

# Distinct Functional and Pharmacological Properties of Tonic and Quantal Inhibitory Postsynaptic Currents Mediated by $\gamma$ -Aminobutyric Acid<sub>A</sub> Receptors in Hippocampal Neurons

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## ABSTRACT

$\gamma$ -Aminobutyric acid (GABA), the principal inhibitory neurotransmitter, activates a persistent low amplitude tonic current in several brain regions in addition to conventional synaptic currents. Here we demonstrate that GABA<sub>A</sub> receptors mediating the tonic current in hippocampal neurons exhibit functional and pharmacological properties different from those of quantal synaptic currents. Patch-clamp techniques were used to characterize miniature inhibitory postsynaptic currents (mIPSCs) and the tonic GABAergic current recorded in CA1 pyramidal neurons in rat hippocampal slices and in dissociated neurons grown in culture. The competitive GABA<sub>A</sub> receptor antagonists, bicuculline and picrotoxin, blocked both the mIPSCs and the tonic current. In contrast, mIPSCs but not the tonic current were inhibited by gabazine (SR-95531). Coapplication experiments and computer simulations revealed that gabazine bound to the receptors responsible for the tonic current but did not

prevent channel activation. However, gabazine competitively inhibited bicuculline blockade. The unitary conductance of the GABA<sub>A</sub> receptors underlying the tonic current ( $\sim 6$  pS) was less than the main conductance of channels activated during quantal synaptic transmission ( $\sim 15$ – $30$  pS). Furthermore, compounds that potentiate GABA<sub>A</sub> receptor function including the benzodiazepine, midazolam, and anesthetic, propofol, prolonged the duration of mIPSCs and increased tonic current amplitude in cultured neurons to different extents. Clinically-relevant concentrations of midazolam and propofol caused a greater increase in tonic current compared with mIPSCs, as measured by total charge transfer. In summary, the receptors underlying the tonic current are functionally and pharmacologically distinct from quantally activated synaptic receptors and these receptors represent a novel target for neurodepressive drugs.

$\gamma$ -Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system, modifies electrical activity in the brain by regulating membrane hyperpolarization and the "shunting" of excitatory input. GABA released from presynaptic terminal binds to GABA<sub>A</sub> receptors clustered at the postsynaptic membrane and activates inhibitory postsynaptic currents (IPSCs). In addition to conventional quantal synaptic transmission, a persistent form of GABAergic inhibition has been described in several brain regions. A small but significant tonic GABAergic current has been observed in the cerebellum (Brickley et al., 1996; Wall and Usowicz 1997), cortex (Salin and Prince, 1996), thalamus (Liu et al., 1995), and hippocampus (Otis et al., 1991). This tonic current has been best characterized in the cerebellum, where glomerular structures that surround synapses onto granule cells serve as a repository for transmitter released from neighboring synapses. Transmitter in the glomerulus may activate high-affinity GABA<sub>A</sub> receptors with minimal

desensitization properties that are located in perisomatic and extrasynaptic regions of granule cells (Rossi and Hamann, 1998).

The mechanisms that regulate the tonic GABAergic inhibition in other brain regions are not well understood. The tonic conductance in the hippocampus may result from the summation of overlapping miniature IPSCs (Soltesz et al., 1995; Salin and Prince, 1996), or the spill-over of vesicular transmitter released from neighboring synapses (Brickley et al., 1996; Rossi and Hamann, 1998). Recently, it was postulated that the tonic current results from the release of GABA from a surface matrix reservoir that becomes exposed during exocytosis (Vautrin et al., 2000). Also, reverse operation of GABA cotransporters (Gaspary et al., 1998) or release of GABA from astrocytes (Liu et al., 2000) might elevate GABA to concentrations sufficient to activate receptors. The in vivo ambient concentration of GABA in the extracellular space, measured using microdialysis ( $0.8$ – $2.9$   $\mu$ M), is sufficient to

**ABBREVIATIONS:** GABA,  $\gamma$ -aminobutyric acid; IPSC, inhibitory postsynaptic currents; mIPSC, miniature inhibitory postsynaptic current; aCSF, artificial cerebrospinal fluid; TTX, tetrodotoxin.

activate GABA<sub>A</sub> receptors (Lerma et al., 1986). Alternatively, the tonic current might result from spontaneous openings of constitutively active GABA<sub>A</sub> channels (Neelands et al., 1999; Birnir et al., 2000).

Regardless of the source of GABA responsible for the tonic current, receptors that mediate this persistent GABAergic conductance are of considerable physiological and pharmacological interest. Small but persistent increases in chloride conductance alter input resistance and membrane time constants; these changes, in turn, modulate synaptic efficacy and synaptic integration. The tonic GABAergic current may also play an important role in the manifestation of disease processes. Certain types of seizures are associated with a decrease in ambient concentrations of GABA and seizure control improves with treatments that increase the concentration of GABA. Modulation of tonic receptors represents a promising strategy for the development of new anticonvulsant, anxiolytic, and anesthetic drugs. Notably, allosteric modulation of GABA<sub>A</sub> receptor function by many compounds strongly depends on the occupancy of the receptor by GABA, as well as the state of receptor activation. The greatest increase in GABA<sub>A</sub> receptor activity by benzodiazepines and anesthetics occurs when receptors are activated by low concentrations of GABA (Harris et al., 1995). Accordingly, it is predicted that receptors underlying the tonic current (activated by low concentrations of GABA) would respond to pharmacological agents differently from receptors activated during quantal synaptic transmission.

