On the Mechanism of the γ-Aminobutyric Acid Receptor in the Mammalian (Mouse) Cerebral Cortex. Chemical Kinetic Investigations with a 10-ms Time Resolution Adapted to Measurements of Neuronal Receptor Function in Single Cells†

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Received June 14, 1991; Revised Manuscript Received December 31, 1991

ABSTRACT: The γ-aminobutyric acid$_A$ (GABA) receptor belongs to a superfamily of proteins involved in chemical reactions that regulate signal transmission between cells of the nervous system and is the target of some of the agents most frequently used in medicine to control disorders of the central nervous system. In contrast to the nicotinic acetylcholine receptor, which initiates signal transmission and is the best characterized member of the superfamily, the GABA receptor forms anion-specific transmembrane channels and inhibits signal transmission. The chemical kinetic experiments described here, in which fast chemical reaction techniques were used, indicate that both receptor proteins may operate by the same mechanism. Also described is the use of a chemical kinetic technique with a 10-ms time resolution that we have developed for making measurements with single cells isolated from specific areas of the nervous system, in this case the cerebral cortex of embryonic mice. A flow device was used to equilibrate receptors on the cell surface with GABA, and the concentration of open transmembrane channels in the cells was then measured by recording the whole-cell currents at pH 7.2, 21-23 °C, and a transmembrane voltage of −70 mV. Two different receptor forms, A_α and A_β, were detected in cerebral cortical cells. Although the ratio of A_α to A_β varied from cell to cell, on average 35% and 65% of the receptor-controlled current was associated with receptor forms A_α and A_β, respectively. At saturating concentrations of GABA, the rate coefficients of desensitization, α and β, associated with these two forms have maximal values of 4.4 and 0.7 s^{-1}, respectively. The constants of a mechanism that accounts for the open transmembrane channels of both receptor forms were evaluated over a 50-fold range of GABA concentration. The dissociation constant of the site controlling channel opening was 40 μM for A_α and 320 μM for A_β. The channel-opening equilibrium constant, Φ^{-1}, was 3.5 for A_α and 20 for A_β. The evaluated constants allow one to calculate P_o, the conditional probability that at a given concentration of GABA the receptor-channel is open. P_o could also be determined in the presence of 100 μM GABA by an independent method in which different assumptions are made in the interpretation of the experimental results, the single-channel current-recording technique. The value of P_o obtained (0.56) was in good agreement with the P_o value (0.61) calculated for receptor form A_α from chemical kinetic measurements at 100 μM GABA. At this GABA concentration the values of the desensitization rate coefficient associated with receptor form A_α determined by both techniques (2.1 and 2.6 s^{-1}) were also in good agreement. It was, therefore, possible to determine the conductance of the transmembrane channel associated with A_α (γ = 22 pS). Preliminary experiments with a well-characterized specific inhibitor of the GABA receptor, picrotoxin, indicated that the method employed can be used to investigate the mechanism of action of activators and inhibitors of this receptor. The experiments also demonstrate the importance of using kinetic techniques with good time resolution in the investigations. In an experiment with a low (500-ms) time resolution, picrotoxin appears to inhibit both receptor forms, whereas in the experiment done with a 10-ms time resolution it was seen that one receptor form, A_α, was not inhibited; in the low time resolution experiment, A_α had merely desensitized before it could be observed. The ways in which activators and inhibitors affect the receptor, the role of subunit composition, and the effect of diseases of the nervous system on the mechanism are all unanswered and interesting subjects for future research. The results obtained so far suggest that rapid chemical kinetic techniques suitable for making measurements with single cells from specific areas of the nervous system may be useful in such studies.

The γ-aminobutyric acid (GABA) receptor is one of many membrane-bound proteins involved in the key reactions that determine whether signals are transmitted between cells of the nervous system (Kandel & Schwartz, 1985). Upon binding their specific neurotransmitter, this and the glycine receptor open anion-specific transmembrane channels transiently (for a few milliseconds) and counteract the effects of receptors like those for acetylcholine and glutamate, which initiate signal transmission between cells (Kandel & Schwartz, 1985). Both types of receptor belong to a superfamily of proteins (Betz, 1990; Stroud et al., 1990), even though the transmembrane channels they form display different ion specificity. Ultimately, the initiation of signal transmission, or its inhibition, depends on the concentration of open transmembrane channels formed as a result of the binding of neurotransmitters (e.g., GABA, acetylcholine) to their specific receptors. Under-

† This work was supported by a grant (NS 08527) from the National Institutes of Health.

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0006-2960/92/0431-5488$03.00/0

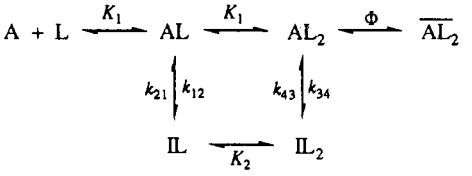
standing the mechanism that allows the concentration of open receptor–channels and, therefore, the signal transmission process to be adjusted in the millisecond time region by a variety of factors, including the concentration of the specific neurotransmitter and many compounds that activate or inhibit the function of the receptors, is one of the unsolved challenges in modern biology. One aim of this study was to determine whether rapid chemical kinetic techniques, successfully used for studying complex biological reactions in solution (Eigen, 1967; Hammes, 1982; Fersht, 1985) and reactions involving the nicotinic acetylcholine receptor in membrane vesicle preparations [Hess et al., 1979; reviewed by Hess et al. (1983) and Ochoa et al. (1989)], can also be useful in investigations of receptor function in single cells isolated from specific parts of the nervous system. The cells we used came from the cerebral cortex of embryonic mice and contain the $GABA_A$ receptor. The preparation of the cells has been described in detail (Frere et al., 1982). We have modified this cell preparation to obtain cells that can be suspended in a stream of GABA solutions flowing over the cells in rapid chemical kinetic measurements.

Tremendous advances have been made in studying the molecular biology of the GABA receptor, beginning with the identification of two receptor subunits, α and β, with a molecular weight of about 55 000 each (Sigel & Barnard, 1984). Since then additional subunits, the γ- and δ-subunits, have been identified (Pritchett et al., 1989; Shivers et al., 1989), as well as many isozymes of the α- and β-subunits [reviewed in Olsen and Tobin (1990), Schofield (1989), Schofield et al. (1990), and Vicini (1991)]. When different combinations of these subunits have been expressed in *Xenopus* oocytes or human embryonic kidney cells, receptors with different properties, as measured by electrophysiological techniques, have been produced (Levitan et al., 1988a,b; Pritchett et al., 1989; Schofield et al., 1990; Sigel et al., 1990; Verdoon et al., 1990).

Various approaches have been used to investigate the function and activation of this receptor. Binding studies in which radioactively labeled activators and inhibitors of the receptor were used [reviewed in Krogsgaard-Larsen et al. (1986)], measurements of receptor-controlled translocation of inorganic ions across the cell membrane (Thampy & Barnes, 1984; Harris & Allan, 1985; Yang & Olsen, 1987; Cash & Subbarao, 1987a–c, 1988; Dunn et al., 1989), and electrophysiological measurements (Sakmann et al., 1983a; Hamill et al., 1983; Twymann et al., 1990), including the use of fast perfusion techniques (Sakmann et al., 1983b; Numann & Wong, 1984; Bormann & Clapham, 1985; Akaike et al., 1986; Bormann & Kettenmann, 1988; Huguenard & Alger, 1986; Verdoon et al., 1990), have all been reported. A great variety of animals, organs, and cells were used in those experiments. There is general agreement regarding the existence of several receptor forms and the requirement for the binding of two GABA molecules for the receptor–channel to open (Nowak et al., 1982; Sakmann et al., 1983b; Bormann & Clapham, 1985; McBurney et al., 1985; Cash & Subbarao, 1987a–c). Except for one study (Cash & Subbarao, 1987a–c), there have been no attempts to determine the constants that account for the concentration of open receptor–channels as a function of GABA concentration, to integrate receptor desensitization into a minimum reaction mechanism, or to consider the existence of rapidly interconverting receptor forms with different properties.

