METHODS

Chemicals. Glutamate, NMDA, Hepes, Na_2ATP, EGTA, D-glucose, and L-glutamine were purchased from Sigma (St. Louis, MO). CsCl, $CaCl_2$, KCl, and $MgCl_2$ were purchased from Fisher Scientific (Pittsburgh, PA), and trypsin-EDTA 10X solution (0.5% trypsin, 5.3 mM EDTA·4NaOH) from Gibco BRL/Life Technologies (Carlsbad, CA). αCNB-caged glutamate (*14*) was kindly provided by Molecular Probes, Inc. (Eugene, OR).

Cell Culture. Neurons from hippocampi of 1-day old Sprague−Dawley rats were prepared as described earlier (*26*). Hippocampi were dissected from the rat brain and then dissociated using 1 mL of trypsin [trypsin-EDTA 10X solution diluted to 1X in a dissecting solution (D1SGH) consisting of Puck's saline (137 mM NaCl, 5.0 mM KCl, 4.0 mM $NaHCO_3$, 10.0 mM Hepes-NaOH (pH 7.35)) with 43 mM sucrose and 38 mM glucose. Cells were separated by triturating with a Pasteur pipet. The neurons were cultured on dishes coated with rat tail collagen and maintained in Minimum Essential Medium (Gibco BRL) containing 5% fetal bovine serum and 5% horse serum. The medium was supplemented with 2.7 mM glutamine and 30.5 mM glucose. The neurons were incubated in 5% CO_2. Neurons used in the experiments had been maintained in culture for 9−13 days; the neurons used were about 4−8 μm in diameter.

Whole-Cell Current Recording, Cell-Flow Measurements, and LaPP. Currents were recorded using a whole-cell configuration (*27*) with a 3−6 MΩ resistance saline-filled pipet. All experiments were done at room temperature (∼22 °C) with the cells voltage-clamped at −60 mV. The intracellular (pipet) solution contained 140 mM CsCl, 2 mM Na_2ATP, 10 mM EGTA/1 mM $CaCl_2$, and 10 mM Hepes-CsOH (pH 7.4). The extracellular bath solution contained 145 mM NaCl, 1 mM $CaCl_2$, 3 mM KCl, 10 mM glucose, and 10 mM Hepes-NaOH (pH 7.4). The capacitance and series resistance were compensated and the cell currents amplified using an Adams & List EPC-7 amplifier. Currents were low-pass filtered using a Bessel filter (Dagan) at 1−5 kHz for the cell-flow experiments and at 20−30 kHz for the LaPP experiments (see the following discussion). Currents were digitized at twice the filter cutoff frequency using a Labmaster DMA board controlled by Axon pClamp 5.5 software.

Once the whole-cell recording configuration was established, the recording pipet was lifted to detach the neuron from the bottom of the culture dish, and the suspended cell was placed in the effluent of the modified U-tube device (*9*, *10*) that was used to apply glutamate to the cell surface. This cell-flow method allows for the equilibration of all receptors on the cell surface with ligands within 4−10 ms. Glutamate was applied by the cell-flow method to establish

the position of the cell that gave the largest maximum current amplitude. The current amplitude was then corrected for receptor desensitization that occurred during the equilibration of the cell surface receptors with glutamate by using eq 1 (*10*, *28*), which is based on the theory of the flow of solutions over spherical objects (*29*, *30*). To take into account the uneven rate at which the ligand solution flows over the cell surface, we divide the current time course into constant time intervals (we have used 3 ms) and then correct the current for the desensitization that occurs during each time interval Δt. After the current $(I_{obs})\Delta t_i$ is measured for each of n constant time intervals ($n\Delta t = t_n$), the corrected current I_A is given by

$$(I_A)_{t_n} = (e^{\alpha\Delta t} - 1)\sum_{i=1}^{n}(I_{obs})\Delta t_i + (I_{obs})\Delta t_n \qquad (1)$$

α represents the rate coefficient for receptor desensitization and t_i the time. $(I_{obs})\Delta t_i$ is the observed current during the ith time interval. $(I_A)t_n$ becomes equal to I_A when the value of t_n is equal to or greater than the current rise time. In the LaPP study (see the following discussion), the cell-flow method was used to pre-equilibrate the cell with the αCNB-caged glutamate (*14*) before the delivery of UV light (337 nm) from a Lumonics excimer laser. This had two purposes: (1) to monitor recording stability for the LaPP study and (2) to determine if the αCNB-caged glutamate was contaminated with free glutamic acid. The physical arrangement of the cell, U-tube, and fiber optic for the LaPP experiments has been described in detail (*13*, *31*, *32*).

