# How Fast Does the γ-Aminobutyric Acid Receptor Channel Open? Kinetic Investigations in the Microsecond Time Region Using a Laser-Pulse Photolysis Technique†

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ABSTRACT: The γ-aminobuytric acid$_A$ (GABA$_A$) receptor is a membrane-bound protein that mediates signal transmission between neurons through formation of chloride ion channels. GABA is the activating ligand, which upon binding to the receptor triggers channel opening in the microsecond time domain and reversible desensitization of the receptor in the millisecond time region. We have investigated the channel-opening mechanism for this receptor in rat hippocampal neurons before the protein desensitizes by using a rapid flow method (cell-flow) with a 10 ms time resolution and a laser-pulse photolysis technique with a ∼30 μs time resolution to determine the rate and equilibrium constants for channel opening and closing. Two different forms of the receptor, namely, a rapidly and a slowly desensitizing form, exist in the rat hippocampal cells and are characterized by their different rates for desensitization. At 250 μM GABA the rate constant for desensitization was 2.3 ± 0.4 $s^{-1}$ for the rapidly desensitizing form and 0.4 ± 0.1 $s^{-1}$ for the slowly desensitizing form. The dissociation constant of GABA from the site controlling channel opening was 100 ± 40 μM for the rapidly desensitizing form and 120 ± 60 μM for the slowly desensitizing form. The rate constants for channel closing did not differ significantly for the two forms, 85 ± 20 $s^{-1}$ for the rapidly desensitizing and 100 ± 60 $s^{-1}$ for the slowly desensitizing form. However, the channel-opening rate constant differed by a factor of 3, 1840 ± 160 $s^{-1}$ for the rapidly desensitizing and 6700 ± 330 $s^{-1}$ for the slowly desensitizing form. This difference in the rate constant for channel opening for the two forms, determined by the laser-pulse photolysis technique, is reflected as a shift in the channel-opening equilibrium constant, which is 7 ± 5 and 20 ± 15 for the rapidly and slowly desensitizing forms respectively, determined by the cell-flow method. These constants, together with the concentration of GABA and the concentration of receptor sites in the membrane, determine the number of channels that open as a function of GABA concentration, and the rate at which they open and close. These constants play an important role in determining the rate of the transmembrane ion flux and, therefore, the receptor-controlled changes in transmembrane voltage that trigger signal transmission.

The γ-aminobuytric acid (GABA)[1] receptor belongs to a family of membrane-bound proteins responsible for transmission of signals at the junctions (synapses) between cells of the nervous system (*1*). Upon binding their specific neurotransmitters, the GABA and glycine receptors form transient anion-conducting transmembrane channels (*1*). These inhibitory anion-conducting channels counteract the excitatory cation-conducting channels formed by receptors activated by acetylcholine and glutamate (*1*). The net movement of ions across the cell membrane leads to a change in voltage, which determines signal transmission (*1*). An investigation of the mechanism of the channel-opening process of neurotransmitter receptors (Figure 1) is a prerequisite to an understand-

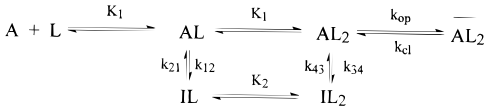


FIGURE 1: Minimum chemical mechanism for the GABA$_A$ receptor based on cell-flow (*16*) and quench-flow experiments (*12*, *13*). A and I represent the active and inactive (desensitized) receptor forms, respectively, and $K_1$ and $K_2$ represent the receptor−neurotransmitter (L) complex dissociation constants for the A and I forms, respectively. Φ is the equilibrium constant between the closed and open forms of the receptor and is equal to $k_{cl}/k_{op}$, where $k_{cl}$ is the rate constant for channel closing and $k_{op}$ is the rate constant for channel opening. $k_{12}$ and $k_{34}$ are the desensitization rate constants for the AL and AL$_2$ forms, and $k_{21}$ and $k_{43}$ are the resensitization rate constants for the AL and AL$_2$ forms. The same mechanism has been shown to account for transient kinetic investigations of the muscle-type nicotinic acetylcholine receptor in BC$_3$H1 cells (*19*, *36*).

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[1] Abbreviations: GABA$_A$, γ-aminobuytric acid$_A$; GABA, γ-aminobuytric acid.