Given the potential physiological and therapeutic importance of GABA<sub>A</sub> receptors that mediate the tonic GABAergic inhibition, we investigated the tonic current in hippocampal neurons. We demonstrate the differential pharmacological properties of tonic and synaptic currents mediated by GABA<sub>A</sub> receptors. Midazolam and propofol produced a greater increase in charge transfer associated with the tonic current compared with that associated with miniature IPSCs. At concentrations that produce equivalent prolongation of IPSCs, the anesthetic propofol had a greater effect on the tonic current than the sedative midazolam. We speculate that modulation of the tonic current may account for differences in the clinical actions of these two classes of compounds. Some of the results were published in abstract form (Bai et al., 1998).

## Materials and Methods

**Cell Culture and Electrophysiological Techniques.** Primary cultures of hippocampal neurons were prepared from embryonic Swiss White mice using aseptic techniques (MacDonald et al., 1989). Cells were maintained in culture for 13 to 18 days before use.

Conventional whole-cell patch clamp recordings were performed at room temperature (21 to 23°C), at a holding potential of -60 mV. The extracellular recording solution contained 140 mM NaCl, 1.3 mM CaCl<sub>2</sub>, 5.4 mM KCl, 2 mM MgCl<sub>2</sub>, 25 mM HEPES, and 33 glucose, with pH adjusted to 7.4 with 1 M NaOH. Tetrodotoxin (TTX, 300 nM) was added to the extracellular solution to block voltage-sensitive Na<sup>+</sup> channels, and 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (10 μM) and 2-amino-5-phosphonovaleate (40 μM) were added to inhibit ionotropic glutamate receptors. Recording electrodes were filled with a solution containing 120 mM CsCl, 30 mM HEPES, 11 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 4 mM MgATP; pH was adjusted to 7.3 with CsOH. Currents were recorded simultaneously on a chart recorder and videotape recorder through a digital con-

verter and a PC computer using Strathclyde Electrophysiological Software (SCAN or SPAN; Strathclyde Electrophysiological Software, courtesy of Dr. J. Dempster, Strathclyde University, United Kingdom; <http://www.strath.ac.uk/Departments/PhysPharm/ses.htm>). Control and drug-containing solutions were delivered to the cultured neurons through glass barrels that were positioned close to the soma of the neuron. Propofol was prepared from Diprivan 1% (Zeneca Pharma, Mississauga, Ontario, Canada) and the solutions for the control experiments contained equivalent concentrations of Intralipid (KabiVitrum Canada Inc., Toronto, Canada). Intralipid did not influence the mIPSCs or tonic current. Midazolam was prepared from a commercial preparation of Versed (Hoffman-LaRoche Ltd., Mississauga, Ontario, Canada). We observed no differences in the actions of midazolam prepared from Versed compared with the pure compound (generously provided by Hoffman-La Roche, Nutley, NJ) dissolved in dimethyl sulfoxide. Bicuculline methobromide was purchased from Sigma (Oakville, Ontario, Canada) and gabazine (also known as SR-95531) was obtained from Research Biochemical International (RBI, Natick, MA).

Whole-cell recordings were also made from the CA1 region of hippocampal slices obtained from 2- to 3-week old Wistar rats. Coronal slices were prepared with a vibratome (VT1000E; Leica, Wetzlar, Germany) and incubated at room temperature for a minimum of 1 h in oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) artificial cerebrospinal fluid containing 124 mM NaCl, 3 mM KCl, 4 mM CaCl<sub>2</sub>, 4 mM MgCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM glucose. Slices were then transferred to a tissue chamber as needed and maintained at 31°C ± 0.5°C at the interface between humidified and oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) aCSF perfused through the chamber at a rate of 0.5 to 1 ml/min. Tight-seal (>5 GΩ) whole-cell recordings were obtained from CA1 pyramidal cells using a "blind" approach. The internal pipette solution consisted of 140 mM CsCl, 10 mM HEPES, 2 mM MgCl<sub>2</sub> (pH 7.2–7.3 using CsOH; osmolality, 270–280 mOsm). Spontaneous miniature IPSCs (see below) were isolated by the addition of 0.5 μM TTX, 10 μM 6-cyano-2,3-dihydroxy-7-nitroquinoxaline and 40 μM 2-amino-5-phosphonovaleate to the aCSF. Drugs tested were dissolved in aCSF and superfused over slices. Spontaneous mIPSCs were recorded using an Axopatch-1D (Axon Instruments, Foster City, CA), filtered at 2 kHz and stored on videotape for subsequent off-line analysis using a digital data recorder (VR-10B; InstruTECH Corp., Port Washington, NY).

**Data Analysis.** Current recordings that demonstrated a stable baseline and distinct mIPSCs were used for the analysis. All experiments were digitized (2 kHz) with a pulse-code modulator and stored on VHS videotapes. For analysis, the recordings were played back and re-digitized using an event detection program (SCAN). For detection of IPSCs, the trigger level was set at approximately three times higher than the level of the baseline noise (~3.4 pA). All events greater than the threshold level were recorded for frequency analysis including those infrequent compound events (<2%) with multiple peaks. When multiple peaks were clearly evident during the visual inspection of the records, the additional peaks were counted as mIPSCs. However, compound events were excluded from the analysis of rise time or decay of synaptic currents. In addition, we manually scrolled through files of detected events to reject spurious events that were caused by excessive noise.