Chemical kinetic investigations of membrane-bound receptors have been greatly aided by the availability of electric organs of fish that are extremely rich in nicotinic acetylcholine receptors, making it possible to prepare receptor-rich membrane vesicles in which receptor-controlled ion flux could conveniently be studied [Kasai & Changeux, 1971; Sachs, A. B., et al., 1982; reviewed by Changeux (1990)] using rapid mixing techniques like the quench- and stopped-flow methods [reviewed by Hess et al. (1983)]. The minimum reaction scheme for the acetylcholine receptor in the electric organ of *Electrophorus electricus* based on these studies has been considered as a guideline in quench-flow investigations of the GABA receptor in rat brain membrane vesicles (Cash & Subbarao, 1987a–c, 1989) and is considered as a model in our investigations of the GABA receptor in cerebral cortical cells. The minimum reaction scheme for the acetylcholine receptor in *E. electricus* electric organ was formulated (Cash & Hess, 1980) as

mechanism I



A represents the receptor in its active form; L represents acetylcholine, and the subscript indicates the number of ligand molecules that are bound to the receptor molecule. The binding of at least two acetylcholine molecules to the nicotinic acetylcholine receptor in frog muscles prior to channel opening had been suggested earlier (Katz & Thesleff, 1957). $\overline{AL_2}$ represents the open-channel form of the receptor through which inorganic ions are exchanged across the membrane, thus initiating an electrical signal and signal transmission. K_1 is the intrinsic dissociation constant of the receptor site that controls channel opening, and Φ^{-1} is the channel-opening equilibrium constant (Cash & Hess, 1980). Two first-order transitions to inactive (desensitized) receptor forms have been observed: a rapid process (milliseconds) was first discovered for the receptor from the electroplax of *E. electricus* (Hess et al., 1978, 1979, 1983) and a slow process (seconds to minutes), not shown in the mechanism, was first discovered in investigations of the frog muscle receptor (Katz & Thesleff, 1957). In the case of the *E. electricus* receptor, the slow process has a time constant of hours (Aoshima, 1984). k_{12}, k_{21}, k_{34}, and k_{43} are the rate constants for the interconversion between active and inactive receptor forms. The ligand-binding properties, characterized by the dissociation constant K_2, of the inactive, desensitized receptor forms (IL and IL_2) are different from those of the receptor form involved in signal transmission (Katz & Thesleff, 1957; Cash & Hess, 1980; Hess et al., 1983; Changeux et al., 1984) and dominate the measurements once the receptor has desensitized (Cash & Hess, 1980; Hess et al., 1983).

The use of the quench-flow technique, however, is restricted to only the few receptors that occur in sufficient abundance in nature to enable one to prepare membrane vesicles conveniently. Direct measurements of receptor function in cells overcome the problem of preparing and purifying membrane vesicles. However, in measurements with cells the time interval between the mixing of cells with a ligand and tracer ions and the first measurement, the time resolution, has been on the order of many seconds (Catterall, 1975; Stallcup & Cohen, 1976), during which time the receptors are likely to be converted to desensitized forms whose properties dominate the measurements (Hess et al., 1983). To overcome this problem, the rapid flow of solutions containing receptor-activating ligands over a cell was introduced by Krishtal and Pidoplichko

(1980). In their experiments the concentration of open receptor–channels was measured by a whole-cell current-recording method (Hamill et al., 1981; Marty & Neher, 1983). We first developed cell culture conditions that allowed us to obtain nearly spherical cells without processes that could be suspended in the stream of solution emerging from the cell-flow device. This allowed us to flow solutions at a moderate rate over a cell surface so that many measurements, with different concentrations of ligand, can be made with the same cell (Hess et al., 1987; Udgaonkar & Hess, 1987). The hydrodynamic theory for solution flowing over submerged spherical objects (Landau & Lifshitz, 1959; Levich, 1962) can then be used to correct the observed concentration of open receptor–channels for the desensitization that occurs during the time the receptors equilibrate with the activating ligand (Hess et al., 1987; Udgaonkar & Hess, 1987). The technique has already been used with the muscle (Udgaonkar & Hess, 1987) and neuronal (Matsubara & Hess, 1992) types of acetylcholine receptor in the clonal, mammalian BC_3H1, and PC12 cell lines respectively. Under certain restricted conditions of ligand concentration, it is possible to evaluate the fraction of receptors in the open-channel form $(\overline{AL}_2)_o$, and the rate of receptor desensitization, by an entirely independent method, the single-channel current-recording technique (Neher & Sakmann, 1976). Comparison of the results obtained by the two independent techniques, with BC_3H1 and PC12 cells and in the experiments reported here, demonstrates good agreement.

The question addressed here is, What information about receptor mechanism can one obtain using cells isolated from a specific part of the brain, the cerebral cortex, and a cell-flow technique with a 10-ms time resolution? Are the mechanism and constants describing the function of the cerebral cortex GABA receptors significantly different from those of the nicotinic acetylcholine receptor from the *E. electricus* electric organ or the GABA receptor in membrane vesicles prepared from an adult rat brain? Is the cell-flow technique adequate to investigate the mechanism by which activators or inhibitors affect the function of the GABA receptor?

Experimental Procedures

Cell Culture. Cerebral cortical cells were prepared from 15–16-day-old mouse embryos as described earlier (Frere et al., 1982). In brief, 35-mm Falcon dishes were coated with 0.1 mL of collagen solution [1 mg of rat tail collagen (Sigma)/2 mL of 1:1000 aqueous acetic acid] and sterilized for 4–6 h with ultraviolet radiation from a germicidal UV lamp (G30T8, Sylvania from The Baker Company Inc., Sanford, ME) in a laminar flow hood. Two milliliters of a suspension of approximately (6–10) × 10^5 cells were added to each dish. The cells were cultured in fetal bovine serum (FBS) (10%) + horse serum (HS) (10%) made in minimum essential medium (all from GIBCO) with freshly added glucose (30.5 mM) (Sigma) and glutamine (268 mM) (Sigma) and incubated with 5% CO_2. 0.2% 5′-fluoro-2′-deoxyuridine + 0.5% uridine (FUDR) (Sigma) was added to control the mitosis of glial cells. On day 3, 1 mL of old medium was exchanged for 1 mL of medium containing 10% FBS + 10% HS + 1% FUDR. Subsequently, the medium was changed twice a week with only horse serum added. An important aspect of these investigations was to modify the cell culture conditions to obtain cells that could be suspended in the stream of solution emerging from the flow device. The length of time UV radiation was used for sterilizing the collagen-coated dishes had a pronounced effect on the extent of attachment of the cells to the dish. A 24-h exposure resulted in 100% nonadhered cells that died eventually. However, a 4–6-h irradiation gave the optimum attachment so that cells could be detached from the culture dish. Experiments were carried out with cells that were 4–6 days old (counting the cell-plating day as day 1) so that they were loose enough to be lifted with the electrode used for recording whole-cell currents. The cells suspended in the solution stream were nearly spherical and had no processes.

Whole-Cell Current Recording. An amplifier (List L/M-EPC 7) was used for the current-recording measurements (Hamill et al., 1981). The intracellular solution inside the electrode contained 140 mM CsCl, 1 mM $CaCl_2$, 10 mM EGTA, and 10 mM Hepes (pH 7.2), and the extracellular solution in which the cells were suspended contained 145 mM NaCl, 1.8 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes (pH 7.2). The solutions emerging from the flow device (see below) had the same composition. The glass pipettes (electrodes), made from borosilicate glass (1.5-mm o.d.) (World Precision Instruments, CT), were pulled on a L/M 3P-A pipette puller (Adam & List, New York) and fire-polished; the electrode resistance was typically 3–4 MΩ when filled with the pipette solution. The series resistance was typically 6–10 MΩ, which was compensated up to 70%. The transmembrane voltage of −70 mV in the experiments could be held constant (±7 mV) by the procedure described in detail by Sigworth (1983).

In whole-cell current recording measurements (Hamill et al., 1981), the current signal from the amplifier was passed through a low-pass filter (Krohn-Hite, MA, model 3322) with a cutoff frequency of 4 kHz (−3 db point) and was amplified and digitized at a 200-Hz sampling frequency using either a PDP 11/23 minicomputer or an IBM personal computer, AST, using the pClamp-clampex software from Axon Instruments. The data were then transferred to a floppy disk and analyzed on an AST-PC using GENPLOT version 1.01 (Computer Graphics Service, Ithaca, New York). A nonlinear least-square program (Bevington, 1969) was used to fit the multiexponential decays of the current (Figure 1), and a linear prediction method (Millhauser et al., 1989) was used as an independent technique to evaluate the exponentials of the decaying phase of the current. The whole-cell current varied from 0.3 to 4 nA. Because the whole-cell current differed from cell to cell, measurements from different cells were normalized to the current obtained with 250 μM GABA. All experiments were performed at a transmembrane voltage of −70 mV, at room temperature (21–23 °C) and pH 7.2.