Before and after each LaPP experiment, the energy of the laser was determined by using a Gentec joule meter. The amount of free glutamate released from the 5 mM solution of αCNB-caged glutamate was adjusted during the LaPP protocol by varying the energy of the laser pulse (~10 ns) delivered at the cell surface over a range from 0.15 to 0.5 mJ/mm^2. Before and after each laser-pulse measurement, the response stability of the cell was monitored by the cell-flow application of 0.5 mM glutamate. The responses to 0.5 mM L-glutamate were corrected for desensitization according to eq 1 (*10*) and the results used to calibrate the concentration of glutamate released from the αCNB-caged glutamate. If the response to 0.5 mM glutamate decreased by more than 10% in the repeated cell-flow applications, the cell was discarded, and a new cell was used. Three cells were considered stable enough to obtain the LaPP data reported here. These cells each received 3−5 single laser pulses at a given energy level, and each was exposed to 2−4 different energy intensities. Responses were analyzed offline; linear regression and nonlinear least-squares fitting (Marquardt algorithm) were performed using MicroCal Origin version 3.0 software. [Hippocampal pyramidal neurons express a mixture of non-NMDA receptor subunits (*33*−*35*), and our measurements may, therefore, reflect a population of different non-NMDA receptor types.]

RESULTS

A typical cell-flow experiment with a rat hippocampal neuron exposed to 500 μM L-glutamate at room temperature and pH 7.4 and with the cell voltage-clamped to −60 mV is shown in Figure 2. The dark line represents the observed current, which reaches its peak value within 4 ms. The thin line giving rise to the wavy trace parallel to the abscissa represents the whole-cell current, I_A, after it has been corrected for receptor desensitization occurring during the rising response (*10*). Only the rapid desensitization process (see the following discussion) is pertinent for correcting the rising phase of the current response, which occurs within a few milliseconds (see Figure 2). Without this correction, the observed current amplitude reflects the concentration of open channels, the rise time of the current, and the rate of receptor desensitization (*10*, *28*).

The decay phase of the currents recorded from 0.1 to 10 mM L-glutamate was best fitted by the sum of two exponential functions (solid line in Figure 2) with time constants of 5 ms (or desensitized rate of 200 s^{-1}; ~80% of total current) and 20 ms (or desensitized rate of 50 s^{-1}; ~20% of total current). This current decay, seen in the continued presence of the ligand, was attributed to receptor desensitization. Just as the concentration of receptors varies from cell to cell, so does the ratio of the percent of receptors

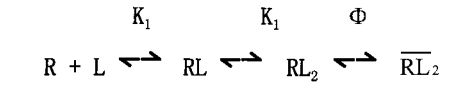


FIGURE 1: Minimum mechanism for ionotropic glutamate receptors. R represents the receptor in the cell membrane, L the ligand or neurotransmitter, and K_1 the dissociation constant for the receptor site controlling channel opening. The channel-opening equilibrium constant $\Phi^{-1} = k_{op}/k_{cl}$, where k_{op} and k_{cl} are the rate constants for channel opening and closing, respectively. RL_2 and $\overline{RL_2}$ represent the ligand−receptor complex in its closed and open forms, respectively. The binding of two ligand molecules prior to channel opening is consistent with the data. For clarity, the steps leading to the reversible desensitization of the receptor with time constants in the 5−20 ms range are not shown.

