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Properties of $GABA_A$ Receptors Underlying Inhibitory Synaptic Currents in Neocortical Pyramidal Neurons

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Rapid applications of GABA (from 10 μM to 10 mM) to outside-out patches were used to study the role that the kinetic properties of $GABA_A$ receptors play in determining the time course of IPSCs in neocortical pyramidal neurons. Currents induced by rapid applications of brief (1 msec) pulses of GABA (1 mM) showed a biexponential decay phase that seems to involve the entry of $GABA_A$ receptors into desensitized states. This conclusion is based on the similar fast decay kinetics of the response to brief and prolonged pulses of GABA and on the correlation between the degree of paired-pulse depression and the decay rate of the currents induced by brief pulses.

Under nonequilibrium conditions we found that the concentration–response curve of pyramidal $GABA_A$ receptors has an EC_{50} of 185 μM (GABA pulse of 1 msec). The decay time course of the patch currents in response to brief applications of GABA was insensitive to agonist concentrations at the range from 50 μM to 10 mM. Faster decay rates were observed only in response to pulses of 10 μM GABA. These data are compatible with the suggestion that briefer openings derive from a monoliganded state and that these are negligible when receptor activation is >2%. Assuming that GABA transients at neocortical synapses are fast, a several millimolar GABA concentration would be needed to saturate the postsynaptic $GABA_A$ receptors.

Key words: cerebral cortex; inhibitory synapse; GABA; $GABA_A$ receptor; desensitization; saturation; inhibition; rapid agonist application; pyramidal neuron; synaptic transmission; miniature IPSC

GABA is thought to mediate most fast inhibitory synaptic currents in the mammalian cerebral cortex through the activation of postsynaptic $GABA_A$ receptor channels. GABA-mediated IPSCs play a crucial role in neuronal processing, and changes in their kinetics, like those generated by widely used drugs (Gage and Robertson, 1985; Harrison et al., 1987; Otis and Mody, 1992), result in important therapeutic effects.

Previous studies on the mechanisms underlying the time course of IPSCs led to the proposal that these mostly reflect the functional properties of the postsynaptic $GABA_A$ receptors rather than the clearance of GABA from the synaptic cleft (Maconochie et al., 1994; Puia et al., 1994; Jones and Westbrook, 1995; Tia et al., 1996). The $GABA_A$ receptor channel is thought to be a heteropentameric complex assembled from at least 16 potential subunits (Macdonald and Olsen, 1994; Sieghart, 1995), and different neuronal types have been shown to express distinct combinations of $GABA_A$ receptor subunits (Fritschy et al., 1992; Wisden et al., 1992; Ruano et al., 1997) (for review, see McKernan and Whiting, 1996). Because subunit composition determines $GABA_A$ receptor kinetic properties (Verdoorn et al., 1990), this structural heterogeneity could provide cell-type specificity in the kinetic characteristics of the IPSCs (Puia et al., 1994).

To investigate the factors responsible for the time course of the IPSCs that impinge on neocortical pyramidal neurons (Salin and Prince, 1996; Ruano et al., 1997), we have studied the kinetic properties of the native postsynaptic $GABA_A$ receptors expressed by these cells. To address this issue, we analyzed the responses generated by rapid application of GABA to outside-out patches excised from the soma of pyramidal cells in brain slices, and we compared these responses with the miniature IPSCs (mIPSCs).

The actual time course of GABA concentration in the synaptic cleft remains unknown, but it has been estimated to be very fast (Destexhe and Sejnowski, 1995; Clements, 1996). Assuming that brief pulses of agonist would mimic transmitter transients during synaptic transmission better than prolonged ones, we have examined the kinetic properties of pyramidal $GABA_A$ receptors in response to brief pulses of GABA. Under these conditions we have studied the possible role of receptor desensitization in shaping the decay of the IPSCs (Jones and Westbrook, 1995; Tia et al., 1996), the kinetics of the outside-out patch responses at different concentrations of GABA, and the levels of agonist required for receptor saturation.

MATERIALS AND METHODS

Cerebral slices. Parasagittal slices (300 μm thick) of the cerebral cortex were obtained from 13- to 18-d-old male Wistar rats with a vibroslicer (D.S.K. Microslicer, Ted Pella, Redding, CA). Ice-cold standard solution (see Solutions below) was used during slicing. After sectioning, the slices were placed in a holding chamber containing standard solution at 32–34°C for 30–60 min and then stored at room temperature until used. After an incubation period of at least 1 hr, one slice was transferred to a submersion-type recording chamber, where it was kept at room temperature (22–24°C). Solutions bathing the slices (≈2 ml/min) were bubbled continuously with a gas mixture of 95% O_2/5% CO_2.

Identification of the cells. Cells were identified as pyramidal neurons according to their morphological appearance, using infrared-differential interference contrast (IR-DIC) video microscopy, and their pattern of firing in response to intracellular injection of depolarizing current pulses was recorded in the current-clamp mode (McCormick et al., 1985; Connors and Gutnick, 1990; Kawaguchi, 1993). In addition, a subset of 20 pyramidal neurons was identified morphologically after being filled with

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biocytin during the whole-cell recording period (Horikawa and Armstrong, 1988). Pyramidal cells fired relatively wide action potentials (first spike half-width 1.5 ± 0.4 msec, mean ± SD, $n = 20$) that exhibited a small fast afterhyperpolarization (3.7 ± 4.0 mV) and frequency accommodation. Both large and medium-sized pyramidal neurons were included in this study.