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ing of (i) the integration of excitatory and inhibitory chemical signals arriving at one cell that determines whether a signal is transmitted to another cell, (ii) the mechanism of abnormal receptor function in diseases of the nervous system (e.g., epilepsy) (*2*), and (iii) the effects of therapeutic agents (e.g., tranquilizers) (*3*) and abused drugs (e.g., cocaine) (*4*) on the elementary steps of the receptor-mediated reactions. Investigation of the mechanism involves the determination of the reaction pathway starting with neurotransmitter and the receptor in the cell membrane and leading to the formation of an open receptor channel and desensitized receptor forms and of the rate and equilibrium constants pertaining to these steps (*5*−*8*). Included in our studies is a determination of the dissociation constant of the neurotransmitter and of the rate constants for channel opening and closing and receptor desensitization.

The $GABA_A$ receptor, upon binding GABA, forms transient transmembrane channels permeable to chloride ions (*1*, *3*, *9*). The function and activation of the $GABA_A$ receptors have been studied extensively by various techniques, such as equilibrium binding measurements (*10*), quench-flow (*11*, *12*), single-channel current recordings (*10*, *13*−*15*), and flow measurements (*16*, *17*). These techniques indicated a requirement for the binding of two GABA molecules for channel opening to occur (*11*, *12*, *16*, *17*), and the quench-flow and cell-flow measurements indicated the existence of at least two distinct forms of the $GABA_A$ receptor (*11*, *12*, *16*). The dissociation constant ($K_1$) of GABA from the receptor, as well as the desensitization rates for the $GABA_A$ receptor, were dependent on the type of neuronal cells used (*11*, *12*, *16*). In embryonic cortical cells from mice the values of $K_1$ for the two forms of the $GABA_A$ receptors were 40 $\mu$M and 320 $\mu$M and the desensitization rate constants were 4.4 $s^{-1}$ and 0.7 $s^{-1}$ at saturating concentrations of GABA (*16*). On the other hand, in membrane vesicles prepared from postnatal rat cortical cells, the concentration of GABA that gives the half-maximal response was approximately 100 $\mu$M for both receptor forms and the desensitization rates were 21 $s^{-1}$ and 4 $s^{-1}$ at saturating concentrations of GABA (*11*, *12*). The above parameters from quench-flow (*11*, *12*) and cell-flow measurements (*16*) make it possible to separate the important steps leading to channel opening and desensitization (Figure 1). However, the time resolution of methods in which the cell surface receptors are equilibrated with the neurotransmitter in solutions flowing over the cell is not sufficient to measure the opening ($k_{op}$) and closing ($k_{cl}$) rate constants of the transmembrane channel (*19*) (see Figure 5). The ratio of these rate constants determines the concentration of open receptor channels at a given concentration of neurotransmitter (*20*, *21*). This information is of interest because the neurotransmitter concentration and time-dependent concentration of open receptor channels in the cell membrane, determined in these studies, together with the conductance of the receptor channel, determines the rate of transmembrane flux of inorganic ions (*21*). The rate of this transmembrane flux determines the change in the voltage (*22*−*24*) across the cell membrane and, therefore, whether a signal is transmitted to another cell (*1*). Furthermore, the mechanism of action of inhibitors and potentiators, as well as changes due to a change in the transmembrane voltage, can be best understood by studying their effect on these rate constants (*25*, *26*). Specifically, the approach used in this study can be extended to determine the mechanism of $GABA_A$ receptor potentiators such as barbiturates and benzodiazepines, which are used as anxiolytics and as sedatives (*27*).

The value of the rate constant for channel closing ($k_{cl}$) (Figure 1) can be obtained from mean channel-open durations using single-channel current recordings (*10*, *28*). However, the presence of two receptor subtypes for the $GABA_A$ receptor *(11*, *12*, *16*) complicates the interpretation of single channel current measurements (*14*, *15*). Recent measurements, in which small outside-out membrane patches were used, have increased the time resolution for the flow measurements from milliseconds to submilliseconds (*17*). However, as shown in the Results section, the time resolution of these measurements is not sufficient to establish the relationship between the observed rate constant for channel opening and GABA concentration (*17*). Furthermore, only one receptor form was observed in these studies, because only a very small sample of the receptors in the membrane of a whole cell is present in the outside-out membrane patches. It may, therefore, not be possible to detect the different receptor forms that may be present in the cell membrane.

The recently developed laser-pulse photolysis technique (*19*, *29*), wherein the whole cell is equilibrated with a biologically inert photolabile precursor of the neurotransmitter (caged neurotransmitter) and the neurotransmitter is photolytically released from an inactive precursor (*19*, *29*−*35*), has proven to be useful in determining the rate constants for channel opening and closing of nicotinic acetylcholine receptors in $BC_3H1$ cells (*19*, *25*, *26*). In this report the dissociation constant for GABA and the rate constants for channel opening, closing, and desensitization for two receptor forms (the rapidly and slowly desensitizing forms) of the $GABA_A$ receptors in rat hippocampal neurons were determined by using a combination of cell-flow (*36*) and laser-pulse photolysis (*19*, *21*, *29*, *37*) techniques.