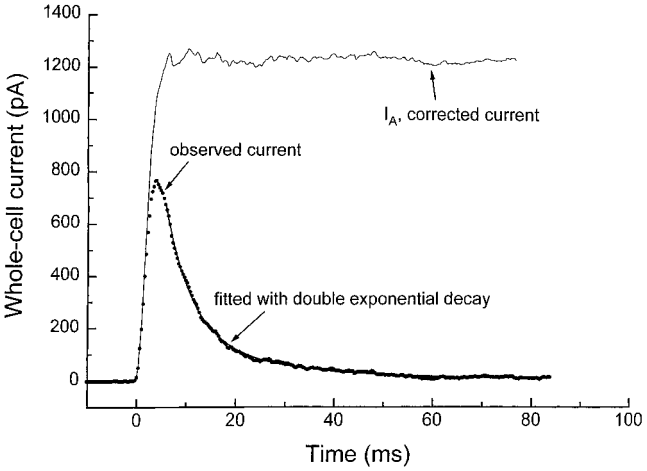


FIGURE 2: Whole-cell current recorded (dotted line) from an isolated rat hippocampal neuron at −60 mV, ~22 °C, and pH 7.4 when 0.5 mM glutamate solution was applied at time 0 using the cell-flow technique (*9*, *10*, *28*) The current rapidly rises and then decays (desensitizes). The desensitization can be fitted by a double exponential decay function using a nonlinear least-squares method. The time constants are 5 ms (80% of the total current) and 20 ms (20% of the total current) for the fast and slow forms, respectively. The current rise time for this trace is about 4 ms. The diameter of the cell used in this experiment was about 6 μm. The corrected current (solid thin line) was calculated to give the current in the absence of receptor desensitization according to eq 1 (*10*).

desensitizing rapidly and slowly. This excludes mechanisms in which a single receptor form desensitizes by two parallel reactions or by a consecutive reaction scheme, as such mechanisms require a constant ratio of the reactions characterized by different rate processes. The results indicate, therefore, that two different receptor forms, characterized by different desensitization rates, are present in rat hippocampal neurons.

The correction of the current amplitude at a given concentration of neurotransmitter was verified for the nicotinic cholinergic receptor (*28*) by determining the fraction of receptors in the open-channel form at the same neurotransmitter concentration using two independent techniques: (1) single-channel current recordings (*36*, *37*) and (2) LaPP (*12*, *13*). When the LaPP technique is used, the current rise is much faster than the rate of receptor desensitization. Correction of the current amplitude for receptor desensitization is, therefore, not required. However, as it is necessary to estimate the free glutamate concentration applied during the LaPP protocol, the cell-flow method is used together with the LaPP technique (see the following discussion) to take advantage of the different information obtained from each method of ligand application.

A plot of the corrected current amplitude, I_A, as a function of glutamate concentration, obtained in cell-flow experiments, is shown in Figure 3.

For the minimum mechanism shown in Figure 1, the relationship between I_A and the dissociation constant of glutamate from the receptor sites controlling channel opening, K_1, and the channel-opening equilibrium constant, Φ^{-1}, is given by eq 2 (*38*)

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

$$\left[\frac{I_M R_M}{I_A} - 1\right]^{1/2} = \Phi^{1/2} + \frac{\Phi^{1/2} K_1}{L} \tag{2B}$$

I_M represents the current produced by 1 mol of open receptor channels, R_M is the moles of receptors on the cell surface, and L is the concentration of neurotransmitter. Equation 2B and a nonlinear least-squares fitting program was used to evaluate the constants, with the assumption that the ligand-binding sites are equivalent: $\Phi = 0.15 \pm 0.17$, $K_1 = 0.6 \pm 0.4$ mM, and $I_M R_M = 2600 \pm 600$ pA. These values for the constants and eq 2A were used to draw the solid line through the experimental points in Figure 3. Because different cells contain different numbers of receptors, the results from different cells were normalized to each other to obtain a complete concentration−effect curve. An I_A value of 1480 pA obtained in the presence of 500 μM glutamate was used for this normalization.

As mentioned previously, the cells contain a minor receptor form that desensitizes at a much slower rate than the main receptor form, which contributes, on average, 80% of the total current. The evaluated constants, therefore, contain contributions from this minor receptor form. In the case of the *Electrophorus electricus* nicotinic acetylcholine receptor (*39*) and of the $GABA_A$ receptor in rat hippocampal neurons (*23*), it has been possible to obtain cells that contain either a rapidly or a slowly desensitizing receptor form. In the case of these two receptors, the two forms have, within experimental error, the same receptor−neurotransmitter dissociation constant K_1. So far, we have not succeeded in obtaining cells that contain only one or the other of the two forms of the receptor in this study. In the analysis of our experiments, we have assumed that K_1 has the same value for both forms.