Spontaneous postsynaptic currents recorded in the presence of tetrodotoxin (TTX) are referred to as miniature IPSCs (mIPSCs). Miniature IPSCs with a rapid onset (10 to 90% rise time < 5 ms) and decay phase that were not contaminated by other mIPSCs were selected for further kinetic analysis. At least 100 individual mIPSC events were recorded under each experimental condition. Peak amplitude, charge transfer (Q, the integrated area under mIPSCs), and the time constant of current decay (τ<sub>off</sub>) were analyzed. The decay phase was well described by a single exponential equation in the form  $I(t) = A_0 \exp(-t/\tau_{off}) + C$ , where  $I(t)$  is the current amplitude at any given time  $t$ ,  $C$  is the residual current, and  $A_0$  is the current amplitude at time 0. Change in the charge transfer (ΔQ<sub>mIPSC</sub>) asso-

ciated with mIPSC was analyzed according to Brickley et al. (1996) using the equation  $\Delta Q_{\text{mIPSC}} = f_{\text{drug}} \times Q_{\text{drug}} - f_{\text{con}} \times Q_{\text{con}}$ , where  $f_{\text{drug}}$  and  $f_{\text{con}}$  are the frequencies (Hz) of mIPSCs and  $Q_{\text{drug}}$  and  $Q_{\text{con}}$  are the average charge transfer (pC) per mIPSC during drug and control conditions, respectively. Under our experimental conditions, we assumed that the change in charge transfer reflected a proportional change in membrane conductance. The amplitude of the tonic current was calculated as the difference between the holding current measured before and after the application of bicuculline (10  $\mu\text{M}$ ) (Brickley et al., 1996; Wall and Usowicz, 1997). The increase in the tonic current that was observed after the application of midazolam or propofol was measured from the chart record (Astro-Med, West Warwick, RI). The charge transfer associated with the tonic current was calculated according to the equation:  $\Delta Q_{\text{TC}} = I_{\text{TC}} \times \Delta t$ , where  $\Delta Q_{\text{TC}}$  is the charge transfer produced by the tonic current,  $I_{\text{TC}}$  is the current amplitude at steady-state, and  $\Delta t$  is time.

Variance analysis was used to estimate the single channel current ( $i$ ) from the mean current ( $I_{\text{mean}}$ ) and current variance ( $\sigma^2$ ). Variance ( $\sigma^2$ ) was calculated according to the formula:

$$\sigma^2 = \sum_{n=1}^n (AC_i - AC_m)^2 / (n - 1)$$

where  $n$  is the number of samples per record,  $AC_i$  is the current mediated by GABA<sub>A</sub> receptors at sample  $i$ , and  $AC_m$  is the mean AC current. The plot of  $\sigma^2 - I_{\text{mean}}$  follows a parabolic relationship:  $\sigma^2 = i(1 - P_o)I_{\text{mean}}$ , where  $P_o$  is the channel open probability, which varies from 0 to 1. If we assume that the channel open probability of receptors mediating the tonic current is small under our experimental conditions (concentrations of exogenous GABA = 0.1 – 1  $\mu\text{M}$ ), then the following equation holds:  $i = \sigma^2 / I_{\text{mean}}$ . Single channel conductance ( $\gamma$ ) was estimated according to the equation:  $\gamma = i / (V_H - V_R)$ , where  $V_H$  is the holding potential and  $V_R$  is the reversal potential for chloride.

After establishing the whole-cell configuration, 10 – 20 min were allowed to elapse before the application of drug to allow the membrane patch to stabilize and exchange of ions between the recording electrode and the cytosol to occur. Under these conditions, the frequency of mIPSCs remained stable. In six cells, the frequency of mIPSCs was measured during the first minute of recording ( $0.67 \pm 0.08$  Hz) and 10 min later ( $0.68 \pm 0.09$  Hz). Thus, the frequency of the mIPSCs was stable before the application of the drugs ( $102 \pm 10\%$ ,  $n = 6$ ,  $P = 0.93$ ).

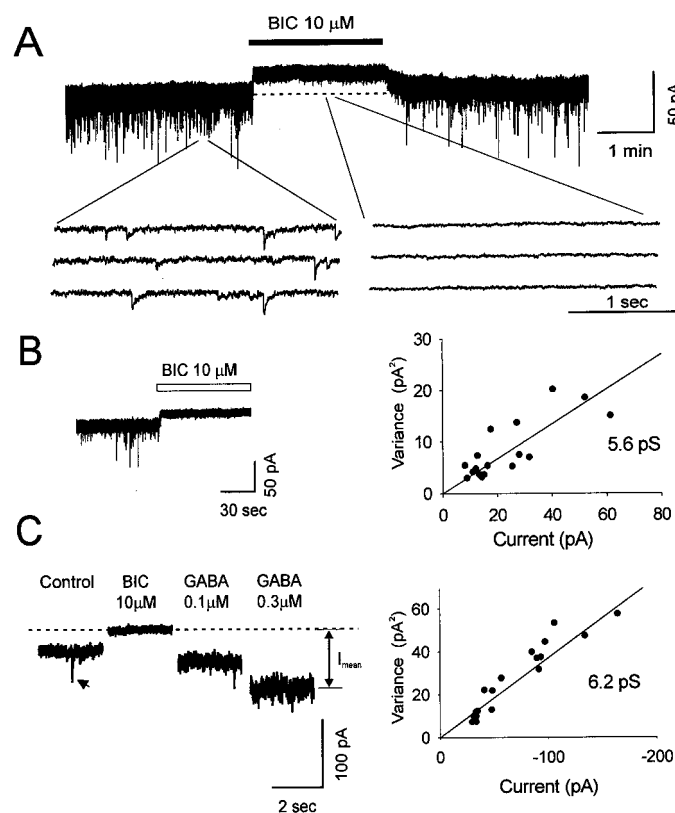
**Simulation.** A general simulator program, Axon Engineer (Aeon Software, Fort Lauderdale, FL; <http://www.pompano.net/~aeonsoft/>) was used to simulate the data. This program allows kinetic states to be defined and linked together by rate constants that can be a function of voltage, ion, and drug concentration. The differential equations implicit in the kinetic scheme are then integrated and driven by user-defined stimuli. The distribution of states in time is converted to open probability by assigning conductance weights to the individual states and summing the system at each time point.

**Statistics.** Results are presented as mean  $\pm$  S.E.M. Differences between groups are considered significant for  $P < 0.05$ , using a paired Student's  $t$  test, unless otherwise indicated.