## MATERIALS AND METHODS

GABA was purchased from Sigma and the caged GABA derivative, *N*-(α-carboxy-2-nitrobenzyl)GABA (*31*), was a gift from Molecular Probes (Eugene, OR). Hippocampal neurons were obtained from 1-day-old Sprague−Dawley rats (*16*). These cells were mechanically isolated and cultured on dishes coated with rat tail collagen and in minimum essential medium containing 5% fetal bovine serum and 5% horse serum and supplemented with 2.7 mM glutamine and 10 mM glucose (*16*). Cells used in the experiments had been maintained in culture for 5−8 days.

*Cell-Flow and Laser-Pulse Photolysis*. The flow device used for the rapid exchange of solution around a single cell has been described in detail by Krishtal and Pidoplichko (*38*) and its use in a cell-flow method by Udgaonkar and Hess (*36*). In brief, the flow device is a U-tube with a 100 $\mu$m aperture. At a flow rate of 5 cm/s and when the cell is placed 50 $\mu$m from the aperture, the equilibration of the cell surface receptors with the ligand solution occurs within tens of milliseconds. The laser-pulse photolysis experiments were performed as described by Matsubara et al. (*19*). The photocleavage of the caged GABA was initiated with a pulse of laser light generated by a flash lamp-pumped dye laser (Candela SLL 500) using a rhodamine 640 dye together with frequency doubling of the laser output ($\lambda$ = 320 nm). The

output of the laser was coupled into an optical fiber (~200 $\mu$m in diameter), which delivered the light near the cell. The concentration of caged GABA used was 1 mM, and the laser energy for the photolysis was in the range of 2−10 mJ. The cell-flow method was used before and after each laser-pulse experiment to determine the concentration of the GABA released from the caged GABA and to detect if there was any cell damage. We have previously shown (*31*) that exposing fetal mouse cortical neurons to 500 $\mu$M caged GABA does not affect the properties of the GABA receptor as determined in cell-flow experiments. We have ascertained that exposing the hippocampal neurons to 1 mM caged GABA for 1 s, before flowing 1 mM caged GABA together with 20 $\mu$M GABA over the cell, gives the same current versus time trace as experiments in which the caged GABA was omitted (experiments not shown).

For both the cell-flow and the photolysis experiments, the electrode solution contained 140 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 2 mM $Na_2ATP$, and 10 mM HEPES (pH 7.4); the extracellular bath solution contained 145 mM NaCl, 1.8 mM $MgCl_2$, 1 mM $CaCl_2$, 3 mM KCl, 10 mM glucose, and 10 mM HEPES (pH 7.4). Whole-cell currents (*39*, *40*) were amplified with an Adams and List EPC-7 amplifier, low-pass-filtered at 1−5 kHz for the cell-flow experiments and at 20−30 kHz for the laser-pulse photolysis experiments. The filtered signal was digitized at 0.5−2 kHz for the cell-flow measurements and 10−20 kHz for the laser-pulse photolysis experiments by a Labmaster DMA digitizing board controlled by Axon PClamp software. The time constants for the rising and decaying phases for the whole-cell current were obtained by using a nonlinear least-squares fitting program with Microcal Origin 3.0 software. All the experiments were performed at room temperature (~22 °C), at pH 7.4 and at −60 mV.

*Outside-out patches* were obtained from the cells as described by Hamill et al. (*39*). The diameter of the pipets used was ~1−2 $\mu$m. GABA was delivered from the same flow device and at the same flow rate as was used for the whole-cell recording measurements. The currents for the above three measurements were amplified with an Adams and List EPC-7 amplifier and filtered at 10 kHz. The filtered data were digitized at 10−20 kHz and analyzed with Microcal Origin 3.0 software.

## RESULTS AND DISCUSSION

*Cell-Flow Experiments*. Figure 2 shows a GABA- (250 $\mu$M) induced whole-cell current recorded from a rat hippocampal neuron at a membrane potential of −60 mV. The maximum current reflects the concentration of the open channel and the decay of the current represents receptor desensitization (Figure 1). The desensitization can be best fit by two exponentials, 0.4 $s^{-1}$ and 2.3 $s^{-1}$, in the experiment shown. The fraction corresponding to each of the two phases varied from cell to cell, with some cells exhibiting only the fast or only the slow desensitization reaction. Further, only the component corresponding to the slow desensitization is inhibited by picrotoxin (*16*). For these reasons, the two desensitization phases have been assigned to two $GABA_A$ receptor forms (*16*).