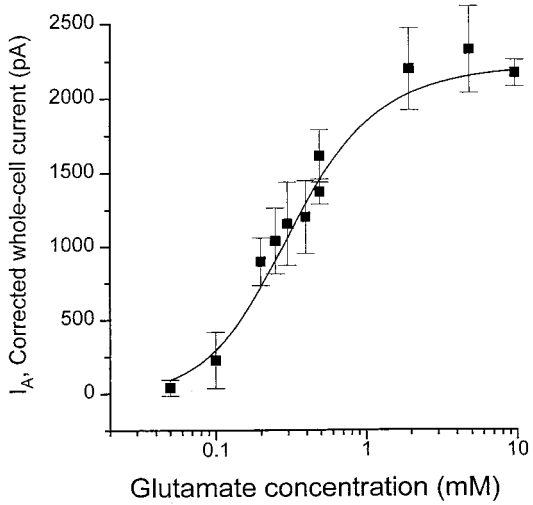


FIGURE 3: Dependence of I_A, the corrected whole-cell current, on the glutamate concentration used. The whole-cell current was recorded from isolated rat hippocampal neurons at −60 mV, ~22 °C, and pH 7.4. The data points for the corrected currents (solid squares) were obtained from different cells. For each of the two cells used, the corrected current amplitude was determined in the presence of 0.5 mM glutamate. The current amplitudes obtained at this glutamate concentration are shown. The current amplitudes obtained at all other concentrations were normalized to a value of 1480 pA at a glutamate concentration of 0.5 mM. The solid line is the nonlinear least-squares fitting of the data based on eq 2B. The values of the dissociation constant of glutamate ($K_1 = 0.6 \pm 0.4$ mM) and the channel-opening equilibrium constant ($\Phi = 0.15 \pm 0.17$) were determined. Each symbol represents 3−5 measurements from 1−2 cells.

A typical result obtained with the LaPP protocol applied to glutamate receptors expressed in a rat hippocampal neuron is shown in Figure 4. In this experiment, the neuron was equilibrated with 5 mM αCNB-caged glutamate. The compound was then photolyzed with a pulse of laser light at 337 nm at 0 time to liberate 0.4 mM free glutamate. The current thus induced first rises, because of the opening of glutamate receptor channels, and then falls because of receptor desensitization. Over the range of glutamate concentration (10−600 μM) investigated using the LaPP protocol, the rise time of the response was faster than receptor desensitization, and the uncorrected current amplitude was taken as a measure of the concentration of open glutamate receptor channels. In the range of glutamate concentrations applied by LaPP, the current rise followed a single exponential rate equation over 90% of the reaction (solid line, Figure 4). This observation is consistent with the assumption that the neurotransmitter-binding steps are fast as compared to the channel-opening step in the range of glutamate concentration used. We also assumed that the constants pertaining to the channel-opening process of the minor, slowly desensitizing receptor form are not different from those of the major, rapidly desensitizing receptor form. With these assumptions,

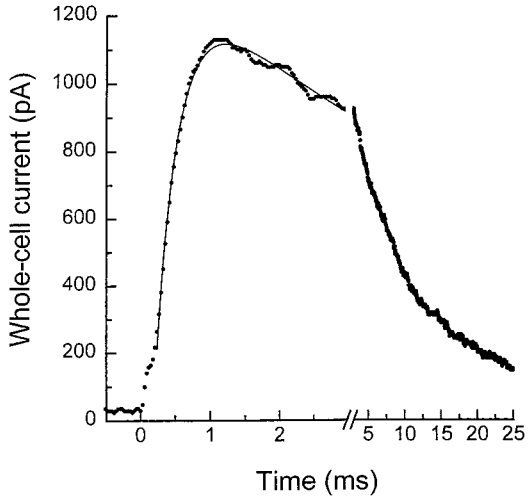


FIGURE 4: Whole-cell current (dotted line) recorded at −60 mV, ~22 °C, and pH 7.4 from an isolated rat hippocampal neuron. The current was induced by LaPP of 5 mM αCNB-caged glutamate at time 0. The current rises very rapidly, reaching its maximum within 2 ms (the formation of open channels), and then in a slower time region, the current decreases as the channels close (desensitization). In this trace, the concentration of glutamate released was estimated to be 0.4 mM, by comparing the maximum amplitude of the corrected whole-cell current in the presence of 0.5 mM glutamate applied using the cell-flow technique before and after the laser-pulse trace was obtained. The rising phase of the current can be fitted by a single exponential (eq 3), and in this trace, the observed rate coefficient, k_{obs}, was determined to be ~3000 s^{-1}.