## Results

**Characteristics of mIPSCs and the Tonic Current in Cultured Hippocampal Neurons.** Miniature IPSCs (Fig. 1A) recorded using whole-cell methods had a mean amplitude of  $40.8 \pm 2.1$  pA ( $n = 44$  neurons) at frequencies ranging from 0.06 to 2.5 Hz ( $0.61 \pm 0.09$  Hz). The mIPSCs had a rapid onset (10 to 90%; rise time,  $2.4 \pm 0.1$  ms;  $n = 44$ ) then decayed with a time course that was generally well fit by a single exponential function ( $\tau_{\text{decay}} = 30.9 \pm 1.1$  ms). Under

control conditions, the frequency of mIPSCs remained constant over the 10 min before drug application. In addition to the transient postsynaptic currents, a persistent or tonic current was revealed after the application of bicuculline (Fig. 1A). Bicuculline (10  $\mu\text{M}$ ) consistently caused an outward current as indicated by an  $18.1 \pm 1.0$  pA, ( $n = 40$ ) outward shift in the holding current. Bicuculline also reduced the variance of the baseline noise from  $11.8 \pm 0.9$  pA<sup>2</sup> to  $6.3 \pm 0.5$  pA<sup>2</sup> ( $n = 9$ ;  $P < 0.01$ ) suggesting that the outward current was in fact caused by the inhibition of a tonic inward current. The tonic current was attributed to activation of GABA<sub>A</sub> receptors (Valeev et al., 1993) because it was also inhibited by another GABA<sub>A</sub> receptor antagonist, picrotoxin (100  $\mu\text{M}$ ;  $19 \pm 3$  pA;  $n = 7$ ), and reversed polarity close to the Nernst potential for chloride ions ( $-3.0 \pm 7$  mV;  $n = 6$ ). This 20 pA current is  $\sim 0.6\%$  of the maximum current recorded in these



**Fig. 1.** Tonic and synaptic GABAergic currents in cultured hippocampal neurons under different experimental conditions. A, the upper trace illustrates currents recorded from cultured neurons in the absence or presence of bicuculline (BIC, 10  $\mu\text{M}$ ). Bicuculline abolished mIPSCs and induced an outward shift of the holding current (20 pA). The dashed line depicts the holding current in the absence of bicuculline. The lower traces are temporal expansions of two short segments. B, the unitary conductance of channels underlying the tonic current was estimated using variance analysis. The segments of the records that contained miniature IPSCs were removed to calculate the current variance. The amplitude of the current ( $I_{\text{mean}}$ ) was measured as the difference in the holding current measured before and after the application of bicuculline as indicated in panel 1C. Data were obtained from 17 neurons and the variance (pA<sup>2</sup>) value was plotted against the amplitude to the bicuculline-sensitive current. The solid line is a linear regression fit to all the data points. The estimated conductance was  $\sim 5.6$  pS. C, inward current was activated by the application of low concentrations of GABA (0.1, 0.3 and 1  $\mu\text{M}$ ) applied to the neurons. The arrow indicates a mIPSC evident under control conditions. The variance was plotted against the mean current amplitude. Data were obtained from seven different neurons and the unitary conductance was estimated to be  $\sim 6.2$  pS.



cells (Orser et al., 1994) and represents activation of  $\sim 0.4\%$  of the receptors (Bai et al., 1999).

It was observed previously (Valeyev et al., 1993) that the tonic GABAergic current in hippocampal neurons was reduced in amplitude when cells were perfused with a stream of saline, suggesting that a diffusible ligand activated the persistent chloride conductance. Under our experimental conditions, we have observed a similar phenomenon. To avoid fluctuations in the ambient concentration of GABA, a constant low perfusion rate was maintained throughout the experiments.

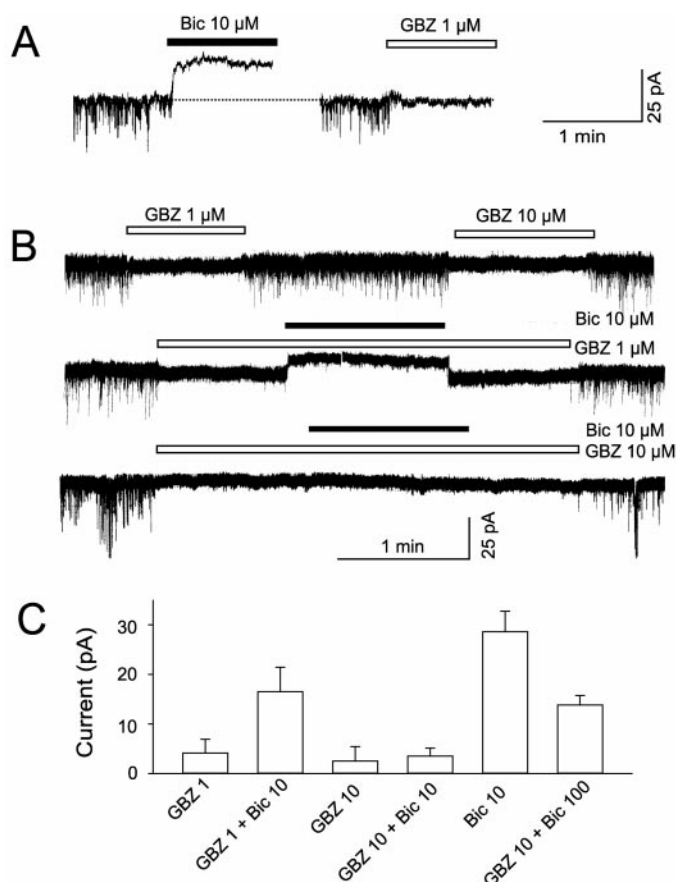
To investigate the biophysical properties of the GABA<sub>A</sub> receptors underlying the tonic current, the mean elementary conductance of the channels ( $\gamma$ ) was estimated from the relationship:  $\gamma = \sigma^2/[I_{\text{mean}} \times (V_H - V_R)]$ . This elementary conductance was then compared with the value for current activated by low concentrations of exogenous GABA (0.1–1  $\mu\text{M}$ ). The relationship between mean current amplitude and current variance is illustrated in Fig. 1, B and C. The unitary conductance for the tonic current was  $\sim 5.6$  pS. This value was similar to the unitary conductance, estimated in the same way, for GABA<sub>A</sub> receptors activated by low concentrations of exogenous GABA ( $\sim 6.2$  pS).