Recording and data analysis. Patch pipettes (3–5 MΩ when filled with the intracellular solution) were made from thin-wall (1.5 mm outer diameter, 1.17 inner diameter) borosilicate glass (GC150T-7.5, Clark, Reading, UK), using a horizontal electrode puller (P-87, Sutter Instruments, Novato, CA). Whole-cell recordings from neurons located in layer V of the visual cortex were made under visual control by an upright microscope (Axioskop, Zeiss, Oberkochen, Germany) equipped with Nomarski IR-DIC optics and a water immersion lens (40×). Whole-cell recordings in current-clamp or voltage-clamp mode and outside-out patch recordings (Hamill et al., 1981) in voltage-clamp mode were obtained with a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). Both synaptic and patch currents were recorded at a holding potential of −70 mV. Because of the presence of a chloride-rich solution in the recording pipette (see Solutions below), $GABA_A$-mediated responses were recorded as inward currents at this holding potential. No correction was made for the pipette junction potential. The voltage and current output were filtered at 2 kHz and digitized at 16-bit resolution (National Instruments, Austin, TX). The series resistance ranged from 6 to 17 MΩ and was monitored throughout the experiments. Traces were stored on a video recorder device (Vetter, Rebersburg, PA). Data were digitized off-line at 10 or 20 kHz and transferred to a PC. Detection of mIPSCs was done by setting a threshold of −8 pA. We restricted the analysis to mIPSCs with rise time (20–80%) ≤ 0.6 msec, which are less likely to be attenuated by dendritic filtering. Under these conditions the lack of correlation ($r < 0.2$) between mIPSC rise times and their decay rates, measured as the fraction of decay at 7 msec from peak, indicated that the waveform of the inhibitory synaptic currents does not represent electrotonic filtering. mIPSCs were aligned at the 50% crossing of the rising phase before averaging. Curve fitting for averaged mIPSCs and patch currents was performed with a double exponential equation:

$$I(t) = \sum_{i=1}^{n} ai \exp(-t/\tau_i),$$

where I is the current as a function of time (t), and ai and τi are the amplitude and time constant of each component, respectively. The best fit was selected by using a least sum of squares algorithm. In a few cases the addition of a third exponential function improved the fit, but for the sake of comparison double exponential fits were used. Data are presented as mean ± SD, unless otherwise noted. Statistical analysis testing two-sample hypothesis was performed with unpaired, two-tailed Student's t test. In Table 1 multiple comparisons were performed with ANOVA and the Newman–Keuls test. The level of significance was $p \leq 0.05$.

Solutions. The standard solution contained (in mM): 126 NaCl, 2.5 KCl, 1.25 KH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 glucose, and 0.4 ascorbic acid, pH 7.4 (315 mOsm). mIPSCs were recorded in the presence of the sodium channel blocker tetrodotoxin (TTX, 0.5 μM; Sigma, St. Louis, MO) and the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; RBI, Natick, MA). Bicuculline methiodide was purchased from Sigma and picrotoxin from RBI. Drugs were dissolved in the standard solution.

Patch pipettes were filled with a chloride-rich intracellular solution containing (in mM): 80 K-gluconate, 40 KCl, 10 HEPES, 4 MgATP, 20 phosphocreatine(Na), 0.3 NaGTP, and 10 EGTA, pH 7.3 (295 mOsm). In some experiments the serine–threonine phosphatase inhibitor okadaic acid (1–5 μM; LC Laboratories, Woburn, MA), and/or the actin depolymerization inhibitor phalloidin (1 μM; Sigma) were added to the internal solution. Because no effect was observed on the GABA-mediated responses, data from all the experiments have been pooled together. For labeling neurons, biocytin (0.3%; Sigma) was added to the pipette internal solution.

The control solution used for the rapid perfusion system contained (in mM): 135 NaCl, 0.5 $CaCl_2$, $MgSO_4$, 10 HEPES (NaOH), and 40 sucrose, pH 7.3 (320 mOsm). The GABA-containing solution was diluted by 10% with respect to the control one.

Rapid application to outside-out patches. Rapid applications of GABA to outside-out membrane patches were performed with a piezoelectric element that displaced a pipette made from theta tubing, as previously described (Hestrin, 1992). Rapid solution exchange at the outside-out membrane patch was obtained by positioning the tip of the patch pipette in the control solution stream near the interface with the GABA-containing solution stream (see Solutions above). In each patch experiment a series of GABA pulses separated by 8 or 10 sec was applied to obtain an average patch current. A progressive decrease in the peak response, probably caused by the accumulation of receptors in desensitized state, was observed when intervals ≤5 sec were used. So that the pulse duration could be measured, the membrane patch was blown away from the tip of the pipette at the end of each experiment, and the current generated by the liquid junction potential, caused by the 10% dilution between the control and the GABA containing solution, was recorded. The solution exchanged with a rising/falling time (20–80%) of 150 μsec. The reversal potential for the $GABA_A$ receptor-mediated responses was calculated by measuring the peak amplitude of the patch response at a range of holding potentials (from −70 to +50 mV). The value obtained in four experiments was −11 ± 1 mV. To obtain nucleated patches, we applied suction through the patch pipette after obtaining the whole-cell configuration and while the pipette was withdrawn slowly from the cell (Sather et al., 1992).

GABA dose–response curves were constructed for GABA applications ranging from 10 μM to 10 mM. The peak amplitude of the response generated by 1 msec pulses was normalized with respect to the maximal response elicited in every patch. In a subset of experiments this maximal response was obtained by recording the patch current generated by the application of a 1 msec pulse of a saturating (10 mM) concentration of GABA (see Fig. 6*A–C*). To accomplish this, we switched the GABA-containing stream to a 10 mM GABA solution after recording several responses at the test concentration. A full solution exchange was reached in 30–40 sec. Because a run-down of the patch currents usually was observed during the first applications, we waited for an apparent stabilization of the response before switching to the 10 mM GABA solution, and, in some cases, the measurement was verified by switching back to the test concentration. When the response was unstable, the patch was discarded. In a second group of experiments the maximal GABA-mediated patch response was obtained by prolonged (>20 msec) applications of the agonist at any given concentration (see Fig. 5*A*). Brief and prolonged GABA applications were applied alternatively during these experiments. The dose–response curve was fit with the logistic equation: $I = 1/[1 + (EC_{50}/c)^h]$, where I is the normalized peak amplitude of the current, c is the GABA concentration, EC_{50} is the GABA concentration to generate the half-maximal current, and h is the Hill coefficient.