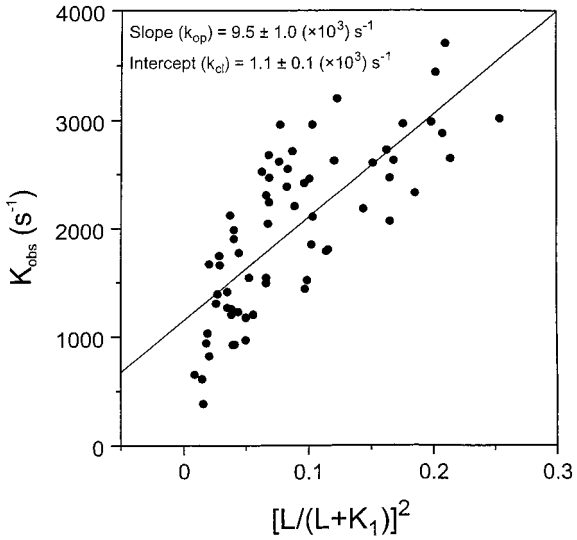


FIGURE 5: Determination of rate constants for the opening and closing of the glutamate receptor channel. The observed rate coefficient, k_{obs}, obtained from laser-pulse experiments is plotted as a function of $[L/(L + K_1)]^2$ (eq 3B) where L is the glutamate concentration and K_1 is the dissociation constant of glutamate. On the basis of eq 3B, a straight line is plotted through all the data points, and from its slope and intercept, the rate constants for glutamate channel opening and closing, k_{op} and k_{cl}, are evaluated to be $(9.5 \pm 1.0) \times 10^3$ s^{-1} and $(1.1 \pm 0.1) \times 10^3$ s^{-1}, respectively. Each point in this Figure represents one trace in a LaPP experiment. The experiment was repeated 3−5 times at each glutamate concentration. All LaPP data were recorded from three cells.

the equations pertaining to the reaction scheme (Figure 1) are

$$I_{(t)} = I_{max}[1 - \exp(-k_{obs}t)] \quad (3A)$$

$$\text{where} \quad k_{obs} = k_{cl} + k_{op}[L/(L + K_1)]^2 \quad (3B)$$

$I_{(t)}$ and I_{max} represent the measured current at time t and when the current reaches its maximum value, respectively. A plot of k_{obs} for the current rise time versus $[L/(L + K_1)]^2$ according to eq 3B is shown in Figure 5. The k_{obs} value for the current rise obtained in the LaPP experiments and the concentration of glutamate (L) were used to obtain the least-squares line in Figure 5. The value of k_{cl} obtained from the ordinate intercept of the line is $(1.1 \pm 0.1) \times 10^3$ s^{-1}. From the slope of the line, a value for k_{op} of $(9.5 \pm 1) \times 10^3$ s^{-1} is obtained. A value for K_1 of 600 μM gives the best fit of the kinetic measurements to the least-squares line shown (Figure 4). This is the same as the value obtained from the cell-flow method (Figure 3).

DISCUSSION

Here, we have determined the dissociation constant, K_1, for glutamate and the rate constants for channel opening, k_{op}, and closing, k_{cl}, for the non-NMDA glutamate receptors in rat hippocampal neurons. The assumption that we are studying non-NMDA receptors is based on the fact that our recording solutions were not supplemented with the NMDA receptor co-agonist glycine, and that the kinetics of NMDA receptor channel activation and desensitization are relatively slow (*3*). The channel-opening equilibrium constant, Φ^{-1}, was defined as k_{op}/k_{cl}. We have also presented evidence that the cells contain two forms of the glutamate receptors on the basis of the observation that the ratio of the current amplitudes associated with the two desensitization reactions characterized by different rate processes was not constant. This excludes reaction schemes in which a single receptor form desensitizes either in two parallel reactions or in consecutive reactions. How do these results compare to those obtained previously by different techniques?

Our concentration−effect data suggested that the activation of glutamate receptors in hippocampal neurons requires the binding of two molecules of L-glutamate. Recent reports indicate that the glutamate receptor is tetrameric (*40*) and that the conductance level depends on the number of subunits occupied by glutamate (*40*, *41*). However, these experiments are also consistent with the requirement of the occupancy of two binding sites to initiate channel opening (*40*). This interpretation is consistent with most reports where the apparent Hill coefficient for activation of glutamate responses, recorded under conditions favoring activation of non-NMDA receptor channels, varied between 1.1 and 2.0 (*42*−*44*).