**Gabazine Inhibits mIPSCs but not Tonic Current.** We next tested a series of GABA<sub>A</sub> receptor antagonists to determine whether the tonic and synaptic currents could be distinguished pharmacologically. Notably, the classical GABA<sub>A</sub> receptor antagonists, bicuculline and gabazine, had similar effects on mIPSCs but different effects on the tonic current. Bicuculline abolished the mIPSCs and evoked a large outward shift in the holding current. In contrast, the high-affinity antagonist gabazine (1  $\mu\text{M}$ ) produced no significant shift in the holding current; nonetheless, it completely abolished the mIPSCs (Fig. 2A) ( $n = 12$  cells). These observations suggest that the tonic current does not result from the simple summation of unresolved mIPSCs. Gabazine has a higher affinity for GABA<sub>A</sub> receptors than bicuculline. However, despite this high affinity high concentrations of gabazine (10–20  $\mu\text{M}$ ) did not inhibit the tonic current (Fig. 2B). Analysis of the tonic noise recorded during the application of gabazine (10  $\mu\text{M}$ ) revealed that the unitary conductance of the underlying channels was  $\sim 4.3$  pS ( $n = 15$  cells), comparable with the channels responsible for the tonic current recorded in the absence of gabazine (see above).

If the gabazine-insensitive tonic current were caused by the activation of a population of GABA<sub>A</sub> receptors with subunit composition distinct from synaptic receptors with a low affinity for gabazine, bicuculline should block the tonic current in the presence of gabazine. In an additional series of experiments, when gabazine (10  $\mu\text{M}$  and 1  $\mu\text{M}$ ) was applied alone, it caused no appreciable decrease in the holding current ( $2.5 \pm 2.9$ ,  $n = 5$ , and  $4.1 \pm 2.8$  pA,  $n = 8$ , respectively). Bicuculline (10  $\mu\text{M}$ ) alone caused an outward current of  $28.6 \pm 4.1$  pA ( $n = 8$ ). However, when bicuculline (10  $\mu\text{M}$ ) was coapplied with gabazine (1  $\mu\text{M}$ ), the outward current ( $16.5 \pm 4.9$  pA;  $n = 5$ ;  $P < 0.05$ ) was less than that observed when bicuculline was applied alone. Thus, it seems that gabazine reduced the inhibition by bicuculline. Increasing the concentration of gabazine to 10  $\mu\text{M}$  caused a further reduction in the inhibitory effects of bicuculline as the tonic current was reduced to  $3.5 \pm 1.6$  pA ( $P < 0.05$ ; Fig. 2C). A higher concentration of bicuculline (100  $\mu\text{M}$ ) partially inhib-

ited the current recorded in the presence of gabazine 10  $\mu\text{M}$  ( $13.8 \pm 1.6$  pA;  $n = 6$ ;  $P > 0.05$ ) suggesting a competitive interaction between bicuculline and gabazine. Taken together, the coapplication experiments indicate that gabazine has an affinity for tonic GABA<sub>A</sub> receptors that is approximately 10 times that of bicuculline (see below). The lack of blockade of the tonic current by gabazine, while producing a substantial block of mIPSCs, could not be attributable to gabazine acting as a weak partial agonist. Applications of gabazine at either high (1 mM) or low (1  $\mu\text{M}$ ) concentrations failed to evoke an inward current in low density regions of hippocampal neuron cultures ( $n = 4$ ).

**Gabazine Effects on Tonic Current in Rat Hippocampal Brain Slice.** The complement of GABA<sub>A</sub> receptor subunits changes with cell maturation and tissue culture conditions (Laurie et al., 1992). Consequently, the apparent lack of effect of gabazine on the tonic current might occur only in immature hippocampal neurons grown in dissociated cul-



**Fig. 2.** Gabazine blocks mIPSCs but not the tonic current and reduces the inhibition by bicuculline. A, the tonic current was sensitive to bicuculline (10  $\mu\text{M}$ ) but not inhibited by gabazine (1  $\mu\text{M}$ ). Gabazine (1 or 10  $\mu\text{M}$ ) when applied alone did not cause a significant shift in the baseline current but abolished the mIPSCs. In contrast, bicuculline abolished the mIPSCs and caused an outward shift in the baseline. Current traces were filtered at a high cut-off frequency of 100 Hz. B, coapplication of gabazine and bicuculline caused the baseline to shift less than that observed in the presence of bicuculline alone. C, the bar graph illustrates the change in current amplitude after the application of bicuculline (10  $\mu\text{M}$ ,  $n = 8$ ) or gabazine (1  $\mu\text{M}$ ;  $n = 8$ ;  $P < 0.05$ ). The changes in the amplitude of the current when bicuculline (10  $\mu\text{M}$ ) was coapplied with gabazine 1  $\mu\text{M}$ , ( $n = 5$ ), 10  $\mu\text{M}$  ( $n = 5$ ) or 100  $\mu\text{M}$  ( $n = 6$ ) are also shown. Note that the inhibition of the tonic current was reduced when bicuculline coapplied with gabazine 1 and 10  $\mu\text{M}$  ( $P < 0.05$ ) but not when bicuculline was applied alone.

ture. To determine whether the tonic current was evident in postnatal hippocampal neurons, we next recorded from the hippocampal slice preparation.