Rapid Application of Ligand Solution. The flow method used to apply a ligand solution rapidly to cells, and the recording of the whole-cell current at a constant transmembrane voltage, have been described (Krishtal & Pidoplichko, 1980; Udgaonkar & Hess, 1987; Matsubara & Hess, 1992). In the present experiments, a cell (~10-μm diameter) attached to a whole-cell current recording electrode was placed about 75 μm from the porthole of the flow device that had a diameter of 100 μm. The flow rates of the extracellular solutions emerging from the flow device were between 4 and 5 cm s^{-1}, and the time taken for the current to reach its maximal value was between 10 and 70 ms. On average, four measurements, at different ligand concentrations, were made with each cell. Between each measurement the cells were washed for 5 min with extracellular solution flowing over the cells at a rate of approximately 5 cm s^{-1}. The experimental error in measurements made with the same GABA concentration and the same cell was about 10%. When GABA, picrotoxin, and bicuculline (Sigma) were used, they were added to the extracellular solution.

Single-Channel Recording. The composition of the pipette (electrode) solution was the same as that of the extracellular

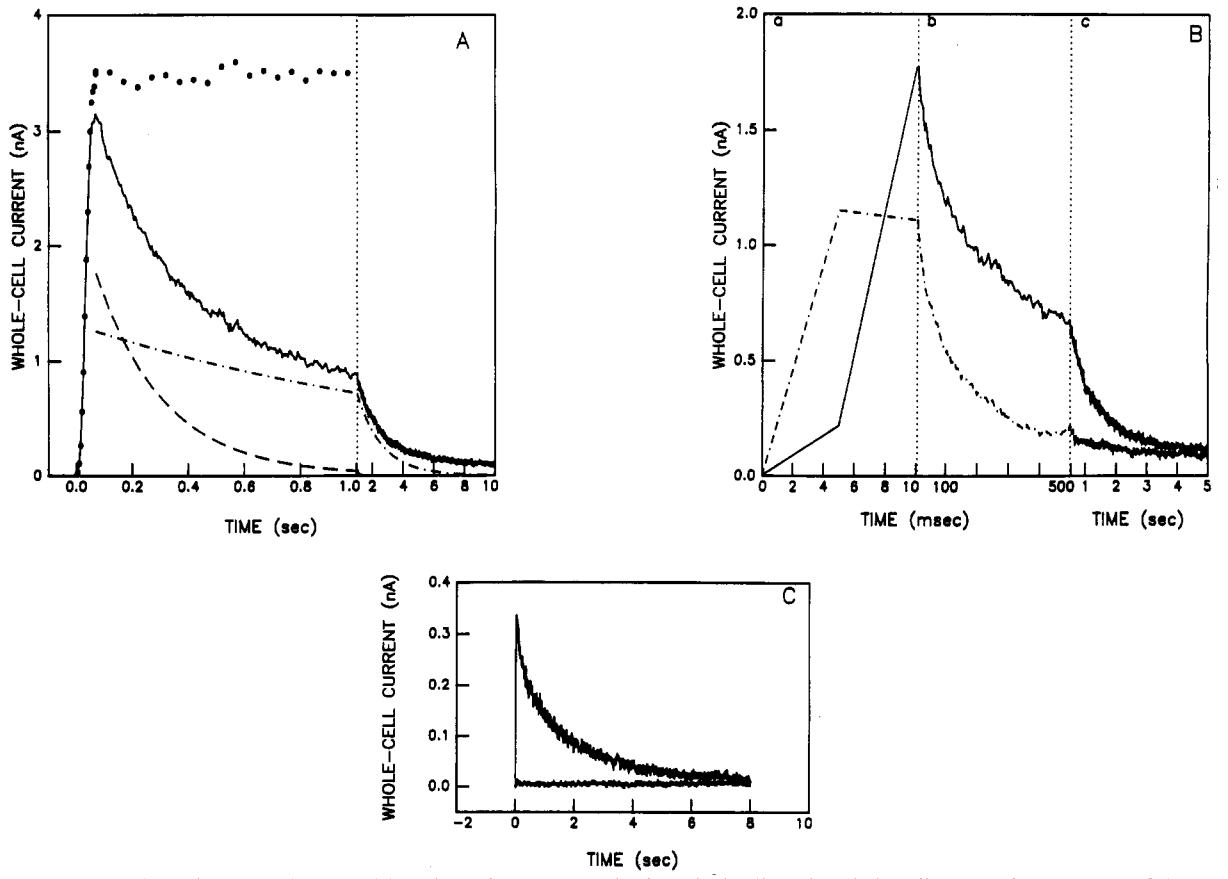


FIGURE 1: Examples of cell-flow experiments with embryonic mouse cerebral cortical cells. The whole-cell current is a measure of the number of receptors in the cell membrane in the open-channel form. The measurements were made at a transmembrane voltage, V_m, of −70 mV, at pH 7.2 and 21–23 °C. A cell-flow technique was used (see Experimental Procedures). (A) The GABA concentration was 250 μM, and the flow rate of the solution was approximately 4.5 cm s^{-1}. The observed current (solid line) reached its maximal value within 70 ms. Approximately 60% of the current decayed with a rate coefficient α of 4.1 s^{-1}, and approximately 40% of the current decayed with a rate coefficient β of 0.6 s^{-1}. The dots parallel to the time axis represent the current corrected for receptor desensitization that occurs during the rising phase of the current. The curves below the whole-cell current represent the current versus time curves for the rapidly (---) and slowly (-·-) desensitizing receptor forms. The dotted line parallel to the ordinate indicates the change on the time axis. (B) The GABA concentration was 500 μM (upper curve), and the flow rate of the solution was approximately 4.5 cm s^{-1}. The rise time of the current was approximately 10 ms. The current was corrected for receptor desensitization. $I_{A\alpha}$ was 1.0 nA and $I_{A\beta}$ was 0.75 nA. The values of α and β were 4.4 and 0.6 s^{-1}, respectively. The lower trace was obtained with the same cell, but picrotoxin (5 μM) was added to the GABA solution flowing over the cell. Only a single exponential decay of the current is observed. The amplitude of the rapidly decaying current component $I_{A\alpha}$ is the same as that observed in absence of picrotoxin, 1.0 nA, but the value of α has increased to 7.6 s^{-1}. The slowly decaying component, 43% of the corrected current in absence of picrotoxin (upper line), cannot be observed in the presence of 5 μM picrotoxin (lower line). The dotted lines parallel to the ordinate of the graph indicate changes on the time axis. (C) The GABA concentration (upper curve) was 250 μM, and the flow rate of the solution was approximately 4.5 cm s^{-1}. The rise time of the current was approximately 45 ms. Approximately 40% of the current decayed with a rate coefficient α of 4.8 s^{-1} and approximately 60% of the current decayed with a rate coefficient of 0.5 s^{-1}. The lower trace was obtained with the same cell, but 100 μM bicuculline was added to the GABA solution.

solution. In the single-channel current measurements, the cell-attached mode was used (Hamill et al., 1981; Sakmann et al., 1983b) and the data were stored on an FM analogue tape or on a video cassette. The data were then passed through a low-pass filter (Krohn-Hite 3322) with a cutoff frequency (−3 db point) adjusted such that the average baseline deviation was 5–7 times less than the unit amplitude of the main state single-channel current. After filtering, the data were digitized on an IBM-AST personal computer using the software pClamp-fetchex, at a 5–10-kHz sampling frequency, which is at least 5 times the cutoff frequency of the low-pass filter. The data were then transferred to a Convex Computer (Material Science Center, Cornell University) and analyzed using an automated analysis program developed by Sachs, F., et al. (1982) and modified by Udgaonkar (1986). Open channels characterized by different current amplitudes were observed, in agreement with previous investigations of the mouse spinal cord GABA receptor (Hamill et al., 1983; Bormann et al., 1987; Twymann et al., 1990). Only the GABA-activated channel that showed the highest current amplitude could be analyzed, and the fraction of the time the receptor is in the open-channel state (P_o) was determined as has been described in detail (Colquhoun & Hawkes, 1982; Neher, 1983). A burst of channel activity was accepted for analysis only if it had no overlapping open states and if it was separated from the next burst by at least 3 times the mean intraburst closed time period (Neher, 1983). Under the experimental conditions described in this paper, a period during which no channel activity was observed had to be 60 ms or longer to qualify as an interburst interval. In order to compare whole-cell and single-channel current-recording measurements, we subtracted the E_{Cl}, the equilibrium potential (−36 ± 4 mV), from the transmembrane voltage in single-channel current measurements.