Some receptor molecules begin to desensitize before the current reaches the maximum amplitude; the current amplitude was, therefore, corrected for desensitization by the method described by Udgaonkar and Hess (*36*). The relationship between the corrected current ($I_A$), the GABA concentration, and the constants pertaining to the mechanism (Figure 1) of channel opening is shown in (*36*)

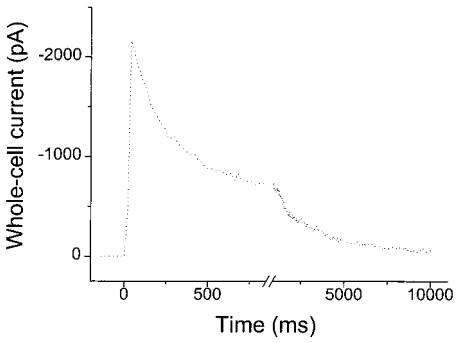


FIGURE 2: Whole-cell current recording from a rat hippocampal cell induced by 250 $\mu$M GABA in a cell-flow experiment, at pH 7.4, 20 °C and −60 mV. The flow of the GABA solution over the cell was initiated at time 0 and continued for 10 s. The rising phase of the current represents formation of the open channel. About 60% of the current decay, considered to be due to desensitization, is characterized by a rate coefficient of 0.4 ± 0.1 $s^{-1}$ and 40% by a rate coefficient of 2.3 ± 0.4 $s^{-1}$. The break in the abscissa occurs at 900 ms.

$$I_A = I_M R_M L^2 [L^2 + (L + K_1)^2 \Phi]^{-1} = I_M R_M (\overline{AL_2}) \quad (1)$$

$\overline{AL_2}$ is the fraction of the receptor molecules in the open-channel form; $L$ represents the molar concentration of the ligand, namely, GABA; $I_M$ is the current due to 1 mol of open receptor-channels; and $R_M$ represents the moles of receptors in the membrane. $K_1$ is the dissociation constant of GABA and $\Phi^{-1}$ is the channel-opening equilibrium constant.

The corrected maximum current obtained in the cell-flow experiments reported here for the rapidly and slowly desensitizing forms of the $GABA_A$ receptor in rat hippocampal neurons at various GABA concentrations is shown in Figure 3. Because the concentration of the receptors, as measured by the whole-cell current, differs considerably from cell to cell, the maximum current was normalized to the value obtained at 250 $\mu$M GABA. The constants $K_1$, $I_M R_M$, and $\Phi^{-1}$ (listed in Table 1) were evaluated from the dependence of the corrected maximum current on the GABA concentration (eq 1). The $K_1$ values of 100 ± 40 $\mu$M for the rapidly desensitizing form and 120 ± 60 $\mu$M for the slowly desensitizing form are in good agreement with those determined by quench-flow measurements with postnatal rat cortical cells (*11*, *12*). It must be noted that the values of the dissociation constants depend on the type of cells studied, since in embryonic cortical cells from mice the $K_1$ values for the two forms of the $GABA_A$ receptors were determined to be 40 $\mu$M and 320 $\mu$M (*16*). The channel-opening equilibrium constant, $\Phi^{-1}$, differs by a factor of 3 between the rapidly and slowly desensitizing receptor forms, indicating a difference in the rate constants for channel closing or opening or both for the two forms of the receptor.

*Laser-Pulse Photolysis Experiments*. In the experiment shown in Figure 4a, 1 mM caged GABA was allowed to equilibrate with the receptors on the surface of a hippocampal cell before photolysis was induced by a laser pulse at time

Table 1: Constants Determining the Formation of Open Channels in the $GABA_A$ Receptor in Rat Hippocampal Neurons at −60 mV, pH 7.4, and at Room Temperature

| parameter | rapidly desensitizing type | slowly desensitizing type | method |
|---|---|---|---|
| $K_1$ ($\mu$M) dissociation constant of site controlling channel opening | 100 ± 40 | 120 ± 60 | cell-flow[a] |
| $EC_{50}$ ($\mu$M) half-maximum response | 100 | 100 | quench-flow[b] (*11*) |
| $\Phi^{-1}$ channel-opening equilibrium constant | 7 ± 5 | 21 ± 10 | cell-flow[a] |
| | 20 ± 15 | 67 ± 30 | laser-pulse photolysis[a] |
| $I_M R_M$ (nA) | 1.3 ± 0.4 | 2.7 ± 0.5 | cell-flow[a] |
| desensitization rate constant ($s^{-1}$) at 250 $\mu$M GA | 2.3 ± 0.4 | 0.4 ± 0.1 | cell-flow[a] |
| | 21 | 4 | quench-flow[b] (*11*) |
| $k_{op}$ ($s^{-1}$) rate constant for channel opening | 1840 ± 160 | 6700 ± 330 | laser-pulse photolysis[a] |
| | | 6000 | flow[c] (*17*) |
| $k_{cl}$ ($s^{-1}$) rate constant for channel closing | 85 ± 20 | 100 ± 60 | laser-pulse photolysis[a] |
| | | 10,[c,d] 4[c,e] | flow[c] (*17*) |
| | 181, 32 | | single-channel[c] (*14*) |