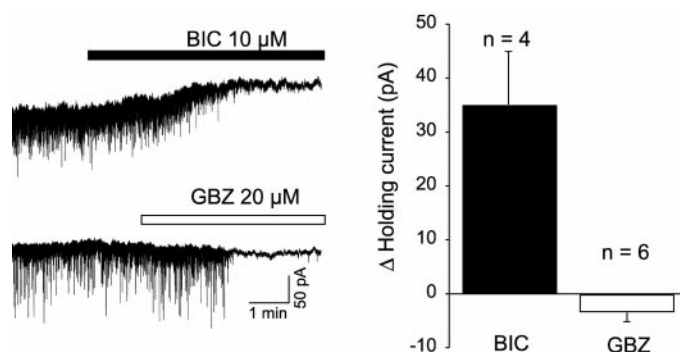
Whole cell recordings from CA1 pyramidal neurons revealed spontaneous mIPSCs as illustrated in Fig. 3. The application of bicuculline (10  $\mu$ M) abolished the mIPSCs and induced an outward shift of the baseline (35.1  $\pm$  9.9 pA) in all four slices tested as described previously (Otis et al., 1991). As in cultured neurons, applications of gabazine (20  $\mu$ M) abolished mIPSCs while causing only a slight, outward shift of the baseline tonic current (3.5  $\pm$  1.7 pA,  $P$  > 0.05). Whole-cell currents were also recorded from acutely isolated neurons obtained from the hippocampal slice to rule out the possibility that the tonic current in postnatal hippocampal neurons was caused by spontaneous opening of GABA<sub>A</sub> channels (Birnir et al., 2000). This preparation provides excellent concentration-clamp conditions that eliminate the exposure to neurotransmitter released from neighboring cells. Both gabazine (1  $\mu$ M–1 mM) and bicuculline (10  $\mu$ M) failed to activate a current in isolated neurons (data not shown) suggesting that spontaneous channel openings did not account for the tonic current. In addition, the lack of response to gabazine again indicates that gabazine does not act as a weak partial agonist.

**The Tonic Current Is Enhanced by Midazolam in Cultured Neurons.** We next tested whether the tonic current evident in cultured neurons was sensitive to a sedative-hypnotic benzodiazepine, as recently reported in granule cerebellar neurons (Leao et al., 2000). Classical benzodiazepines, including midazolam, do not directly activate native GABA<sub>A</sub> receptors in the absence of GABA, but potentiate GABA-evoked channel opening by increasing agonist affinity (Lavoie and Twyman, 1996). The application of midazolam produced an inward current, as illustrated in Fig. 4A. Flumazenil, a specific benzodiazepine antagonist at the GABA<sub>A</sub> receptor, produced no effect when applied in the absence of midazolam but reversed the baseline shift induced by midazolam (Fig. 4B). These results suggest that ambient GABA activates tonic current.

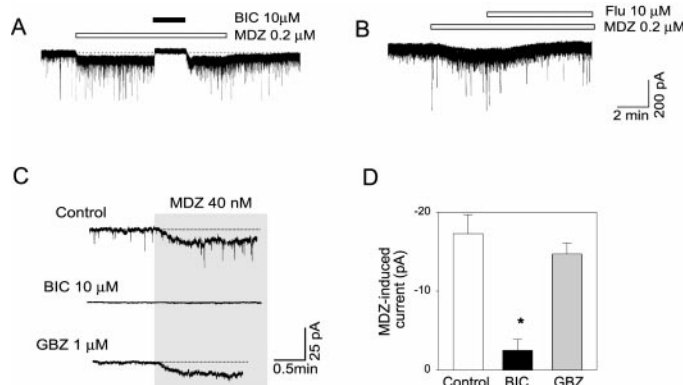
The tonic inward current was increased to a similar extent when midazolam was applied in the absence (17  $\pm$  2.4 pA,  $n$  = 8) or presence (15  $\pm$  1.4 pA,  $n$  = 12,  $P$  > 0.05) of gabazine (Fig. 4, C and D). Furthermore, no mIPSCs were detected in the presence of gabazine despite the increase in the tonic

current by midazolam (Fig. 4C). Examination of the concentration-response relationship (Fig. 5 B) for the enhancement of the tonic current by midazolam indicated that concentrations of greater than 0.2  $\mu$ M caused no further increase in current amplitude. The concentration of midazolam that produced half the maximal enhancement was  $\sim$ 28 nM. This value is consistent with the high affinity of benzodiazepines for GABA<sub>A</sub> receptors identified by binding assays (Johnston, 1996). Maximal enhancement of the GABAergic current was observed with concentrations of midazolam within the nM range whereas the enhancement was reduced at higher concentrations (>1  $\mu$ M), as described previously (Rogers et al., 1994). The increase in the tonic current by midazolam was blocked by bicuculline (Fig. 4A).

We also examined the effects of the intravenous anesthetic, propofol, on the tonic and synaptic currents. Propofol increases the affinity of the GABA<sub>A</sub> receptor for GABA, decreases the rates of dissociation, reduces desensitization and, at higher concentrations, directly activates channel opening (Orser et al., 1994; Bai et al., 1999). We reasoned that if the tonic current results from persistent low concentrations of GABA, then a fraction of this population would desensitize and thus be enhanced by compounds that reduce desensitization. It was predicted that low concentrations of propofol that reduce desensitization but do not directly activate the receptor would produce a greater increase in the tonic current compared with benzodiazepines that do not reduce desensitization but simply increase the apparent affinity for GABA. Applications of propofol induced a shift in the baseline tonic current (Fig. 5, C and D) and the tonic inward current increased in amplitude with increasing concentrations of propofol. Unlike midazolam, the response to propofol did not saturate but continued to increase with concentrations over the range tested (0.2–5  $\mu$ M), as described previously (Orser et al., 1994).