Results

Figure 1A shows the results of a cell-flow experiment in which a mouse cerebral cortical cell was suspended in a 250 μM GABA solution streaming from the flow device and the resulting whole-cell current was measured. The current (solid line) reaches a maximum value within 70 ms (Figure 1A); the

time-dependent decay of the current, considered to be due to receptor desensitization, is shown in two different time regions, less than 1 s and in the 1–10 s range. About 3% of the current does not return to the baseline during the period of the measurement; this current probably reflects the channels that are still open after the receptor has desensitized. Two exponentials and a constant (amounting to 3% of the whole-cell current discussed above) are required to fit the decaying phase of the current, corresponding to the rate coefficients for receptor desensitization, α and β, of 4.1 and 0.6 s^{-1}, respectively.

Calculation of the Corrected Current Amplitude, I_A. Because some receptor molecules begin to desensitize before the current reaches a maximum amplitude, and the effect is not always negligible, the current amplitude corrected for desensitization was calculated using (Udgaonkar & Hess, 1987; Hess et al., 1987)

$$I_A = (e^{\alpha\Delta T} - 1)\sum_{i=1}^{n}(I_{obs})_{\Delta ti} + (I_{obs})_{tn} \quad (1)$$

The symbol I_A is the current amplitude corrected for receptor desensitization where I represents the current and A refers to the active, nondesensitized receptor form (mechanism I); $(I_{obs})_{\Delta ti}$ is the observed current during the ith time interval and $(I_{obs})_{tn}$ the observed current during a time interval equal to or greater than the time to reach the maximum current; α is the rate coefficient for receptor desensitization, and Δt is the sampling time interval, which was 5 ms in these experiments.

The major correction of the current is associated with the rapidly desensitizing receptor form. Prior to correcting this current using eq 1, two procedures are followed: (i) the current that appears not to decay during the period of measurement (between 0 and 7% of the observed current in different experiments) is subtracted from the total current, and (ii) the current corrected for desensitization of the slowly desensitizing receptor form, $I_{A\beta}$, is determined. To obtain the value of $I_{A\beta}$, where β refers to the rate coefficient for receptor desensitization associated with this form, the decaying phase of the current due to this receptor form is extrapolated to zero time. The procedure is valid because this receptor form desensitizes to the extent of less than 5% during the rising phase of the current. The value of $I_{A\beta}$ is then divided by the sampling time interval used in recording the rising phase of the current, and this current value is subtracted from the observed current during the sampling time interval. Equation 1 is then used to calculate $I_{A\alpha}$, where α refers to the rate coefficient for receptor desensitization associated with the rapidly desensitizing receptor form.

The dashed and dashed/dotted current versus time curves represent the observed current associated with the two receptor forms present in cerebral cortical cells, A_α and A_β. The dots parallel to the time axis represent the current corrected for receptor desensitization. The percentage of the whole-cell current associated with $I_{A\alpha}$ and $I_{A\beta}$ in the experiment in Figure 1A is 60% and 40%, respectively.

Evaluating the Constants That Allow One To Determine the Concentration of Open Receptor–Channels as a Function of GABA Concentration. The relationship between I_A, the current corrected for receptor desensitization, which is a measure of the concentration of open receptor–channels, and the constants pertaining to the mechanism has been derived in terms of the mechanism (mechanism I) for the acetylcholine receptor from the electric organ of *E. electricus* (Cash & Hess, 1980; Hess et al., 1983) and modified to apply to cell-flow measurements (Udgaonkar & Hess, 1987). When the initial concentration of the activating ligand is much larger than the moles of receptor molecules in the membrane,

$$I_A = I_M R_M L^2[L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi]^{-1} = I_M R_M(\overline{AL_2})_o \quad (2a)$$

$(\overline{AL_2})_o$ is the fraction of receptor molecules in the open-channel form, L represents the molar concentration of ligand, i.e., GABA, I_M is the current due to 1 mol of open receptor–channels, R_M represents the moles of receptors in the cell membrane, K_1 is the dissociation constant of the receptor site controlling channel opening, and Φ^{-1} is the channel-opening equilibrium constant. All four constants in eq 2a can be evaluated. Equation 2a can be written in linear form (Cash & Hess, 1980):

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

A nonlinear least-squares computer program was used to evaluate K_1, Φ, and $I_M R_M$ using eq 2b. $I_M R_M$ corresponds to the whole-cell current that would be observed if all the receptor–channels opened in the presence of a saturating concentration of activating ligand. Use of the single-channel current-recording technique (Neher & Sakmann, 1976) allows one to determine the current that passes through a single open receptor–channel (Figure 5B). The single-channel current-recording technique also allows one to determine P_o, the conditional probability that the channel is open while the receptor is in a nondesensitized state (Sakmann et al., 1980). P_o is determined by measuring the fraction of time the channel is open during a burst of channel activity (Figure 5A). Thus, the value of P_o is given by $(\overline{AL_2})_o$ (Udgaonkar & Hess, 1987), and eq 2a becomes

$$I_A = I_M R_M(\overline{AL_2})_o = I_M R_M P_o \quad (2c)$$

Therefore, $I_M R_M$ can be evaluated by two independent techniques.

Evaluation of the Rate Coefficient for Receptor Desensitization, α. The relationship between ligand concentration and the rate coefficient for receptor desensitization based on mechanism I has been derived (Aoshima et al., 1981), with the following assumptions. The concentration of ligand L, is much larger than R_M, and the reaction leading to the open-channel form, $\overline{AL_2}$, is rapid compared to the formation of inactive, desensitized receptor species, IL and IL_2 (mechanism I):

$$\alpha = \frac{Lk_{43} + 2k_{21}k_3}{L + 2K_2} + \frac{(L^2k_{34} + 2k_{12}K_1)\Phi}{L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi} \quad (3a)$$

When the rate constant k_{34} dominates the measurement, eq 3a can be simplified (Udgaonkar & Hess, 1987)

$$\alpha = \frac{k_{34}\Phi L^2}{L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi} \quad (3b)$$

Figure 1B shows a representative cell-flow experiment in which 500 μM GABA (upper line) or 500 μM GABA and 5 μM picrotoxin (lower line) flowed over a mouse cortical cell suspended in solution and the resulting whole-cell current was measured. The experiment shows that the time resolution of the cell-flow technique is sufficient to resolve the individual sequential steps of the reaction. The rising phase of the current in the 5–10-ms time region is shown in part a of Figure 1B, and the decaying phase of the current, occurring in two different time regions, is shown in parts b and c. The rising phase of the current reflects mainly the equilibration of the receptors on the cell surface with GABA, but the decaying phases of the current give information about the concentration of the two receptor forms present in the membrane and the rate

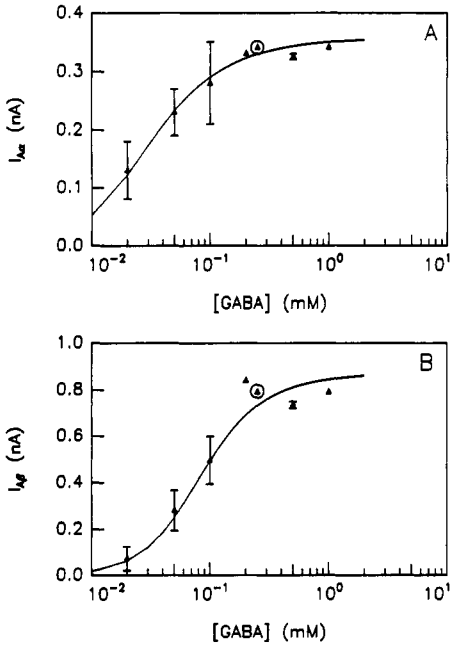


FIGURE 2: Relationship between I_A and GABA concentration at −70 mV, pH 7.2, at 21–23 °C. The data were normalized to the I_A values obtained in the presence of 250 μM GABA and are indicated by the circled solid triangles. The data were obtained from measurements made with 12 cells; 4–10 measurements with different GABA concentrations were made with each cell. The relative error in measurements made with the same cell was ±10%. The parameters used for computing the solid line ($I_M R_M$, K_1, Φ) are listed in Table I and were computed as described under Results. (A) $I_{A\alpha}$ versus GABA concentration. (B) $I_{A\beta}$ versus GABA concentration. The error bars represent plus or minus standard deviations.

coefficients for the inactivation (desensitization) of the two forms induced by GABA. About 55% of the receptors present in the membrane are associated with a desensitization rate coefficient α of 4.4 s^{-1} and 45% with a rate coefficient β of 0.6 s^{-1}. When 5 μM picrotoxin, a well-characterized, specific inhibitor of the GABA receptor (Elliot & Florey, 1956; Alger & Nicoll, 1980; Allen et al., 1985; Levitan et al., 1988a; Engblom et al., 1989), is added to the GABA solution flowing over the cell (lower current trace), the receptor is inhibited. It should be noticed that if one employs a low time resolution in the cell-flow measurements, picrotoxin will appear to inhibit the GABA receptor completely (lower trace, panel c), in agreement with previous observations [reviewed by Ticku (1986)]. However, when one utilizes a 10-ms time resolution (lower trace, panels a and b), one notices that the current due to the rapidly desensitizing receptor form is the same, ~1 nA, in the absence and presence of 5 μM picrotoxin; but the rate coefficient for receptor desensitization has increased from 4.4 s^{-1} in the absence to 7.6 s^{-1} in the presence of picrotoxin. It can be seen from Figure 1B that the reason that the effect of picrotoxin on the rapidly desensitizing receptor form cannot be observed when a low time resolution is used is that this form is inactivated (desensitized) within 500 ms.