[a] Using rat hippocampal neurons. [b] Using membrane vesicles from rat cortical cells. [c] Using membrane patches. [d] From rat cerebellar granule neurons. [e] From purkinje neurons. [f] Using mouse spinal cord cells.

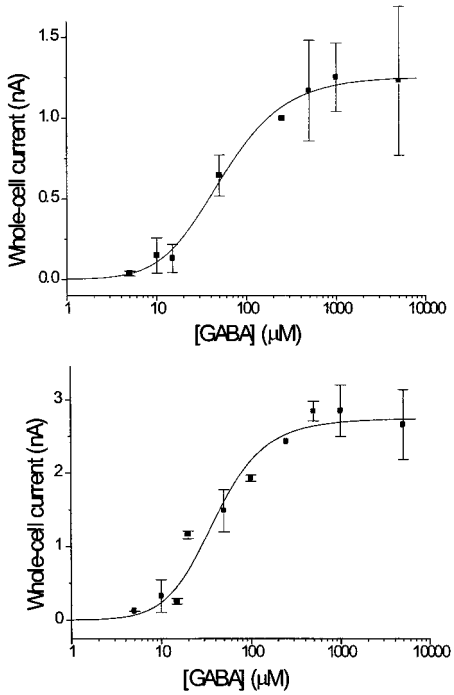


FIGURE 3: Concentration dependence of the current amplitude ($I_A$) corrected for receptor desensitization, obtained at pH 7.4, 20 °C and −60 mV, for the (top panel) rapidly desensitizing form and (bottom panel) slowly desensitizing form of the $GABA_A$ receptor. The solid squares in both panels represent the corrected current obtained from cell-flow measurements (*36*). The data were normalized to the $I_A$ value obtained in the presence of 250 $\mu$M GABA. A value of 1.1 nA was taken as normalization point for the results in the top panel and 2.4 nA for the results shown in the bottom panel. These current values represent the mean value of the current, for all the cells used in the present experiments, induced by 250 $\mu$M GABA. Each data point shown in the figures is the average of 2−4 measurements made with 1−4 cells. The parameters used to compute the solid line ($I_M R_M$, $K_1$, and $\Phi$) are listed in Table 1 and were obtained by using the relationship shown in eq 1 with a nonlinear least-squares fitting program.

zero, leading to the liberation of 170 $\mu$M free GABA. In the experiment shown, the current rise follows a single exponential with a $k_{obs}$ value of 830 $s^{-1}$ ($\tau$ = 1.2 ms). The current rise represents formation of the open channel and the maximum current reflects the concentration of the open channel. For comparison, Figure 4b shows a cell-flow experiment with the hippocampal cell in which 200 $\mu$M GABA was used. The time constant $\tau$ for the current rise time of ~60 ms is approximately 50 times larger than in the laser-pulse photolysis experiment (Figure 4a). The current corrected for desensitization (*36*) is shown by the dotted line. About 50% of the current decay due to receptor desensitization is characterized by a rate coefficient of 2.9 $s^{-1}$ and 50% by a rate coefficient of 0.4 $s^{-1}$.

The current from a 2 $\mu$m diameter membrane patch obtained by flowing 200 $\mu$M GABA over the patch at a rate of 5 cm/s is shown in Figure 4c. The time constant for the current rise of 8 ms is about 7 times longer than in the laser-pulse photolysis experiment (Figure 4a; see also Figure 5 inset). Only a single desensitization process, characterized by a rate coefficient of 2.3 $s^{-1}$, characteristic of the fast-desensitizing receptor form is observed.