RESULTS

Whole-cell recordings and rapid application of GABA to somatic outside-out patches excised from layer V pyramidal neurons were performed. Pyramidal cells were identified in slices of the rat visual cortex according to their morphological appearance, using IR-DIC video microscopy, and according to their pattern of firing in response to intracellular injection of depolarizing current pulses (see Materials and Methods).

Kinetics of $GABA_A$-mediated mIPSCs in neocortical pyramidal neurons

$GABA_A$-mediated mIPSCs recorded from neocortical pyramidal neurons have been shown to decay with either a mono- or with a biexponential time course (Salin and Prince, 1996; Ruano et al., 1997). Because the kinetic components of synaptic currents have important mechanistic implications (Jones and Westbrook, 1995), we first analyzed the kinetics of the mIPSCs generated in layer V pyramidal neurons of the rat visual cortex. mIPSCs, thought to originate at single synaptic contacts, were selected because they represent elementary synaptic currents.

mIPSCs were recorded at a holding potential of −70 mV in the presence of CNQX (10 μM) and TTX (0.5 μM) (Figs. 1, 3). Under these conditions AMPA-mediated excitatory events and action potential-driven postsynaptic currents were blocked. Bath application of the $GABA_A$ receptor antagonists bicuculline methio-

dide (10 μM; $n = 3$) or picrotoxin (100 μM; $n = 2$) abolished any detectable spontaneous synaptic activity (data not shown), indicating that the mIPSCs are mediated by the activation of $GABA_A$ receptors.

Pyramidal neurons receive inhibitory synaptic inputs on their soma, axonal initial segment, and dendrites (Peters, 1985). Because mIPSCs exhibiting faster rise times would be less likely to be attenuated by the dendritic filtering, we restricted our analysis to events for which the rise times (20–80%) were faster than 0.6 msec. mIPSCs exhibited variable peak amplitudes that generated skewed histograms. The average amplitude of mIPSCs recorded from 25 pyramidal cells was 27.8 ± 7.4 pA. Considering that the reversal potential for $GABA_A$ receptor-mediated currents was −11 mV (see Materials and Methods), the mIPSC conductance was estimated to be 470 pS. The decay time course of individual mIPSCs usually exhibited two components and could not be described by a single exponential function (Fig. 1*A*). The average mIPSC recorded in a given neuron was fit with a biexponential function (Fig. 1*B*). In 25 pyramidal cells the mean fast time constant (τ_{fast}) was 7.5 ± 1 msec (76.3 ± 10% of amplitude), and the slow time constant (τ_{slow}) was 33.2 ± 11 msec.

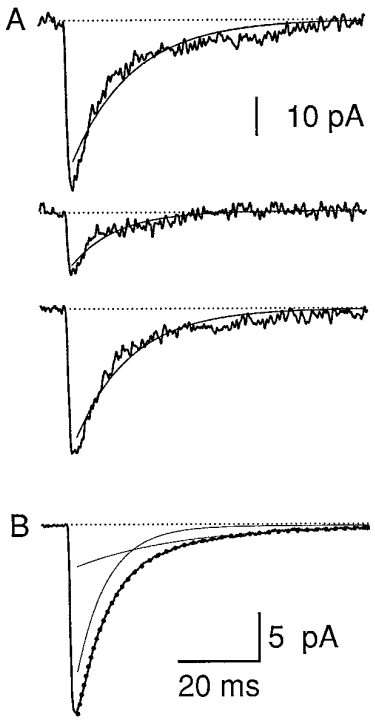

Figure 1. Kinetics of $GABA_A$-mediated mIPSCs in a pyramidal neuron from the rat visual cortex. *A*, Example of three mIPSCs recorded at a holding potential of −70 mV. A monoexponential function did not provide an adequate fit of the individual mIPSCs (*thin continuous lines*). *B*, The average of 248 mIPSCs recorded from the same cell was fit with a double exponential function (*dots*): τ_{fast} = 7.7 msec (79.9%) and τ_{slow} = 26.6 msec. *Thin lines* represent individual components of the fit. The events were detected and aligned as described in Materials and Methods.

Fast desensitization of neocortical GABA receptor channels

Recent experimental results led to the proposal that the biexponential decay of IPSCs recorded from cultured hippocampal cells (Jones and Westbrook, 1995) and granule cerebellar neurons (Tia et al., 1996) could be generated by a rapid entry and exit of the $GABA_A$ receptors through desensitized states (i.e., nonresponsive states). To test if this model also could apply to the mIPSCs recorded from neocortical pyramidal neurons, we studied the properties of their $GABA_A$ receptors, using rapid applications of GABA to excised patches. First, to assess how quickly $GABA_A$ receptors may desensitize, we examined patch currents generated by rapid application of prolonged (100–250 msec) pulses of GABA (1 mM). In the sustained presence of the agonist, we found that GABA-mediated currents exhibited a very rapid component of desensitization, followed by a much slower one (Fig. 2*A, thick trace; B, filled circles*). In 10 patches biphasic decay was described with a τ_{fast} = 3.6 ± 1.5 msec (37.6 ± 17%) and a τ_{slow} = 402 ± 317 msec. Responses induced in the same patches by brief (1 msec) pulses of GABA also had a biexponential decay [τ_{fast} = 4.6 ± 1.5 msec (47.2 ± 13%) and τ_{slow} = 61.2 ± 16 msec, $n = 10$], and, interestingly, their initial decay time course was comparable to the rapid desensitization observed with prolonged pulses (Fig. 2*A,B*). The difference between mean τ_{fast} values of brief and prolonged GABA pulses was not statistically significant ($p > 0.1$). Moreover, a plot of τ_{fast} values for brief GABA applications versus those for prolonged pulses applied to the same patch showed a correlation between these two parameters ($r = 0.82$; $n = 10$ patches; data not shown).