**Fig. 3.** The effects of gabazine and bicuculline on the tonic and synaptic currents recorded in rat hippocampal slices. The application of bicuculline (10  $\mu$ M) abolished the mIPSCs and consistently caused a decrease in the holding current as indicated by an outward shift in the baseline and decrease in the noise. In contrast, gabazine (20  $\mu$ M) inhibited the transient synaptic currents but caused no outward shift, as indicated in the current traces and summarized in the bar graph.



**Fig. 4.** Midazolam increases the tonic current in the absence and presence of gabazine. A, the bicuculline-sensitive tonic current observed after the application of midazolam (bar). The dashed lines indicate the holding current under control conditions and the transient downward deflections represent IPSCs. B, flumazenil (10  $\mu$ M) reversed the increase in the tonic current caused by midazolam. C, current traces from a single neuron illustrate that midazolam (40 nM) produced the same increase of the tonic current when applied in the absence or presence of gabazine (1  $\mu$ M). Midazolam did not change the current amplitude in the presence of bicuculline. D, the effects of midazolam on the tonic current recorded under control conditions or in the presence of bicuculline and gabazine are summarized in the bar graph. Note that the values are compared with the amplitude of the midazolam-induced current. No significant decrease in the tonic current was observed in the presence of gabazine (1  $\mu$ M), whereas bicuculline significantly reduced the effects of midazolam on tonic current ( $P$  < 0.05).

The concentration-response relationships for enhancement in the tonic current by midazolam or propofol are summarized in Fig. 5B, D. Propofol, compared with midazolam, had a lower potency but higher efficacy for increasing the amplitude of the tonic inward current. Unlike midazolam, gabazine (1  $\mu$ M) inhibited responses activated by 1  $\mu$ M propofol by  $\sim 30\%$  ( $70 \pm 8\%$  residual current;  $n = 6$ ;  $P < 0.05$ ). This is consistent with the partial inhibition by gabazine of currents activated by the anesthetics, pentobarbital and alphaxalone (Uchida et al., 1996; Ueno et al., 1997).

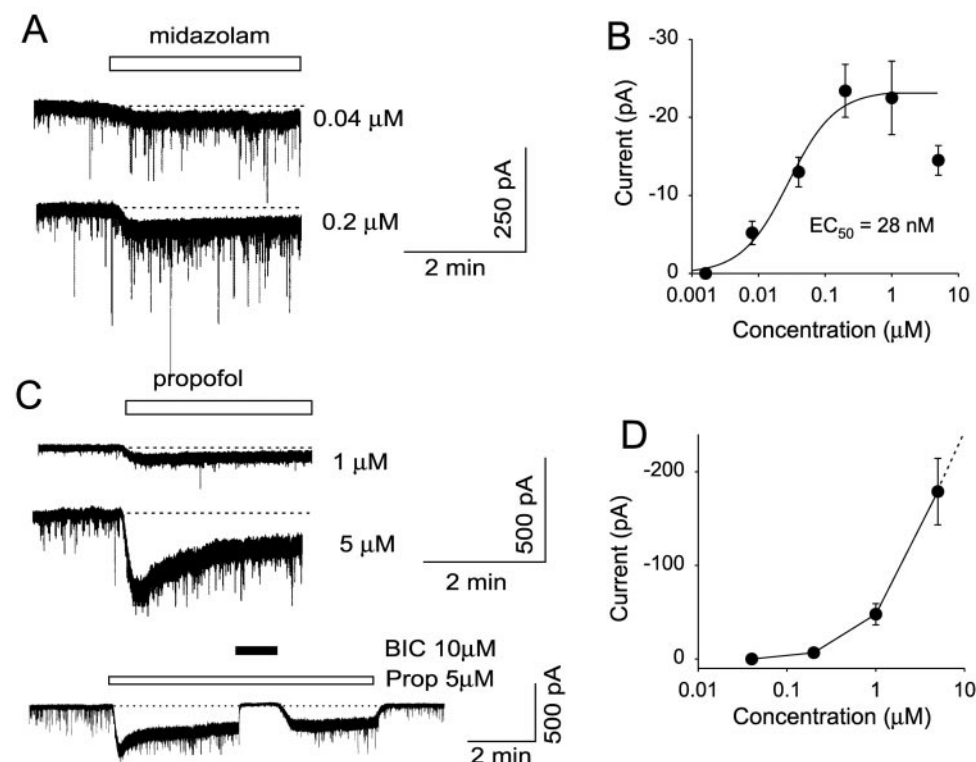
**Comparison of the Relative Increase in the Tonic Current and IPSCs Caused by Midazolam and Propofol in Cultured Neurons.** Compounds that reduce desensitization should enhance the tonic current more than compounds that simply slow dissociation of the agonist. To highlight the influence on deactivation and desensitization, we next compared the effects of midazolam and propofol on the charge transfer associated with the tonic and quantal postsynaptic currents. Changes in quantal charge transfer associated with the mIPSCs are dominated by alterations in the dissociation rate of agonist (Bai et al., 1999). In contrast, changes in deactivation as well as desensitization rates of the receptor should influence the charge transfer associated with the tonic current.

As reported previously, clinically relevant concentrations of midazolam (Otis and Mody, 1992; Poncer et al., 1996; Rovira and Ben-Ari, 1999) and propofol (Orser et al., 1994) produced a concentration-dependent prolongation in the duration of mIPSCs (Fig. 6A). The threshold concentrations of midazolam and propofol that increased the decay time of mIPSCs were approximately 0.04  $\mu$ M and 0.2  $\mu$ M, respectively (Fig. 6A, Table 1). Discrete mIPSCs could be clearly resolved even in the presence of saturating concentrations of midazolam (0.2  $\mu$ M). In contrast, when propofol was applied at concentrations equal to or greater than 5  $\mu$ M, the baseline

noise and tonic current increased such that mIPSCs could no longer be clearly resolved. Table 1 summarizes the changes in the amplitude and time course of the mIPSCs caused by the various concentrations of midazolam and propofol and Table 2 summarizes the effects of these drugs on the frequency of mIPSCs. In addition to slowing the time course of current decay, higher concentration of midazolam, and intermediate concentrations of propofol, increased the frequency of the mIPSCs. This effect of midazolam was not reported for mIPSCs investigated in the CA3 region of the hippocampal slice culture preparation (Poncer et al., 1996).