Figure 1C shows a cell-flow experiment in which 250 μM GABA (upper line) or 250 μM GABA, and 100 μM bicuculline (lower line), another well-known inhibitor of the GABA receptor [reviewed by Simmonds (1983)], flowed over the cell. At least at this concentration of this inhibitor, both receptor forms, characterized by desensitization rate coefficients α of 4.8 s^{-1} and β of 0.5 s^{-1} in this experiment, are completely inactivated by bicuculline.

The effect of GABA concentration on the two receptor forms present in mouse cerebral cortical cells (Figure 2) was

Table I: Constants Determining the Formation of GABA Receptor Transmembrane Channels in Embryonic Mouse Cerebral Cortical Cells, at 21–23 °C, pH 7.2, −70 mV

	A_α, rapidly desensitizing receptor form		A_β, slowly desensitizing receptor form, chemical kinetics[a]
	chemical kinetics[a]	single-channel current	
K_1 (μM) dissociation constant of site controlling channel opening	40		320 (400)[b]
$(1 + \Phi)^{-1}$ maximal fraction of open receptor–channels at saturating GABA concentration	0.75		0.95 (0.95)[b]
$I_M R_M$ (nA)	0.46	0.50	0.92
observed maximal current amplitude (nA)	0.34		0.79
desensitization coefficient (s^{-1})			
(i) at saturating GABA concentrations	4.4		0.7
(ii) at 100 μM GABA	2.1	2.6	0.4
average number of GABA receptors per cell		330	

[a] Average error in constants was ±50%. [b] Determined from the effect of a 50-fold range of GABA concentration on the rate coefficient β; k_{34} (mechanism I, eq 3b) = 20 s^{-1}.

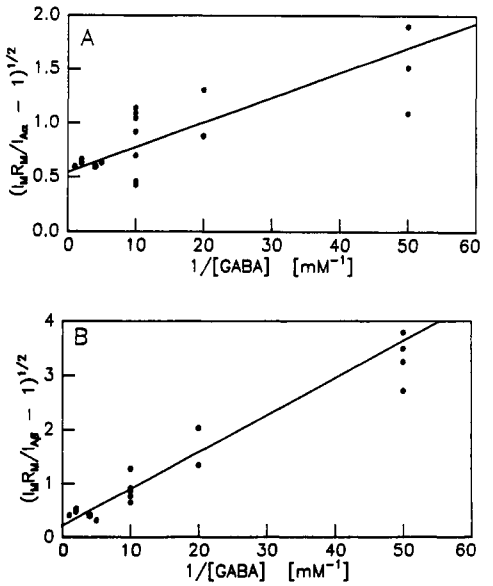


FIGURE 3: Linear fits of I_A data. The data in Figure 2 were replotted according to eq 2b. The values of $I_M R_M$ for receptor forms A_α and A_β were 0.46 and 0.92 nA, respectively, and were obtained by use of a nonlinear least-squares program and eq 2b. The values of K_1 and Φ are listed in Table I. Twelve cells were used, and 30 measurements of $I_{A\alpha}$ and $I_{A\beta}$, respectively, were made. Each data point represents measurements made with one or more cells. (A) A plot of the data for receptor form A_α. (B) A plot of the data for receptor form A_β.

analyzed in order to evaluate the parameters that determine the formation of transmembrane channels. The values of $I_{A\alpha}$ (Figure 2A) and $I_{A\beta}$ (Figure 2B) were determined as a function of a 50-fold range of GABA concentration (20 μM to 1 mM) from experiments such as the one illustrated in Figure 1A. Because the concentration of receptors, as measured by the whole-cell current, differs considerably from cell to cell, all I_A values were normalized to the value obtained at 250 μM GABA. The values of $I_{A\alpha}$ and $I_{A\beta}$ at this GABA concentration were 0.34 and 0.79 nA, respectively. The coordinates for the solid lines in Figure 2, K_1, Φ, and $I_M R_M$, listed in Table I, were

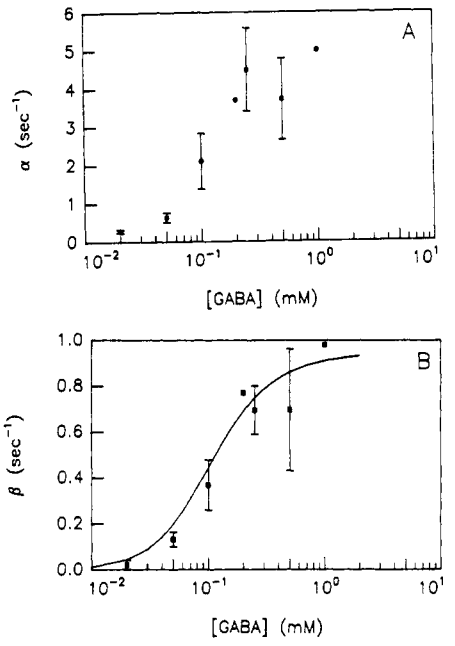


FIGURE 4: Relationship between the rate coefficients for receptor desensitization and GABA concentration. The same cells and numbers of measurements as were used to evaluate $I_{A\alpha}$ and $I_{A\beta}$, respectively (Figure 2), were used to evaluate the rate coefficients for receptor desensitization. The error bars represent plus or minus standard deviations from the mean. (A) The values of α as a function of GABA concentration. (B) The values of β as a function of GABA concentration. The coordinates of the solid line are $k_{34} = 20\ s^{-1}$, $K_1 = 400$ μM, and $\Phi = 0.05$.

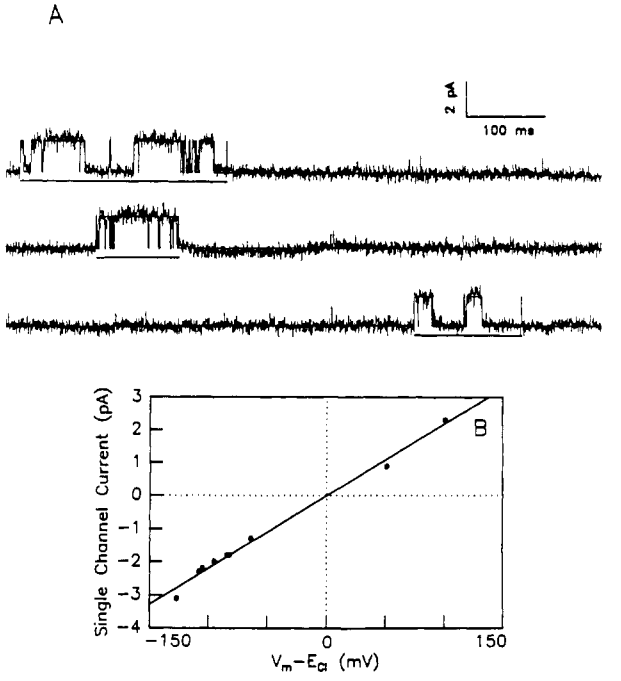


FIGURE 5: Single-channel current recordings. (A) An example of single-channel currents recorded in the presence of 100 μM GABA at a transmembrane potential $V_m - E_{Cl}$ of −84 mV ($E_{Cl} = -36 \pm 4$ mV), pH 7.2, at 21–23 °C. Three bursts of channel activity separated by two long silent periods are shown; a line has been drawn under each burst of channel activity to denote the duration of the burst, and idealized data have been superimposed on the channel openings for identification purposes. A total of 154 bursts of channel activity were measured using five cells. The mean length of the burst was 378 ± 226 ms. $\alpha = 2.6 \pm 1.5\ s^{-1}$; $P_o = 0.56 \pm 0.26$. (B) The current passing through a single receptor–channel as a function of transmembrane voltage. A total of 15 684 channel-opening events were measured using nine cells. The slope of the line gives the conductance, γ, of the single channel; $\gamma = 22 \pm 1$ pS. On the abscissa the reversal potential, E_{Cl}, has been subtracted from the V_m values.

determined from plots of the $I_{A\alpha}$ and $I_{A\beta}$ values according to eq 2b (Figure 3A,B), which is a linear transformation of eq 2a.