By carefully screening many cells it is possible to find cells that contain only the rapidly desensitizing receptor form (~14%) and cells that contain only the slowly desensitizing form (~20%). To determine the rate constants for rapidly and slowly desensitizing receptor forms, only cells that had either of these forms were used in the laser-pulse photolysis experiments. The rise in the current for both types of cell followed a single-exponential rate equation:

$$I_t = I_{max}\,[1 - \exp(-k_{obs}t)] \qquad (2)$$

$I_t$ is the current observed at time $t$, and $I_{max}$ is the maximal observed current in the absence of desensitization. For the mechanism in Figure 1, the relationship between the first-order rate coefficient for the channel-opening process and the constant of the mechanism has been derived (*19*). When the ligand-binding steps are fast compared to the channel-opening process and $L$ represents the molar concentration of the ligand, namely, GABA,

$$k_{obs} = k_{cl} + k_{op}\,[L/(L + K_1)]^2 \qquad (3)$$

The assumption that the ligand-binding steps are faster than the channel-opening steps is based on the fact that the current rise is well represented by a single exponential at all concentrations of GABA. The single-exponential rise will also be observed in the case where the ligand-binding steps are rate limiting. However, under these conditions the concentration dependence of $k_{obs}$, as shown in eq 3, will not

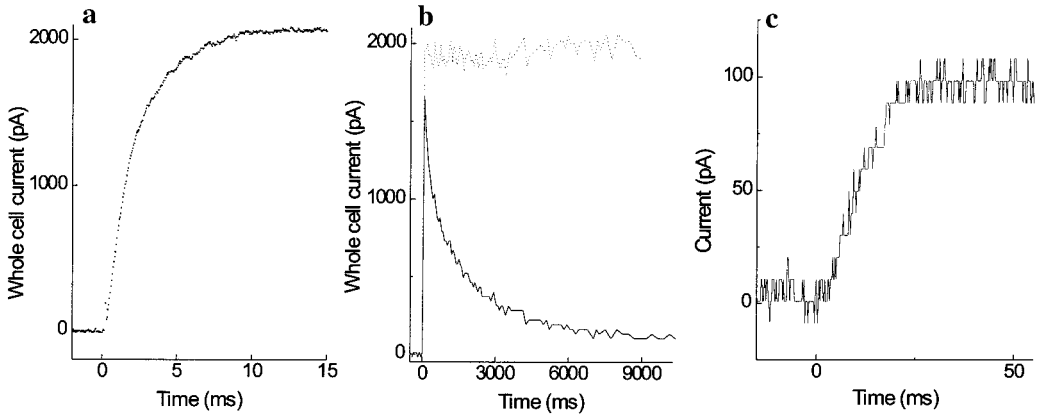


FIGURE 4: Experiments with hippocampal cells, and outside-out membrane patches from them, at pH 7.4, −60 mV, and room temperature. (a) Whole-cell current induced by 170 $\mu$M GABA, which was released by laser-pulse photolysis of 1 mM caged GABA; the cell was equilibrated with 1 mM caged GABA for 1 s prior to photolysis. The $k_{obs}$ for the current rise was 830 $s^{-1}$. (b) Whole-cell current induced by 200 $\mu$M GABA, applied with a flow device and a flow rate of 5 cm/s. The $k_{obs}$ value for the current rise was 17 $s^{-1}$. About 50% of the current decay, due to desensitization, is characterized by a rate coefficient of 0.4 $s^{-1}$ and 50% by a rate coefficient of 2.9 $s^{-1}$. The current corrected for desensitization is shown as a dotted line. (c) Current from a membrane patch ~2 $\mu$m in diameter induced by the application of 200 $\mu$M GABA. The flow rate of the solution was about 5 cm/s and was initiated at time 0. The small (~10 pA) positive and negative current spikes are due to electrical noise and are also observed in the absence of GABA. The $k_{obs}$ for the current rise was 125 $s^{-1}$. The current decay, which occurs in a different time region and is not shown, has a rate coefficient of 2.3 $s^{-1}$. For other details see Materials and Methods.