The K_1 value for the binding of glutamate to the non-NMDA receptors in hippocampal cells may be compared to the EC_{50} values obtained in recordings from whole cells and small membrane patches using other perfusion techniques. Using the values of K_1 and Φ determined in our studies and eq 2, we calculated that the neurotransmitter concentration at which half of the maximum current is reached, the EC_{50} value, is 380 μM. This value is lower than the mean EC_{50} value of 480 μM for the non-NMDA responses evoked by rapidly applied glutamate in whole-cell hippocampal neuron recordings (*42*) and is in good agreement with EC_{50} values of 342 and 424 μM glutamate for outside-out patches excised from CA3 and CA1 neurons, respectively (*43*). In outside-out patches excised from cell bodies of Purkinje neurons,

which lack functional NMDA receptor channels, an EC_{50} value of 432 μM was obtained for glutamate applied by rapid perfusion (*45*).

The value for k_{cl} that we obtained in LaPP experiments, 1100 ± 100 s^{-1}, was comparable to the *deactivation rates* for glutamate responses in outside-out patches excised from rat dentate gyrus neurons, 300−1250 s^{-1} (*35*) and from rat Purkinje neurons, 800−900 s^{-1} (*45*). It is in close agreement with values derived from the single-channel mean open time (660 μs) observed for non-NMDA channels in rat cerebellar granule neurons (*46*).

The k_{op} value of $(9.5 \pm 1) \times 10^3$ s^{-1} that we determined can be compared to the current rise time obtained in rapid perfusion experiments with outside-out membrane patches excised from hippocampal neurons. A mean value of 0.5 ms for rat hippocampal neurons has been reported (*43*) for the 20−80% current rise time in the presence of 3 mM glutamate. This compares well with the value of 0.6 ms for the 20−80% current rise time of CA3-mossy-fiber synaptic currents (*47*). The 20−80% current rise obtained in our experiments is shorter, by a factor of about 3. It is 0.18 ms at 3 mM glutamate and reaches a final value of 0.13 ms. These values are calculated by using eq 4

$$\ln[I_{max} - I_{t_1}][I_{max} - I_{t_2}]^{-1} = -k_{obs}[t_1 - t_2] \qquad (4)$$

I_{t_1} has 80% of the value of I_{max}, and I_{t_2} has 20% of the value of I_{max}. t_1 and t_2 represent the time it takes for the current to reach 80% and 20% of the current maximum, respectively. k_{obs} is the observed first-order rate constant for reaching the current maximum at a particular glutamate concentration. In the LaPP experiments presented (Figures 4 and 5), a 20−80% current rise time of 0.18 ms is obtained at 3 mM glutamate. On saturation of the receptors with glutamate, a 20−80% rise time of 0.13 ms is obtained.

The LaPP method we have used permits an improved measurement of the rise time for direct agonist application as compared with the most rapid perfusion methods, piezoelectric driven devices, because they are limited to time constants of 200−400 μs for solution exchange (*43*, *44*). The time constant for the photolysis of caged glutamate is only 30 μs (*14*).

The results obtained using LaPP and those obtained using rapid perfusion and outside-out membrane patches have been compared previously. In investigations of the $GABA_A$ (*23*) receptor, it was observed that, with either technique, similar results are obtained only at very low concentrations of neurotransmitter, when the current rise times are slow, and at supersaturating concentrations. The suggestion is that, at supersaturating concentrations of neurotransmitter, a small fraction of the applied neurotransmitter, but sufficient to saturate the binding site (*23*), can diffuse to the receptor sites rapidly as compared to channel opening. In the intermediate range of neurotransmitter concentration, the rates obtained by flowing solutions over outside-out membrane patches are lower than the rates observed in LaPP experiments (*23*). These experiments cannot, therefore, be used to evaluate the dissociation constant of the neurotransmitter, K_1, or the channel-opening equilibrium constant, Φ. Another disadvantage of using small membrane patches and rapid perfusion, in contrast to using whole cells in LaPP experiments, is that the surface area, and presumably the number of receptor sites that can be sampled, can be 2 orders of magnitude lower than those found in whole cells. In experiments with membrane patches, only one form of the $GABA_A$ receptor was observed (*48*). Two receptor forms were observed in experiments with whole cells, and the values of k_{op} and k_{cl} for both receptor forms could be determined using the LaPP technique (*23*).