This result suggests that neocortical $GABA_A$ receptors could be entering into a desensitized state in response to a brief pulse of agonist. To test this issue, we used a paired-pulse protocol (Jones and Westbrook, 1995; Tia et al., 1996). If $GABA_A$ receptors are desensitized by the first pulse of agonist, then the availability of receptors to a second activation would be diminished. When two brief (1 msec) pulses of GABA (1 mM) were applied to an outside-out patch at an interval of 15 msec, the peak amplitude of the response induced by the second pulse was consistently reduced (Fig. 2*C*). The average degree of paired-pulse depression (PPD) at an interval of 15 msec was 40.4 ± 18.2% ($n = 11$). Recovery from paired-pulse depression required several hundreds of milliseconds (Fig. 2*D*).

Next, we took advantage of the variability in the degree of desensitization exhibited among patches to confirm the involvement of desensitization in shaping the response to a brief agonist application. A comparison in the same patch of the degree of desensitization induced by prolonged pulses of GABA and of paired-pulse depression showed a correlation between both parameters ($r = 0.7$, Fig. 2*E*). More importantly, we observed that those patches with faster decay in response to a brief pulse of GABA showed stronger PPD, whereas those with slower decay phase exhibited weaker PPD (Fig. 2*F*). The correlation coefficient between these two parameters was 0.9.

In summary, the correlation between the degree of desensitization and the decay rate of the currents induced by brief GABA pulses, together with the similar fast decay kinetics in response to brief and prolonged pulses of agonist, indicate that in neocortical pyramidal neurons $GABA_A$ receptor desensitization may underlie the fast decaying component of the currents generated by brief transients of high concentrations of GABA.

Comparison of mIPSCs and patch responses induced by brief pulses of GABA

The responses induced in outside-out patches by rapid applications of GABA resemble in general the time course of IPSCs in different neuronal types (Maconochie et al., 1994; Puia et al., 1994; Jones and Westbrook, 1995; Tia et al., 1996). In neocortical pyramidal neurons we observed that the time course of mIPSCs and patch responses induced by brief pulses of GABA (1 msec, 1

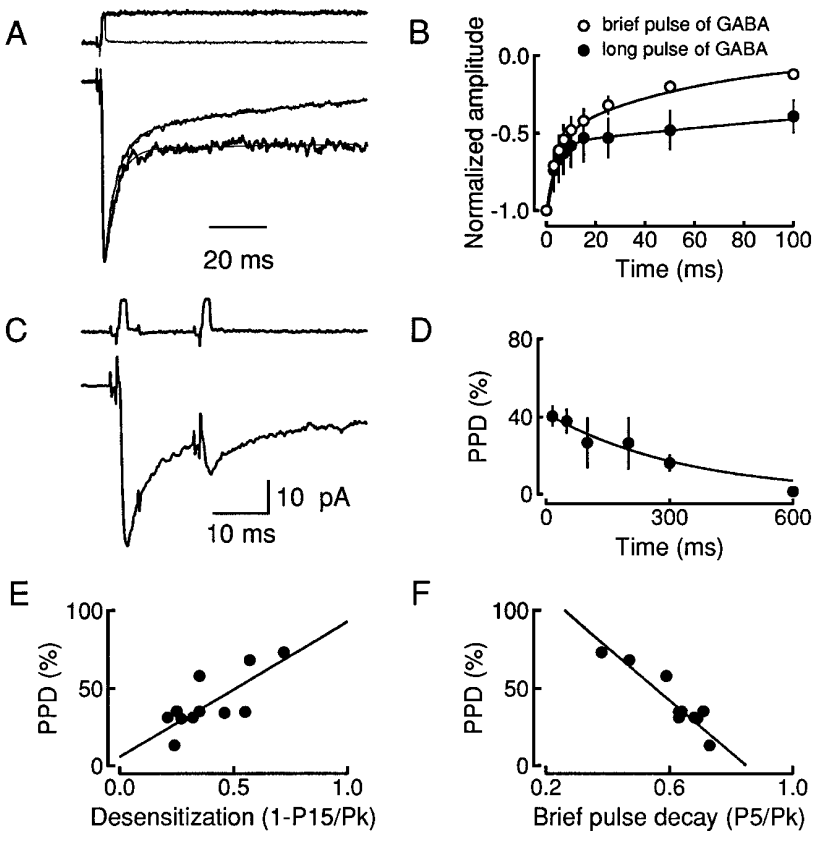

Figure 2. Desensitization induced by brief pulses of GABA in outside-out patches from neocortical pyramidal neurons. *A*, Scaled superimposition of the responses of a patch excised from a pyramidal neuron to brief (1 msec) or prolonged (250 msec) applications of GABA (1 mM). Currents were recorded at a holding potential of −70 mV and were obtained by averaging three to six responses. The duration of the GABA application is shown at the *top* of the panel, by the open pipette recordings obtained at the end of the experiment. Patch responses to a brief [τ_{fast} = 4.2 msec (67%); τ_{slow} = 85 msec] and a prolonged pulse of GABA [τ_{fast} = 4.0 msec (61%); τ_{slow} = 671 msec] were fit by a double exponential function. *B*, Summary plot of the data obtained in similar conditions from a total of 10 outside-out patches. Mean response to brief (1 msec) GABA applications (*open circles*) was fit with the parameters τ_{fast} = 4.6 msec (47%) and τ_{slow} = 61.2 msec, whereas data of prolonged pulses (100–250 msec; *filled circles*) were fit with τ_{fast} = 3.7 msec (43%) and τ_{slow} = 304 msec. Differences between brief and long pulses are statistically significant after 15 msec from the peak. Vertical bars indicate SEM. *C*, Response of the same patch shown in *A* to a pair of brief (1 msec) pulses of GABA (1 mM). The interpulse interval is 15 msec. Note the "glitches" that occur during the application of the command voltage to the perfusion apparatus. *D*, The recovery of the depression induced paired-pulse application of GABA. The degree of paired-pulse depression (PPD) was calculated as $(peak_1 - peak_2)/(peak_1 - onset_2)$, where $peak_2$ and $peak_1$ are the peak amplitudes of the second and first responses, and $onset_2$ is the value of the current at the onset of the second response. Each *dot* represents the mean of 4–11 experiments. Vertical bars indicate SEM. *E*, Relation between the PPD induced by two pulses of GABA (1 msec, 1 mM) applied with an interpulse interval of 15 msec and the degree of desensitization generated by a prolonged pulse (100–250 msec, 1 mM). Every *dot* represents data from a single patch; $r = 0.7$. *F*, Correlation between PPD and the decay time course of the responses induced by a brief (1 msec) pulse of 1 mM GABA; $r = 0.9$. *P5/Pk* means the fraction of decay at 5 msec after the peak.