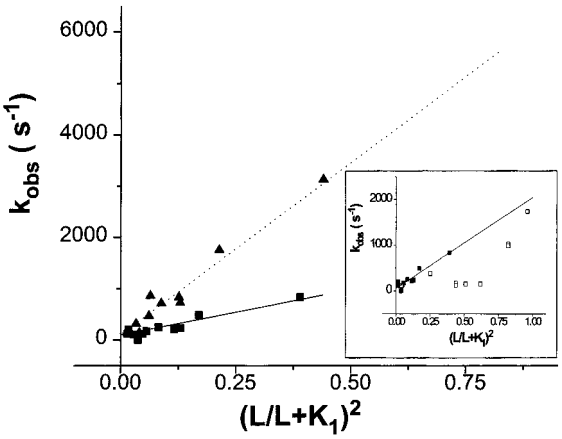


FIGURE 5: Rate coefficient, $k_{obs}$, for the rising phase of the current obtained by the laser-pulse photolysis technique (pH 7.4, −60 mV, and room temperature) is plotted as a function of $(L/L + K_1)^2$, where $L$ is the GABA concentration, according to eq 3. The parameters $k_{op}$, $k_{cl}$, and $K_1$ (Table 1) were evaluated by the relationship described in eq 3 (solid lines) with a nonlinear least-squares fitting program. The solid squares represent the $k_{obs}$ for the rapidly desensitizing form of the $GABA_A$ receptor, and the triangles represent the $k_{obs}$ for the slowly desensitizing form. The inset shows the $k_{obs}$ obtained, with outside-out membrane patches, for flow measurements at various concentrations of GABA.

be satisfied. If the rates for ligand-binding steps were on the same time scale as that of the channel-opening rates, then two exponentials would be required at lower concentrations of GABA. A plot of the observed rate constants for channel opening for the rapidly and slowly desensitizing receptor forms of the $GABA_A$ receptor obtained at various GABA concentrations is shown in Figure 5. The intercept for this plot, as shown in eq 3, represents $k_{cl}$ and the slope $k_{op}$. The rate constants for channel closing, $k_{cl}$, for the two forms of the receptor are 85 ± 20 $s^{-1}$ and 100 ± 60 $s^{-1}$ and are in good agreement with the mean open time of the channel of 10 ms (corresponding to $k_{cl}$ of 100 $s^{-1}$) which was determined from single-channel current recordings in mouse spinal cord cells (*14*, *15*). The rate constant for channel opening, however, differs by a factor of 3 for the two forms of the receptor. This difference in the rate constant for channel opening between the two forms of the receptor accounts for the difference in the channel-opening equilibrium constant ($\Phi^{-1}$) evaluated by flow measurements (Table 1).

It is of interest to compare the values of $k_{obs}$ for channel opening obtained from laser-pulse photolysis with that determined by flow measurements with small outside-out membrane patches from the rat cerebellar cells (*17*). At very high concentrations of GABA the published $k_{obs}$ values obtained in flow measurements (*17*) are in very good agreement with those obtained with the slowly desensitizing receptor form in the laser-pulse photolysis experiments. There is reasonable agreement at very low concentrations of GABA between the flow experiments (*17*) and the laser-pulse photolysis experiments presented here, but not at intermediate concentrations. In the intermediate concentration range the flow measurements (*17*) give lower rate constants than those obtained from the laser-pulse photolysis measurements (see also Figure 4c and Figure 5 inset). We assume that at very low GABA concentrations, where $k_{obs}$ for the channel-opening process is low (~200 $s^{-1}$ in the experiments shown in Figure 5), the flow technique has an adequate time resolution. In the intermediate range of GABA concentrations, at which $k_{obs}$ for channel opening has a lower value in flow experiments than in photolysis measurements (Figure 4c, Figure 5 inset). We assume that the $k_{obs}$ value obtained in flow measurements is determined by both diffusional access of GABA to the membrane-bound receptor and the rate constant for channel opening. When the GABA concentration in the flowing solutions is much higher than is required ($K_1$ = ~100 $\mu$M) to saturate the receptor sites, a small fraction of the GABA will reach the receptor sites in a time that is fast compared to channel opening. Hence, at these supersaturating concentrations of GABA the $k_{obs}$ values determined in flow experiments are in agreement with those obtained by the laser-pulse photolysis technique (Figure 5 inset). Does the difference in $k_{obs}$ values obtained in flow and photolysis measurements reflect differences between

receptor types in the cells used for flow measurements (*17*) (rat cerebellar cells) and those used in laser-pulse photolysis experiments (rat hippocampal neurons)? To answer this question we have made flow measurements with small outside-out patches from the same cells as we have used in the photolysis experiments (Figure 5 inset). At very high and very low concentrations of GABA we find good agreement between the results obtained with the flow measurements and those obtained with the rapidly desensitizing receptor form by the laser-pulse photolysis technique (Figure 5 inset). In the intermediate range of GABA concentration the flow technique gives lower $k_{obs}$ values than the photolysis technique (Figure 4c, Figure 5 inset). We suspect that one reason that the time resolution of the flow technique is less than anticipated is that it is determined by flowing electrolyte solutions over the open end of the glass capillary of the recording electrode (*41*). However, the diffusion coefficients of electrolytes are larger than those of the neurotransmitters and these diffusion coefficients play a critical role in determining equilibration times between reactants (*42*) (GABA and the cell surface receptors in the studies reported here). Another reason may be that the membrane attached to the glass capillary of the recording electrode used in these studies is not flat but invaginated due to contacts of the membrane with the inner wall of the glass capillary (*43*, *44*). It must be noted that the differences we have observed between the flow measurements with membrane patches and the laser-pulse photolysis experiments might have implications in the experiments performed with membrane patches, wherein the rate constants of the ligand binding steps have been determined (*45*, *46*).