In the case of the glutamate receptor, receptor forms that desensitize with different rates were previously detected but only at neurotransmitter concentrations >300 μM (*43*). In the transient kinetic experiments with whole cells presented here, it was possible to observe two desensitization rates, even at a very low (10 μM) glutamate concentration, and to determine the effect of glutamate concentration on these rates and, consequently, to establish the presence of two different receptor forms in the cells. By using the same kinetic techniques as were used here, evidence was obtained previously for the existence of two receptor forms in BC_3-H1 cells containing the muscle type of nicotinic acetylcholine receptor (*10*), in embryonic mouse spinal cord cells containing the strychnine-sensitive glycine receptor (*49*), and in rat hippocampal neurons containing the $GABA_A$ receptor (*23*).

In addition to being able to compare the value of k_{cl} obtained in LaPP measurements to the lifetime of the open channel (e.g., refs *43* and *46*) obtained in single-channel current recordings, results obtained using the transient kinetic techniques can be examined for self-consistency. The value of K_1, obtained from the kinetics of the rise time of the current (eq 3B), is also obtained from measuring the effect of neurotransmitter concentration on current amplitudes in either LaPP or cell-flow experiments (eq 2B). The values of k_{op} and k_{cl} obtained in kinetic measurements of the current rise can be compared to the value of Φ ($=k_{cl}/k_{op}$) determined from the effect of neurotransmitter concentration on the current amplitudes obtained in LaPP or cell-flow experiments (eq 2B). The value of Φ obtained in cell-flow experiments is 0.15 ± 0.17, and the ratio of k_{cl}/k_{op} obtained in LaPP experiments is 0.12.

In contrast to previously available techniques, the LaPP technique allows one to determine the effect of an inhibitor on k_{cl} and also on k_{op} (*11*). Therefore, the affinity of the inhibitor for the open- as well as the closed-channel receptor form can be determined. This information has been used in arriving at a minimum mechanism of inhibition of the muscle nicotinic acetylcholine receptor by cocaine (*22*) and MK801 (*24*) and in the search for and discovery of ligands that prevent this inhibition (*25*, *50*).

The results presented suggest, therefore, that the techniques described are likely to be useful in investigations of the effects of glutamate on non-NMDA receptor channels formed by heterologous recombination, as well as of the effects of different drugs on the mechanism of these glutamate receptors in different neurons. Caged neurotransmitters now exist for all major excitatory and inhibitory neurotransmitter receptors (*11*, *20*). The rapid reaction techniques now available for investigating membrane-bound proteins together with the classical electrophysiological techniques are expected to be useful in investigating how the mechanism of neurotransmitter receptor-mediated reactions are modified by diseases of the nervous system and by therapeutic as well as abused drugs.