Figure 4A,B shows the relationship between the rate coefficients for receptor desensitization and GABA concentration for the rapidly and slowly desensitizing receptor forms, respectively. It can be seen from eq 3a that the relationship between the rate coefficient for receptor desensitization and ligand concentration is complex. When the rate constant k_{34} for the conversion of AL_2 to IL_2 (mechanism I) dominates the desensitization reaction, eq 3a can be simplified to give eq 3b (Udgaonkar & Hess, 1987). Equation 3b gives the relationship between GABA concentration and the rate coefficient in terms of only three constants, k_{34}, K_1, and Φ. The desensitization rate coefficient β (Figure 4B) but not α (Figure 4A) follows the simple relationship given by eq 3b. The values of K_1 and Φ that account for the effect of GABA concentration on $I_{A\beta}$ (Figure 2B) (320 μM and 0.95) are in reasonable agreement with the values of K_1 and Φ that account for the effect of GABA concentration on the desensitization rate coefficient β (Figure 4B) (400 μM and 0.95). The coordinates of the solid line in Figure 4B, k_{34}, K_1, and Φ, are listed in Table I.

The measurement of I_A, and the interpretation of the dependence of I_A on GABA concentration, can be compared to results obtained by an entirely different approach and methodology (Udgaonkar & Hess, 1987; Matsubara & Hess, 1992), namely, measurements of single-channel currents (Neher & Sakmann, 1976). These allow one to determine the conditional probability, P_o, that the channel is open while the receptor is in a nondesensitized state (Sakmann et al., 1980). An example of such measurements made with 100 μM GABA is shown in Figure 5A, where bursts of channel activity are underlined. The bursts are separated by silent periods during which receptor–channels do not open. Determining when these bursts of channel activity begin and end is often difficult and is only possible under certain experimental conditions (Sakmann et al., 1980). In the experiments with GABA and cerebral cortical cells, we observed two types of receptor channels characterized by different conductances; one had a conductance of 22 pA/volt [or 22 picoSiemens (pS)] (Figure 5B), and the other type of channel had a lower conductance. We were able to observe definable bursts of channel activity only with the 22-pS channel. By determining the conductance of a channel only within definable bursts of channel activity, we can determine whether a particular conductance is associated with receptor form A_α or A_β (see below). The conductance of open receptor channels was, therefore, determined only within the definable bursts of channel activity (Figure 5A) at a GABA concentration of 100 μM and a range of transmembrane voltages ($V_m - E_{Cl}$) from −82 to −108 mV. E_{Cl} represents the equilibrium potential of chloride ions of the cell and has a value of −36 mV under the conditions of the experiment (Figure 5B). The mean lifetime of a burst of channel activity (378 ms) corresponds to the mean lifetime of the nondesensitized state and is a measure of the rate coefficient for receptor desensitization (Sakmann et al., 1980). From these measurements, we calculate a value for the desensitization rate coefficient of 2.6 s^{-1}, which appears to be independent of transmembrane voltage in the range measured. In cell-flow measurements at 100 μM GABA, α and β have values of 2.1 and 0.4 s^{-1}, respectively. Thus, the observed burst of channel activity is identified as being due to the rapidly desensitizing receptor form, A_α. The value for P_o of 0.56 also appeared to be independent of the transmembrane voltage at

which the measurements were made. The value of P_o can also be obtained from cell-flow measurements. At 100 μM GABA, $I_{A\alpha}$ has a value of 0.28 nA; using this value, and the value of $I_M R_M$ of 0.46 nA obtained from cell-flow measurements, a P_o value of 0.61 can be calculated by use of eq 2c. Hence, both the burst length and the values of P_o indicate that the bursts of channel activity are associated with receptor form $I_{A\alpha}$. Therefore, under conditions in which comparison was possible the two independent techniques, which involve different assumptions, give similar results.

$I_M R_M$ corresponds to the whole-cell current that would be observed if all the receptor–channels opened in the presence of a saturating concentration of activating ligand. The number of receptor–channels present in the cell membrane can, therefore, be determined by dividing $I_M R_M$ by the current that passes through a single open receptor–channel. This value can be obtained by the single-channel current-recording technique. In Figure 5B is a graph of $(V_m - E_{Cl})$ as a function of the current passing through the channel identified as the receptor form that desensitizes rapidly, where $(I_{obs})_{sc} = \gamma(V_M - E_{Cl})$ and represents the current passing through a single channel; γ represents the single-channel conductance. Each $(I_{obs})_{sc}$ value on the graph (Figure 5B) is obtained from a histogram of the single-channel current amplitudes at the transmembrane voltage given on the abscissa of Figure 5B. A mean current amplitude of 1.5 pA for a single channel of the rapidly desensitizing receptor form observed in the cell-flow experiments is calculated from the conductance of the channel (Figure 5B). From the value for $I_M R_M$ of ~500 pA one calculates a mean value of ~330 rapidly desensitizing GABA receptors in an average mouse cerebral cortical cell.

A summary of the results obtained with the GABA receptor in single cerebral cortical cells of the embryonic mouse, using the cell-flow technique and the single-channel recording technique, is given in Table I.

Discussion

A chemical kinetic technique with a 10-ms time resolution allowed us to evaluate the constants that account for the concentration of open GABA receptor–channels in single cells from the cerebral cortex of an embryonic mouse over a 50-fold range of GABA concentration (Figure 2). To improve the precision of the measurements in cell-flow experiments, we have adopted two strategies that allow us to make measurements using different neurotransmitter concentrations with each cell: (1) We have developed techniques that allow us to obtain nearly spherical cells without processes that can be suspended in the stream of solution emerging from the cell-flow device. This has succeeded with BC_3H1 muscle cells (Udgaonkar & Hess, 1987) and PC12 cells (Matsubara & Hess, 1992) that contain the muscle and neuronal type of nicotinic acetylcholine receptor, respectively, with a mouse spinal cord cell containing glycine receptors (Walstrom and Hess, unpublished), NIE-115 cells containing the type 3 serotonin receptor (Geetha and Hess, unpublished), and the cerebral cortical cells used here (see Experimental Procedures), which contain glutamate receptors in addition to the GABA receptors. (2) We use moderate flow rates (4–5 cm s^{-1}) in the cell-flow experiments and correct the observed current for receptor desensitization that occurs while the receptors equilibrate with ligand, using eq 1. Moderate flow rates are used because the seal between the recording electrode and the cell membrane tends to break as the force exerted on the connection between the recording electrode and the cell membrane is increased by solutions flowing at a rate greater than 5 cm s^{-1}. The criteria used to show that this correction is valid include (i) use of the single-channel current recording technique (Neher & Sakmann, 1976) that allows one to measure P_o, under certain conditions (Sakmann et al., 1980). The value of P_o, which is equal to $(\overline{AL_2})_o$ (eq 2c), can also be obtained from cell-flow experiments by determining the effect of neurotransmitter concentration on I_A (eq 2a); and (ii) determination of the value of I_A at high flow rates, when the rise time is less than 10 ms (Figure 1B) and no, or only minor, correction for desensitization is required, but only a few measurements can be made with each cell. The I_A value obtained when the current rise time is <10 ms can then be compared to the I_A value obtained at low flow rates, when the current rise time is slower (100 ms or more) and considerable correction for receptor desensitization is required. Good agreement between the values of P_o and $(\overline{AL_2})_o$ and the corrected current amplitudes obtained with rapid and slow current rise times is obtained in this and previous experiments (Udgaonkar & Hess, 1987; Matsubara & Hess, 1992).