mM) were comparable, exhibiting a fast rise time and a biexponential decay phase. Figure 3*A* illustrates four representative averaged mIPSCs and outside-out patch currents recorded from different pyramidal cells. To facilitate their comparison, we scaled averaged mIPSCs and patch currents, and it is clear that, in addition to a general resemblance, a consistent quantitative discrepancy also existed between them. In particular, we found that the τ_{slow} of patch responses was slower than that of the mIPSCs. Pooled data from 21 outside-out patches and averaged mIPSCs from 25 neurons, including five cases in which patch responses and synaptic currents were obtained from the same cell, are shown in Table 1.

A possible explanation that could account for this discrepancy between mIPSC and patch response kinetics is that the functional properties of the $GABA_A$ receptors in the outside-out patch may undergo modulation. To explore this possibility, we examined the responses generated by brief pulses of GABA (1 msec, 1 mM) in nucleated patches (Sather et al., 1992). The presence of the cell nucleus in the outside-out patch may keep an internal milieu closer to the physiological one, thus reducing possible receptor modulation. Under these conditions currents were much larger than those obtained in conventional outside-out patches, most likely because nucleated patches have a larger membrane surface. However, their decay kinetics were also slower than those of mIPSCs (Fig. 3*B*). Averaged parameters describing the decay time course of nucleated patch currents induced by brief pulses of 1 mM GABA were τ_{fast} = 10.2 ± 1 msec (55.7 + 9%) and τ_{slow} = 56.1 ± 13 msec (n = 6, Table 1). In addition, we examined the possibility that kinetics of the responses could change with time after the excision of the outside-out patches. In most experiments a "run-down" in the peak of the amplitude response was observed during the first GABA-mediated responses. Nevertheless, when responses obtained several minutes (up to 20) after the excision of the patch were scaled and compared with those obtained at the beginning of the experiment, a similar time course was observed (n = 12). We also found that differences between mIPSCs and patch responses persisted when okadaic acid (a nonspecific phosphatase inhibitor, 1–5 μM) or phalloidin (a cytoskeleton stabilizing agent, 1 μM) was included in the solution filling the patch pipette (data not shown). Thus, these approaches did not demonstrate the existence of significant $GABA_A$ receptor modulation under our experimental conditions; however, it should be emphasized that the possibility that the kinetic properties of the $GABA_A$ receptors in outside-out patches differ from those in intact synapses cannot be ruled out.

Relation between the decay time course of patch currents and GABA concentration

The $GABA_A$ receptor channels are thought to have more than one agonist binding site (Macdonald et al., 1989), and conducting states of multiliganded receptors could generate different kinetics from those of monoliganded receptors (Macdonald et al., 1989; Busch and Sakmann, 1990; Jones and Westbrook, 1995). We addressed this issue by comparing the decay time course of conventional and nucleated patch responses generated by a 1 msec pulse of GABA at a range of different concentrations (10

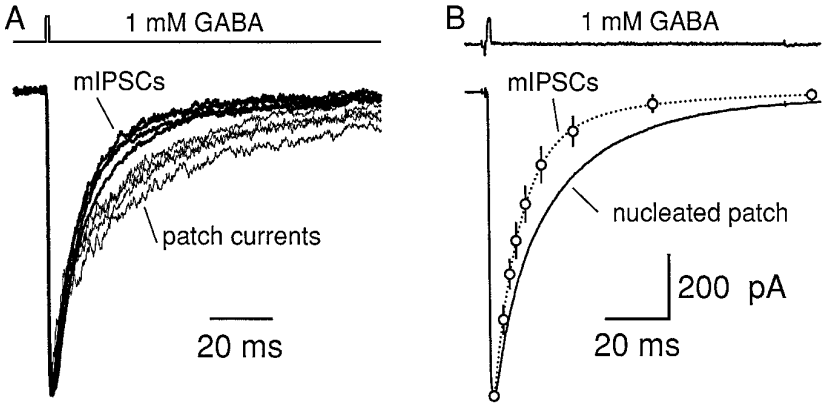

Figure 3. Comparison of neocortical mIPSCs and patch responses induced by brief pulses of GABA to outside-out patches. *A*, Superimposition of four representative outside-out patch currents (*thin traces*) and the averaged mIPSCs (*thick traces*) recorded from four different cells. Patch responses were induced by a brief pulse of GABA (1 msec, 1 mM), and each trace is the average of at least four responses. The peak amplitude of the mIPSCs (26.1 ± 8.7 pA) was smaller than that of the patch currents (75.6 ± 24.7 pA). To facilitate their comparison, we scaled all of the responses to the same peak amplitude. The artifacts generated by the command voltage pulses have been blanked. *B*, The time course of the current induced in a nucleated patch by a brief pulse of GABA (1 msec, 1 mM). The trace, an average of 10 responses, was fit by a double exponential function with the following parameters: τ_{fast} = 9.6 msec (63.8%) and τ_{slow} = 56.0 msec. For comparison, the average decay time course of neocortical pyramidal mIPSCs is represented by the *dotted line* (n = 25). At the *top* of both panels the open pipette recording indicates the duration of the GABA pulse.

μM to 10 mM) (Fig. 4). No significant difference was found among the responses induced by concentrations ranging from 50 μM to 10 mM (Fig. 4*A,C,D*, Table 1). However, application of 10 μM GABA induced responses with a significantly faster τ_{slow}, as compared with the responses to higher concentrations (Fig. 4*B,D*, Table 1). As shown below, the relative activation of $GABA_A$ receptors in response to 10 μM GABA (1 msec) is only 2% (Fig. 6*D*). These results suggest that, when receptor occupancy is very low and $GABA_A$ receptor opening occurs from a monoliganded state, faster deactivation rates are produced (Jones and Westbrook, 1995).