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REFERENCES

1. Watkins, J. C., Krogsgaard-Larsen, P., and Honore, T. (1990) *Trends Pharmacol. 11*, 25−33.
2. Edmonds, B., Gibb, A. J., and Colquhoun, D. (1995) *Annu. Rev. Physiol. 57*, 495−519.
3. Ozawa, S., Kamiya, H., and Tsuzuki, K. (1998) *Prog. Neurobiol. 54*, 581−618.
4. Dingledine, R., Borges, K., Bergles, D., and Traynelis, S. F. (1999) *Pharmacol. Rev. 51*, 7−61.
5. Kiskin, N. I., Krishtal, O. A., and Tsyndrenko, A. Y. (1986) *Neurosci. Lett. 63*, 225−230.
6. Trussell, L. O., Thio, L. L., Zorumski, C. F., and Fischbach, G. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4562−4566.
7. Tang, C. M., Dichter, M., and Morad, M. (1989) *Science 243*, 1474−1477.
8. Silver, R. A., Colquhoun, D. M., Cull-Candy, S. G., and Edmonds, B. (1996) *J. Physiol. 493*, 167−173.
9. Krishtal, O. A., and Pidoplichko, V. I. (1980) *Neuroscience 5*, 2325−2327.
10. Udgaonkar, J. B., and Hess, G. P. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8758−8762.
11. Hess, G. P. and Grewer, J. (1998) *Methods Enzymol.*, 443−473.
12. Milburn, T., Matsubara, N., Billington, A. P., Udgaonkar, J. B., Walker, J. W., Carpenter, B. K., Webb, W. W., Marque, J., Denk, W., McCray, J. A., and Hess, G. P. (1989) *Biochemistry 28*, 49−55.
13. Matsubara, N., Billington, A. P., and Hess G. P. (1992) *Biochemistry 31*, 5507−5514.
14. Wieboldt, 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.
15. Corrie, J. E. T., and Trentham, D. R. (1993) *Bioorg. Photochem. 2*, 243−305.
16. Callaway, E. M., and Katz, L. C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7661−7665.
17. Kotter, R., Staiger, J. F., and Luhmann, H. J. (1998) *Neuroscience 86*, 265−277.
18. Rossi, F. M., Margulis, M., Tang, C.-M., and Kao, J. P. Y. (1997) *J. Biol. Chem. 272*, 32933−32939.
19. Canepari, M., Nelson, L., Papageorgiou, G., Corrie, J. E. T., and Ogden, D. (2001) *J. Neurosci. Methods 112*, 29−42.
20. Gee, K. R., Carpenter, B. K., and Hess, G. P. (1998) *Methods Enzymol. 291*, 30−50.
21. Niu, L., and Hess, G. P. (1993) *Biochemisty 32*, 3831−3835.
22. Niu, L., Abood, L. G., and Hess, G. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12008−12012.
23. Jayaraman, V., Thiran, S., and Hess, G. P. (1999) *Biochemistry 38*, 11372−11378.
24. Grewer, C., and Hess, G. P. (1999) *Biochemistry 38*, 7837−7846.
25. Hess, G. P., Ulrich, A. H., Breiting, H.-G., Niu, L., Gameiro, A. M., Grewer, C., Srivastava, S., Ippolito, J. E., Lee, S., Jayaraman, V., and Coombs, S. E. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13895−13900.
26. Geetha, N., and Hess, G. P. (1992) *Biochemistry 31*, 5488−5499.
27. Hamill, O. P., Marty, E., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) *Pflugers Arch.*, 85−100.
28. Hess, G. P., Udgaonkar, J. B., and Olbricht, W. L. (1987) *Annu. Rev. Biophys. Byophys. Chem. 16*, 507−534.
29. Landau, V. G., and Lifshitz, E. M. (1949) *Fluid Mechanics*, 219.
30. Levich, A. G. (1962) *Physicochem. Hydrodyn.*
31. Billington, A. P., Matsubara, N., Webb, W. W., and Hess, G. P. (1992) *Adv. Protein Chem. 3*, 417−427.
32. Niu, L., Grewer, C., and Hess, G. P. (1996) *Tech. Protein Chem. VII [Symp. Protein Soc.]*, 139−149.
33. Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T. A., Sakmann, B., and Seeburg, P. H. (1990) *Science 249*, 169−171.
34. Mackler, S. A., and Eberwine, J. (1993) *Mol. Pharmacol. 44*, 308−315.
35. Geiger, J. R. P., Melcher, T., Joh, D.-S., Sakmann, B., Seeburg, P. H., Jonas, P., and Monyer, H. (1995) *Neuron 15*, 193−204.
36. Neher, E., and Sakmann, B. (1976) *Nature 260*, 779−802.
37. Sakmann, B., Patlak, J., and Neher, E. (1980) *Nature 286*, 71−73.
38. Cash, D. J., and Hess, G. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 842−846.
39. Huganir, R. L., Delcour, A. H., Greengard, P., and Hess, G. P. (1986) *Nature 321*, 774−776.
40. Rosenmund, C., Stern-Bach, Y., and Stevens, C. F. (1998) *Science 280*, 1596−1599.
41. Smith, T. C., and Howe, J. R. (2000) *Nat. Neurosci. 3*, 992−997.
42. Patneau, D. K., and Mayer, M. (1990) *Neuroscience 10*, 2385−2399.
43. Jonas, P., and Sakmann, B. (1992) *J. Physiol. 455*, 143−171.
44. Clements, J. D., Feltz, A., Sahara, Y., and Westbrook, G. L. (1998) *J. Neurosci. 18*, 119−127.
45. Hausser, M., and Roth, A. (1997) *J. Physiol. 501*, 77−95.
46. Wyllie, D. J. A., Traynelis, S. F., and Cull-Candy, S. G. (1993) *J. Physiol. 463*, 193−226.
47. Jonas, P., Major, G., and Sakmann, B. (1993) *J. Physiol. 472*, 615−663.
48. Maconochie, D. J., Zempel, J. M., and Steinbach, J. H. (1994) *Neuron 12*, 61−71.
49. Walstrom, K. M., and Hess, G. P. (1994) *Biochemistry 33*, 7718−7730.
50. Ulrich, H., Ippoloto, J. E., Pagam, O. R., Eterovic, V. A., Hann, R. M., Shi, H., Lis, J. T., Eledfrawi, M. E., and Hess, G. P. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 14051−14056.

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