An important consideration in interpretation of the cell-flow measurements in Figure 1 is whether the rapid phase of the decaying part of the current represents the conversion of an active receptor form A_α to a desensitized form A_β. If it does, the concentration of receptors in the membrane is given by the maximal observed whole-cell current corrected for the rate of rapid receptor desensitization characterized by the rate coefficient α, and correction of the whole-cell current for the slowly desensitizing receptor form is negligible. The time for the current to reach its maximal value and for the receptors to equilibrate with GABA is 70 ms or less in our experiments. At a saturating concentration of GABA, less than 5% of the slowly desensitizing receptor form desensitizes during the 70-ms current rise time. However, if the two receptor forms are not interconvertible, the observed cell current is treated differently; rather than correcting the maximal observed whole-cell current for receptor desensitization, the current due to the slowly desensitizing receptor form is subtracted from the observed cell current before correcting the current for rapid receptor desensitization characterized by the rate coefficient α. The corrected currents, $I_{A\alpha}$ and $I_{A\beta}$, are then proportional to the concentrations of receptor forms A_α and A_β, respectively, if they both have the same conductance. Two types of GABA receptor forms have been reported to exist in a variety of organs and animals (Guidotti et al., 1979; Sakmann et al., 1983b; Maksay & Ticku, 1984; Akaike et al., 1986; Cash & Subbarao, 1987a,b, 1988; Agey & Dunn, 1989). Cash and Subbarao (1987a,b) concluded from their measurements that the two receptor forms they identified, on the basis of kinetic measurements made with membrane vesicles prepared from rat brain, are not interconvertible on the time scale of their measurements. The following results indicate that the GABA receptors in single cerebral cortical cells of the mouse embryo do not arise in a consecutive reaction in which a rapidly desensitizing receptor form, A_α, characterized by the rate coefficient α, is converted to a slowly desensitizing receptor form, A_β, characterized by the rate coefficient β. This reaction scheme would require that the concentration of the slowly desensitizing receptor form (A_β in this case) can be equal to the concentration of the rapidly desensitizing form A_α but not larger. As determined by the current amplitudes $I_{A\alpha}$ and $I_{A\beta}$, the concentration of A_α is twice that of A_β when the GABA concentration is 20 μM, but at 1 mM GABA concentration the concentration of A_β is twice that of A_α (Figure 2). The conversion of A_α to A_β is not excluded if the conductance of the open receptor–channel is much larger for A_β than it is for A_α. However, in the single-channel current traces, we did not

observe a GABA-activated channel with a conductance greater than 22 pS characteristic of receptor form A_α, but we did observe a channel with a lower conductance, presumably due to receptor form A_β. Since we could not find experimental conditions in which the beginning and ending of bursts of channel activity with the lower conductance could be established, the identification of this lower-conductance channel with receptor form A_β is indirect. We, therefore, used another approach to demonstrate that receptor form A_α is not a precursor of A_β. The concentration of A_α varies from cell to cell. If A_α is a precursor of A_β, then at a given GABA concentration the ratio of A_α to A_β is expected to be constant in all cells investigated even though the concentration of A_α varies widely. In experiments with 31 cells at a GABA concentration of 250 μM, the ratio of $I_{A\alpha}$ to $I_{A\beta}$ varied as much as 20-fold. To assess the experimental error of such measurements, we used seven cells to determine the ratio of $I_{A\alpha}$ at 250 μM and 100 μM GABA and repeated the experiment with $I_{A\beta}$. These ratios measured in the same cell are expected to be constant and independent of the concentration of receptors in the different cells used. A constant ratio with an experimental error of $\pm$24% was obtained.

For the reasons given above, we treated the whole-cell current in flow measurements made with cerebral cortical cells as arising from two different, not interconvertible, receptor forms. The constants listed in Table I (K_1, Φ, $I_M R_M$), which determine the concentration of open receptor–channels, were evaluated by first correcting the current arising from receptor forms A_α and A_β for receptor desensitization, characterized by rate coefficients α and β, respectively, and then measuring the effect of a 50-fold range of GABA concentration on $I_{A\alpha}$ and $I_{A\beta}$ separately. The evaluation of the constants that determine the concentration of open channels of the rapidly desensitizing receptor form obtained using the cell-flow technique was compared to results obtained by an entirely different method, single-channel current recordings (Neher & Sakmann, 1976), and good agreement was obtained (Table I). With the cells used, this comparison has so far been possible only at 100 μM GABA because only at this concentration were we able to clearly determine the beginning and ending of a burst of channel activity of one of the two types of channel detected in the membrane (Figure 5A). The other channel of lower conductance could not be analyzed. The length of the burst of channel activity, considered to be a measure of the rate coefficient for receptor desensitization, identified the open channels within the burst that have a conductance of 22 pS to be characteristic of receptor form A_α. The two methods, the cell-flow and single-channel current-recording techniques, involve entirely different assumptions. The rate coefficients for receptor desensitization are measured by the decaying phase of the current (Figure 1) in the cell-flow technique and by the lifetime of the burst of channel activity in the single-channel current recordings. The fraction of receptors in the open-channel form, $\overline{(AL_2)}_o$, is determined from measurements of the whole-cell current corrected for receptor desensitization over a large range of GABA concentration in the cell-flow technique (eq 2a) and from measurements of the fraction of time the channel is open within a burst of channel activity (P_o) in the single-channel current records. The agreement between the values obtained by these different techniques [at 100 μM GABA, P_o is 0.56, and $\overline{(AL_2)}_o$ is 0.61] suggests that the assumption made in evaluating the constants that determine the concentration of open receptor–channels at a given concentration of GABA are valid and that in the cell-flow technique we are measuring the properties of an active channel-forming receptor form responsible for controlling signal transmission between cells prior to desensitization.

Although in the single-channel current traces we observed GABA-activated channels of a lower conductance than those identified with receptor form A_α, presumably due to receptor form A_β, we were not able to differentiate between periods of channel activity and silent periods associated with these channels. We were, however, able to evaluate the constants K_1 and Φ associated with the receptor form A_β by an independent measurement. Namely, we determined the effect of GABA concentration on the desensitization rate coefficient β characteristic of receptor form A_β, assuming that k_{34} (mechanism I) is the dominant rate constant for receptor desensitization and eq 3b can be used. Evaluation of these constants from the decaying phase of the current does not depend on correcting the current for desensitization that may occur while the ligand equilibrates with the receptors on the cell surface and, therefore, represents another independent evaluation of the constants pertaining to the mechanism. The solid line in Figure 4B was computed using the value for K_1 of 0.40 mM and for Φ of 0.05 (Table I).

We were unable to account for the effect of GABA concentration on α using the simplifying assumption that k_{34} (mechanism I) dominates the desensitization rate of receptor A_α and that eq 3b applies. The more general equation relating GABA concentration to receptor desensitization (eq 3a) requires determination of K_2 (mechanism I) and the rate constants for the recovery of the receptor from desensitization. These determinations can be made providing the receptors on the cell surface can be sequentially equilibrated in the millisecond time region with solutions containing different concentrations of ligand (Aoshima et al., 1981). Unlike the quench-flow technique, the cell-flow method is not yet suitable for such sequential mixing events.

Although the investigations of the minimum reaction scheme of the GABA receptor are in their initial stages, some comparison of results obtained with this receptor and with the nicotinic acetylcholine receptor are of interest. On the basis of cDNA sequences, the two types of receptors are structurally related (Betz, 1990; Stroud et al., 1990) although they have opposing functions in the signal transmission process at the junction between cells. The acetylcholine receptor has a cation-specific transmembrane channel and initiates signal transmission; the GABA receptor has an anion-specific transmembrane channel and counteracts the action of excitatory receptors such as the acetylcholine receptor. Both receptors require two ligand molecules to be bound before the channel opens, and the dissociation constants of the specific neurotransmitters for their respective receptor sites controlling channel opening are all in the micromolar range, between 42 and 320 μM for the GABA receptor in the cerebral cortex of the mouse embryo, between 142 and 169 μM for the GABA receptor in rat brain vesicles (Cash & Subbarao, 1987a,c), and 80 μM for the *E. electricus* acetylcholine receptor (Hess et al., 1983). It has been generally assumed that physiologically active compounds are invariably associated with high-affinity binding constants. The dissociation constant of the receptor site controlling channel opening is given by the dissociation rate of the neurotransmitter from this site divided by the bimolecular rate constant for formation of the neurotransmitter:receptor complex. When the time interval between receptor-controlled sequential transmission of signals between cells is in the millisecond time region, the rate of dissociation of ligand from the site controlling channel opening must also be in the millisecond time region. The requirement to have

dissociation rate constants of this magnitude, and the typical bimolecular rate constants for small molecule–protein interactions, 10^7–10^8 M^{-1} s^{-1} (Hammes, 1978), places limits on the affinity of neurotransmitters for the receptor sites controlling channel opening and closing.