Brief pulses of low concentrations of GABA can generate currents with a fast rise time

Kinetics of activation depend on agonist concentration, and when prolonged pulses of agonist are applied to membrane patches, high concentrations (from 500 μM to 1 mM) of GABA are needed to generate responses with a rise rate as fast as that of the IPSCs (Maconochie et al., 1994; Jones and Westbrook, 1995). Considering, however, that synaptic currents may be better mimicked by brief transients of GABA than by prolonged ones, it is of interest to examine the relationship between the response rise time and GABA concentration when brief pulses of agonist are used (Frerking and Wilson, 1996). Figure 5*A1* shows GABA-mediated responses obtained in the same patch by a brief (1 msec) and a prolonged (100 msec) pulse of GABA at a concentration of 100 μM. Note that the briefer application resulted in a smaller peak response, indicating that only a fraction of channels was driven into open state because of the short pulse length. When these two responses were scaled to the same peak amplitude, a faster rise time in the current generated by a brief application was apparent if compared with the response induced by the prolonged application (Fig. 5*A2*). The average rise time (20–80%) measured from a total of 11 similar experiments was 0.55 ± 0.14 msec for the brief pulses and 2.65 ± 0.67 msec for the prolonged ones. In contrast with this result, when brief and prolonged applications of GABA at higher concentrations (1 and 10 mM) were used, GABA-mediated currents exhibited a similar fast rise time (Fig. 5*B*). We conclude from this group of experiments that if the presence of the agonist is brief enough to prevent channels from reaching their maximum open probability, the rise time of the response would depend mostly on the duration of the transient. Therefore, if GABA time course in the synaptic cleft is very fast, a wide range of concentrations of GABA could generate currents with a rapid rise time similar to that of the synaptic currents (Fig. 5*B*, Table 1).

Table 1. Kinetics of mIPSCs and outside-out patch responses induced by GABA in neocortical pyramidal neurons

	Rise time (20–80%)	τ_{fast} (msec)	% Fast	τ_{slow} (msec)	n
mIPSCs	0.46 ± 0.05	7.5 ± 1	76.3 ± 10	33.2 ± 11	25
Patch currents					
10 μM (N)	0.54 ± 0.15	4.5 ± 2	66.2 ± 8	34.4 ± 12	4
50 μM	0.73 ± 0.09	3.6 ± 2	57.0 ± 8	60.2 ± 12	7
100 μM	0.55 ± 0.04	4.4 ± 2	47.1 ± 7	70.1 ± 20	12
300 μM	0.52 ± 0.03	5.2 ± 3	46.5 ± 16	68.8 ± 27	8
1 mM	0.37 ± 0.03	5.1 ± 2	49.4 ± 10	62.4 ± 14	21
1 mM (N)	0.50 ± 0.07	10.2 ± 1	55.7 ± 9	56.1 ± 13	6
10 mM	0.30 ± 0.04	6.0 ± 3	52.0 ± 8	58.8 ± 17	16

Outside-out patch responses were induced by rapid application of GABA pulses of 1 msec duration. Both mIPSCs and patch responses were recorded at a holding potential of −70 mV. Currents were fit with the sum of two exponential functions, as explained in Materials and Methods. The slow component of patch responses was significantly slower and larger than that of mIPSCs at all of the tested concentrations except 10 μM ($p < 0.05$). In addition, the fast time constants of patch responses and mIPSCs were found significantly different at 10, 50, and 100 μM and 1 mM concentrations. Data are shown as mean ± SD. (N), Nucleated outside-out patches; n, number of experiments.

Concentration–response curve for neocortical $GABA_A$ receptors in nonequilibrium conditions

If the $GABA_A$ receptors are exposed to GABA for a short enough period of time, they will not reach their maximal open probability. Under nonequilibrium conditions, therefore, the concentration–response relation would depend on the binding rate of the receptor. Assuming a brief transient of GABA in the synaptic cleft, we investigated this property in neocortical $GABA_A$ receptors by studying the concentration–response curve generated by brief (1 msec) pulses of GABA. Figure 6*A–C* illustrates examples of the responses generated, in three different patches, by a 1 msec pulse of GABA at a concentration of 100 and 300 μM and 1 mM. When each response is compared with that evoked in the same patch by a 1 msec pulse of 10 mM GABA, it is apparent that a millimolar concentration is necessary to generate a maximal response. The peak of the current evoked by a given concentration of GABA was highly variable from patch to patch. Thus to construct the concentration–response curve (Fig. 6*D*), we normalized data that originated from different patches with respect to the maximal peak current obtained in each patch by the application of 10 mM GABA (*filled symbols*). The concentration–response curve was fit with the logistic equation, and an EC_{50} of 185 μM and a Hill coefficient of 1.3 were obtained. When $GABA_A$ receptors are activated by brief transients of agonist, binding as well as unbinding rates determine the relative fraction of activated receptors. Under these conditions (1 msec pulse) only a small fraction of the receptors is activated at the low micromolar range, and millimolar concentrations are needed to obtain a saturated response. Using the parameters derived from the fit of the logistic equation, we can estimate that to obtain a near-maximal response (>95% of the maximum) a square pulse lasting 1 msec at a concentration higher than 1.8 mM is required.