Furthermore, in the flow measurements with small membrane patches there is no evidence for two $GABA_A$ receptor forms in either the rat cerebellar (*17*) or hippocampal cells (Figure 4c). On average 65% of the rat hippocampal cells used in our experiments contain both rapidly and slowly desensitizing GABA receptor forms. The rapidly desensitizing receptor form on average comprises ~50% of the receptor forms in the cells that contain both receptor forms. The membrane patches carry only a small sample of the receptors on the cell surface; hence in the small outside-out patches from rat cerebellar cells (*17*) or rat hippocampal neurons (Figure 4c) only one of the two $GABA_A$ receptor forms that are usually found in the membrane of neurons of both mice and rats (*1*, *11*, *12*, *16*) can be detected (*17*).

## CONCLUSIONS

The two forms of the $GABA_A$ receptor, namely, the rapidly and slowly desensitizing receptor forms, are characterized by different desensitization rates in postnatal rat hippocampal cells. At 250 $\mu$M GABA the rapidly desensitizing form desensitizes with a rate constant of $2.3 \pm 0.4$ $s^{-1}$ while the slowly desensitizing form desensitizes with a rate constant of $0.4 \pm 0.1$ $s^{-1}$ at 250 $\mu$M GABA. The values of the dissociation constant for GABA for the rapidly and slowly desensitizing receptor forms is similar, $100 \pm 40$ $\mu$M and $120 \pm 60$ $\mu$M, respectively. The dissociation constant values obtained in these experiments with rat hippocampal cells are in good agreement with those obtained by quench-flow measurements on membrane vesicles prepared from the rat cortex (*11*, *12*). However, the values of the dissociation constants are different from those determined in embryonic mouse cortical cells, 40 $\mu$M and 320 $\mu$M for the rapidly and slowly desensitizing receptor forms, respectively (*16*), indicating that the dissociation constant for GABA from the $GABA_A$ receptor is dependent on the type of cells used. The channel-closing rate constant did not differ significantly for the two forms of the receptor in rat hippocampal cells. However, the channel-opening rate constant was three times larger for the slowly desensitizing form relative to the rapidly desensitizing form. This was also reflected in the channel-opening equilibrium constants determined by cell-flow measurements (Table 1). The results in Table 1 form the basis for relating rate and equilibrium constants of the $GABA_A$ receptor-mediated reaction to structural information.

Do the rapidly and slowly desensitizing receptor forms differ in subunit composition and/or in the number of subunits that form the receptor channel? At least 13 $GABA_A$ receptor genes have been identified (*47*). Of these, $\alpha 1-5$, $\beta 1-3$, $\gamma 1-3$, and $\delta$ are present in the hippocampus (*47*). In situ hybridization studies have indicated the colocalization of $\alpha 1$, $\beta 1$, $\gamma 2$, and $\delta$ subunit mRNAs in hippocampal dentate gyrus granule cells and colocalization of the $\alpha 1$, $\beta 1$, and $\gamma 2$ subunits in the pyramidal cells of the hippocampus (*48*). The $\delta$, subunit containing receptors are known to have a reduced desensitization rate associated with them (*48*). In addition, phosphorylation of the nicotinic acetylcholine receptor is known to affect receptor desensitization rates (*49*). The $GABA_A$ receptor is known to have phosphorylation sites on at least three different subunits (*50*). Hence, the two receptor forms could be different in the number of subunits phosphorylated or differ in some other posttranslational modifications.

The determination of the various reaction steps and rate constants for these steps for the $GABA_A$ receptor is a first step toward understanding how a single cell integrates the action of inhibitory and excitatory neurotransmitter receptors (*37*). The determination of the elementary steps of the channel-opening process and receptor desensitization also allows one to determine how the mechanism of the GABA receptor and the associated constants are affected in diseases such as epilepsy (*2*). Furthermore, the mechanism of action of pharmacological drugs such as barbiturates and benzodiazepines, which are potentiators for the GABA receptor (*3*), can now be investigated by determining the effects of such drugs on the elementary steps in channel opening and receptor desensitization. These studies are expected to be useful for the discovery of new drugs that alleviate abnormal receptor function in disease, once the effect of the disease on the elementary steps of the receptor-mediated reaction have been identified.

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