The constant Φ^{-1} characterizes the equilibrium between closed- and open-channel forms of the receptor. It was introduced (Cash & Hess, 1980) to account for the cooperativity observed in the effect of carbamoylcholine concentration on the *E. electricus* receptor-controlled flux of inorganic ions into membrane vesicles and to account for the observation that the maximal receptor-controlled ion flux rates, which are expected to depend on the number and conductance of open receptor–channels (Hess et al., 1984), depended on the type of activating ligand used (Hess et al., 1983), even though the number of inorganic ions that pass through the open receptor–channel is reported to be independent of the activating ligand used (Neher & Steinbach, 1978; Gardner et al., 1984). The constant Φ^{-1} is also required to account for the effect of GABA concentration on the concentration of open transmembrane channels as measured by $I_{A\alpha}$ and $I_{A\beta}$ (eq 2a). It can be seen from the constants in Table I that at saturating concentrations of GABA only 75% of the transmembrane channels of receptor form A_α will be open but 95% of receptor form A_β. The value of Φ^{-1} we obtained is considerably higher than that obtained with the *E. electricus* nicotinic acetylcholine receptor (Cash & Hess, 1980) but falls within the range of Φ^{-1} values for the mammalian muscle (Udgaonkar & Hess, 1987) and neuronal (Matsubara & Hess, 1992) acetylcholine receptors and the rat brain GABA receptor (Cash & Subbarao, 1987a,b).

One form of the GABA receptor in cerebral cortex cells desensitizes in the millisecond time region (maximal value of $\alpha = 4.4$ s^{-1}), as do GABA receptor forms in other cells and animals (Bormann & Clapham, 1985; Akaike et al., 1986; Huguenard & Alger, 1986; Cash & Subbarao, 1987a,b; Bormann & Kettenmann, 1988). The physiological meaning of receptor desensitization is not known (Hess et al., 1983; Changeux, 1989; Ochoa et al., 1989) although slow receptor desensitization (second to minute time region) has been observed for many years (Katz & Thesleff, 1957). Rapid (millisecond) receptor desensitization, however, first discovered in tracer ion flux experiments with acetylcholine receptor-containing vesicles (Hess et al., 1978) prepared from the electric organ of *E. electricus* (Kasai & Changeux, 1971), led to the realization that the constants controlling opening of the receptor–channel form that is primarily responsible for the formation of transmembrane channels and signal transmission cannot be determined when methods with a time resolution that is slow compared to receptor desensitization are used. This in turn led to the development of fast reaction techniques with a 5-ms time resolution suitable for measuring receptor-controlled flux in membrane vesicles prior to receptor desensitization (Hess et al., 1979, 1983). These techniques resulted in the determination of the constants K_1 and Φ (mechanism I) that determine the receptor-controlled flux rates and, therefore, the concentration of open receptor–channels, over a 5000-fold concentration range of acetylcholine and of the rate constants responsible for receptor desensitization in the millisecond time region (Hess et al., 1983). The results obtained also indicated that, in investigations of receptor-controlled processes with a time resolution that is slow compared to receptor desensitization, the properties of inactive receptor forms dominate the measurements (Hess et al., 1983).

The importance of good time resolution in investigations of the GABA receptor is illustrated by the experiments in Figure 1. For instance, in the experiments shown in Figure 1B it can be seen that when the time resolution of the kinetic measurements is less than 500 ms (panel c), one observes only a single receptor form that desensitizes with a $t_{1/2}$ value of about 1 s. In agreement with published experiments, one would observe that 5 μM picrotoxin inhibits the GABA receptor completely (Elliot & Florey, 1956; Alger & Nicoll, 1980; Allan et al., 1985; Levitan et al., 1988a; Engblom et al., 1989). An entirely different result is observed in the 10-ms time resolution experiment. The receptor form responsible for the major component of the whole-cell current in Figure 1B is resistant to 5 μM picrotoxin.

Many important questions regarding the mechanism of the GABA receptor remain, in addition to the desensitization mechanism. For instance, what is the mechanism by which compounds (for instance benzodiazepines) increase the concentration of open receptor–channels in the membrane at a given concentration of GABA? Do these compounds change the value of Φ^{-1} or the dissociation constant for GABA? What is the mechanism of action of compounds, for instance barbiturates, that increase the concentration of open receptor–channels at low concentrations but act as inhibitors at high concentrations? Are the binding constants, or the value of Φ, dependent on the transmembrane voltage, thus making it possible to change the concentration of open receptor–channels at a constant concentration of GABA and, thereby, regulate the signal transmission process? Evidence for regulation of the *E. electricus* acetylcholine receptor by changes in the transmembrane voltage of the cell membrane exists (Shiono et al., 1984; Takeyasu et al., 1986). What additional information can be obtained when one compares chemical kinetic measurements with single-channel current recordings? Single-channel current records have revealed the existence of GABA receptors in a variety of animals, cells, and organs, with different lifetimes of the open channel and different channel conductances (Hamill et al., 1983; Sakmann et al., 1983b; Bromann & Klettenman, 1988; MacDonald et al., 1989; Twymann et al., 1990). What is the relation between these measurements and the constants that determine the concentration of open receptor–channels and the rates for interconversion between active and desensitized receptor forms? The constants determined in cell-flow experiments and the experiment shown in Figure 5A made it possible to relate receptor–channels observed in single-channel records with a certain conductance and/or open-channel lifetime (not measured in the experiments reported) to the rapidly desensitizing receptor form in cerebral cortical cells detected by the cell-flow method. Therefore, one can now relate the constants that determine the concentration of the open receptor–channel over a large range of GABA concentration to the properties of the open channel of one form of the GABA receptor in the cerebral cortical cells of an embryonic mouse. Since the measurements described can be made with single cells from different parts of the nervous system, during various stages of development, the techniques described can also be used to relate mechanism to subunit composition of the receptor and to changes in receptor function during development and in nervous system dysfunction.

Rapid receptor desensitization appears to be a characteristic of the superfamily of proteins that regulate transmission of signals at cell junctions in the nervous system. The importance of good time resolution in kinetic investigations of rapidly interconverting protein forms with different properties is well documented in investigations of protein-mediated reactions in solution (Eigen, 1967; Hammes, 1982; Fersht, 1985) and in

membrane vesicles (Udgaonkar & Hess, 1986; Cash & Subbarao, 1987a,b; Ochoa et al., 1989; Changeux, 1989). However, the use of membrane vesicles in kinetic investigations is restricted not only by the availability of receptor-rich tissues but also by the conductance (a measure of the number of inorganic ions passing through the channel per unit time) of the channel and the value of Φ. In order to observe the active, nondesensitized receptor form in experiments with membrane vesicles, the ion translocation rate must be comparable to the rate of receptor desensitization. The rate of ion flux in membrane vesicles depends on both the number of receptors per internal vesicle volume and the conductance of the channel (Hess et al., 1984). For instance, the density of electric organ *E. electricus* acetylcholine receptor per unit membrane area (Hess et al., 1981) and its conductance (Hess et al., 1984) are about 15 times and 2.5 times greater than the density and conductance respectively of the GABA receptors in mouse cerebral cortical cells. Therefore, even if one could obtain these cells in gram quantities, the rate of flux of inorganic ions into vesicles made from the cell membrane may be too slow for investigations of the rapidly desensitizing GABA receptor form A_α in cerebral cortical cells. The experiments presented indicate that the cell-flow technique has the required time resolution and allows kinetic investigations to be made in which the reaction steps can be separated along the time axis (Figure 1), analytical expressions can be used in evaluation of the constants from the measurements, the concentration of the reactants can be varied over a wide range, and the precision of the measurements is comparable to that obtained in flow measurements with receptor-containing membrane vesicles. The results obtained so far suggest, therefore, that the chemical kinetic approach, using rapid reaction techniques, may be useful in investigations of receptor mechanism in single cells from specific parts of the nervous system.

ACKNOWLEDGMENTS

We are grateful to Dr. Linda Nowak for helpful suggestions regarding the cell culture, Katherine Walstrom for all the encouragement and help with the cell culture, Dr. Robert Oswald, Dr. Glen Millhauser, Alison Carter, and Raymond Wieboldt for the linear prediction program, and Lisa Miller for typing the manuscript.

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