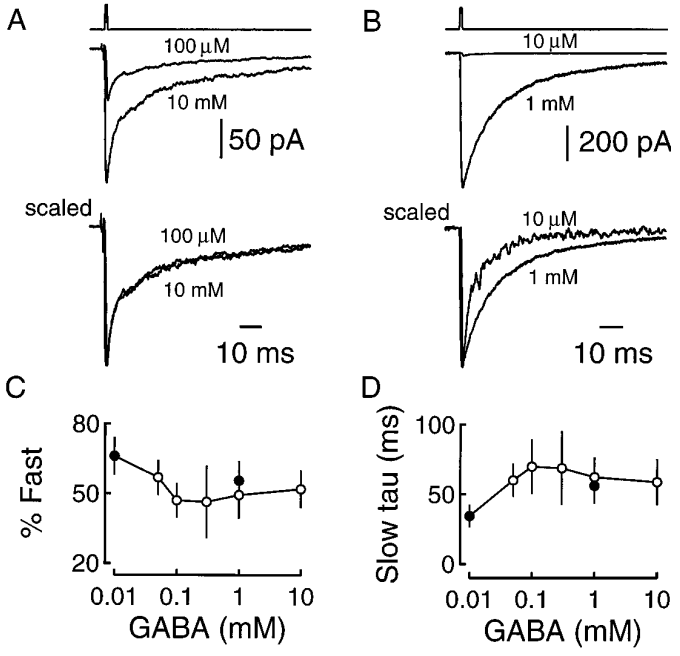

Figure 4. Concentration dependence of the decay time course of GABA-mediated currents in neocortical outside-out patches. *A*, *Top*, GABA-mediated currents recorded from the same outside-out patch in response to a 1 msec pulse of GABA at 100 μM and 10 mM. Data at 100 μM concentration were obtained before 10 mM. Traces are the average of three and seven responses. To facilitate their comparison, we scaled these traces, shown at the *bottom* of the panel. *B*, *Top*, Responses obtained in the same nucleated patch by the application of a 1 msec GABA pulse at 10 μM and 1 mM; average of three and nine traces. *Bottom*, Scaled overimposition of the responses shown in the *top* of the panel. *C, D*, Relation between the decay rate of patch currents induced by brief (1 msec) pulses and the concentration of GABA (from 10 μM to 10 mM). Data obtained from conventional outside-out patches are plotted with *open circles*, whereas *filled circles* represent data from nucleated patches. Each *point* represents the average data from 4 to 21 different patches.

DISCUSSION

Fast desensitization of neocortical $GABA_A$ receptors

Our results, in agreement with previous work in hippocampal and cerebellar neurons (Jones and Westbrook, 1995; Tia et al., 1996), indicate that neocortical $GABA_A$ receptor response to a brief pulse of GABA may be shaped by movement through desensitized states. This conclusion is supported by three pieces of evidence. First, the initial decay time course in the responses induced by prolonged and brief applications of GABA is similar. Second, after a brief pulse of GABA a fraction of receptors remains unresponsive to a second application of the agonist. Third, there is a correlation between the initial decay time course in the responses induced by a brief pulse of GABA and the degree of PPD in different patches.

These results are remarkably different from those obtained at AMPA receptors under similar conditions. The decline of AMPA receptor-mediated currents in response to prolonged applications

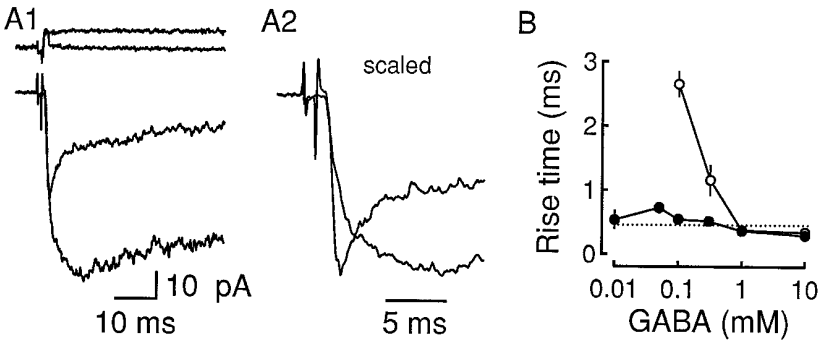

Figure 5. Brief pulses of low concentrations of GABA can generate currents with a fast rise time in neocortical outside-out patches. *A1*, GABA-mediated currents recorded from the same outside-out patch in response to brief (1 msec) and prolonged applications (100 msec) of a low concentration of GABA (100 μM). The duration of the GABA application is indicated at the *top* of the panel with the open pipette recordings. *A2*, Both responses shown in *A1* have been scaled to the same peak amplitude to facilitate the comparison of their rise times (20–80%). The measured values for the brief and prolonged pulses are 0.45 and 2.9 msec, respectively. *B*, Relation between the GABA concentration (from 10 μM to 10 mM) and the rise time of the patch responses. Data obtained in the same patches by brief (*closed circles*) and prolonged pulses (*open circles*) of GABA are compared. Measurements at 10 μM were performed in nucleated patches. *Dotted line* indicates the mean rise time (20–80%) of mIPSCs recorded from pyramidal neurons, which in our experimental conditions was 0.46 msec.

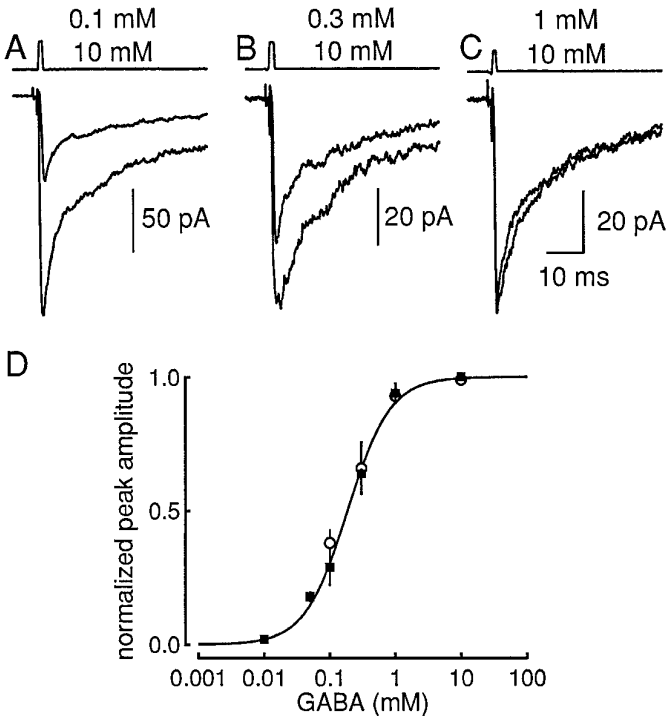

Figure 6. Concentration–response curve of neocortical $GABA_A$ receptors in response to brief pulses of GABA. *A–C,* Representative examples of the responses generated by applying brief (1 msec) pulses of GABA at a concentration of 100 μM (*A*), 300 μM (*B*), and 1 mM (*C*). The variability of the peak current generated by a given concentration of GABA among different patches was minimized by normalizing the data with respect to the peak current obtained in response to 10 mM pulses in the same patch. Data from each concentration were obtained from different patches. Each trace is the average of at least four responses. *D*, Dose–response curve of $GABA_A$ receptor channels in nonequilibrium conditions. *Filled symbols* represent the peak amplitude of the response to a brief pulse of GABA normalized with respect to the peak current in response to 10 mM GABA. Data from pulses at 10 μM GABA, obtained in nucleated patches, were corrected because they were normalized with respect to 1 mM responses. The concentration–response relation was fit with the logistic equation: $I = 1/[1+(EC_{50}/c)^h]$ (see Materials and Methods). EC_{50} was equal to 185 μM, and the Hill coefficient was 1.3. *Open symbols* correspond to the data normalized with respect to maximal current obtained by applying prolonged pulses of the same concentration. Dots and vertical bars represent mean ± SEM. The number of experiments in each point ranges from 4 to 10.

of glutamate is significantly slower than that obtained with brief applications (for review, see Jonas and Spruston, 1994). Thus, whereas the onset of AMPA receptor desensitization is distinctly slower than the decay rate in response to a brief pulse of agonist, at $GABA_A$ receptors the transition from the open state to the desensitized state is rapid and may occur either directly or via a short-lived intermediate closed state (Jones and Westbrook, 1995).

Comparison between mIPSCs and patch responses to brief pulses of GABA

In patches from neocortical pyramidal neurons we found that brief (1 msec) applications of GABA produce currents generally resembling mIPSCs but with consistent discrepancies. In particular, the slow component of the decay phase in patch responses was approximately twofold slower than that in mIPSCs. In hippocampal cultured cells, autaptically evoked IPSCs were found not significantly different from the patch responses induced by pulses of GABA (Jones and Westbrook, 1995), but similar to our data, nucleated patch responses in cerebellar granule cells exhibited a slower slow component as compared with that of spontaneous IPSCs (Puia et al., 1994; Tia et al., 1996). We observed that this discrepancy is maintained when $GABA_A$ receptors are studied in nucleated patches and when serine–threonine phosphatases and actin depolymerization are inhibited. These results do not support the notion that $GABA_A$ receptors are modulated in outside-out patches, but many other alternative modulatory mechanisms should be explored before this possibility could be ruled out completely. In addition, it also should be considered that membrane patch excision unavoidably produces a profound mechanical disruption that could affect $GABA_A$ receptor behavior. Furthermore, the functional properties of synaptic and extrasynaptic $GABA_A$ receptors could differ (Somogyi, 1989). On the other hand, the finding that patch responses induced by low micromolar concentrations of GABA have a faster decay [Jones and Westbrook (1995) and this study] closer to that of the mIPSCs (see Table 1) could be consistent with the proposal that synaptic GABA transients occur in such a low range (Frerking et al., 1995). It should be noted, however, that the concentration of GABA in synaptic vesicles is high (Burger et al., 1991); therefore, its peak cleft concentration could reach the hundreds of micromolar to millimolar range.

Functional implications

Under equilibrium conditions $GABA_A$ receptors exhibit relatively high affinity, and half-maximal responses are obtained when GABA is applied in the low micromolar range (EC_{50} ~20 μM; Schönrock and Bormann, 1993; Maconochie et al., 1994). Considering that synaptic currents are better mimicked by brief pulses of GABA than by prolonged ones, it is possible that $GABA_A$ receptors are not at equilibrium during synaptic transmission. In this scenario the peak of the response would depend on the duration of the GABA transient and on the $GABA_A$ receptor binding and dissociation rates. To estimate this property, we obtained the concentration–response curve of the neocortical $GABA_A$ receptors under nonequilibrium conditions. In response to pulses of GABA of 1 msec we found that the EC_{50} is 185 μM and that GABA concentrations higher than 1.8 mM are needed to obtained a near-saturated response (>95% of maximum). Transmitter transient during synaptic transmission has been estimated to decay with a major component of ~100 μsec (Eccles and Jaeger, 1958; Destexhe and Sejnowski, 1995; Holmes, 1995; Clements, 1996; Wahl et al., 1996). Thus we could predict that the concentration of GABA necessary for saturation could be very high, raising the possibility that neocortical $GABA_A$ receptors may not be saturated during synaptic transmission. In "dinapses" of amacrine cells it has been suggested that $GABA_A$ receptors are not saturated, based on the observation that transmitter concentration is the major determinant of mIPSC amplitude (Frerking et al., 1995). On the other hand, the low quantal variance of evoked IPSCs (Edwards et al., 1990), nonstationary fluctuation analysis, and pharmacological approaches support the hypothesis of saturation in hippocampal granule cells (Otis and Mody, 1992; De Koninck and Mody, 1994). Although differences in the anatomy of the synapses, the amount of GABA released in the cleft, and the kinetic properties of the $GABA_A$ receptors may generate a variety of scenarios (Frerking and Wilson, 1996), it is clear that more experiments and measurement of cleft GABA concentration during synaptic transmission are needed to resolve this issue.

$GABA_A$ receptor desensitization could be involved not only in

shaping individual synaptic currents but also in decreasing the amplitude of the response during repetitive stimulation (Jones and Westbrook, 1996). Thus the depression in the amplitude of the IPSCs observed with paired stimulation of neocortical GABAergic synapses (Deisz and Prince, 1989; Fleidervish and Gutnick, 1995; Thomson et al., 1996) could be explained, at least partially, by accumulation of receptors in desensitized states. It is important to note, however, that the effect of receptor desensitization during repetitive stimulation would be minimized if the $GABA_A$ receptors are not saturated during synaptic transmission.

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