We next calculated the absolute increase in charge transfer (pC) associated with the two sources of current, as well as the relative change ( $\Delta Q_{\text{drug}}/Q_{\text{control}}$ ) produced by the various concentrations of midazolam and propofol. A simple qualitative comparison indicated that both drugs caused a greater increase in the absolute charge transfer associated with the tonic current compared with mIPSCs (Fig. 6C). For example, midazolam (0.2  $\mu$ M) or propofol (1  $\mu$ M) produced a 21- or 33-fold greater increase in the absolute charge transfer, respectively, for the tonic current compared with the mIPSCs ( $P < 0.05$ ). Although the absolute increase in the charge transfer is greater for the tonic current, this is caused in part by the high baseline tonic current. Therefore, the relative changes in the synaptic and tonic current produced by the various concentrations of midazolam and propofol were also examined as illustrated in Fig. 6D.

The above results describe the change in charge transfer associated with miniature synaptic currents recorded in the presence of TTX. Because the amplitude, frequency, and duration of action potential-dependent spontaneous IPSCs may be greater than those of mIPSCs (Otis et al., 1991), we also compared the effects of midazolam and propofol on the tonic current and synaptic currents recorded in the absence of TTX. The peak amplitude ( $45 \pm 5$  pA,  $n = 8$  cells) and area of



**Fig. 5.** Propofol and midazolam cause a concentration-dependent increase in the amplitude of the tonic current. A and B, the concentration-response relationship for the tonic inward current, recorded in the presence of midazolam, is shown. Each data point represents the averaged values ( $\pm$ S.E.M.) obtained from 5 to 9 different cells. The smooth curve represents the data fit using a modified Hill equation ( $I = I_{\text{max}}/(1 + (C/EC_{50})^{n_H})$ ) where  $I_{\text{max}}$  is the maximal response,  $n_H$  is the Hill coefficient, and  $EC_{50}$  is the concentration that produced 50% of the maximal response (for concentrations  $\leq 1$   $\mu$ M). The  $EC_{50}$  was 28 nM,  $I_{\text{max}}$  was 23 pA, and  $n_H = 1.2$ . C, propofol caused a dose-dependent increase in the tonic current. The tonic current produced by propofol (Prop, 5  $\mu$ M) was reversibly blocked by bicuculline (10  $\mu$ M, bottom trace). D, the concentration-response relationship for the tonic current recorded in the presence of propofol is shown. Each point represents the average values ( $\pm$ S.E.M.) for currents recorded from 5 to 7 different cells. The dotted line indicates that higher concentrations of propofol produce an even greater increase in the current, as we previously reported (Orser et al., 1994).

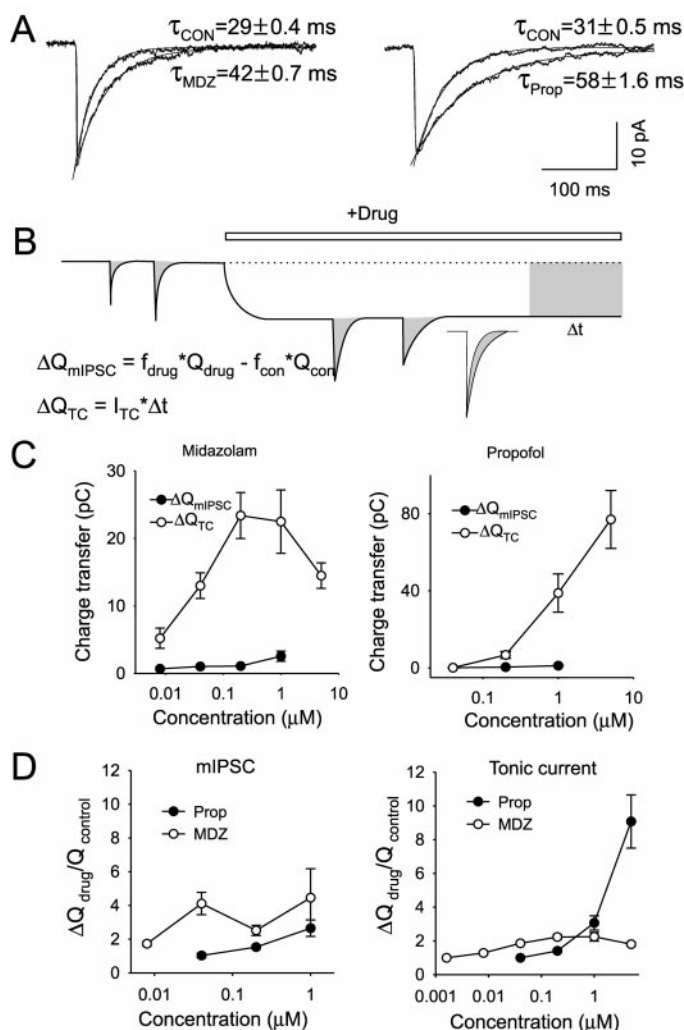


spontaneous IPSC ( $1243 \pm 156 \text{ ms} \times \text{pA}$ ,  $n = 8$ ) were similar to amplitude and area of miniature IPSC ( $40 \pm 2 \text{ pA}$  and  $1243 \pm 80 \text{ ms} \times \text{pA}$ ,  $n = 38$ ,  $P = 0.99$ , respectively). However, the frequency of spontaneous IPSCs ( $1.4 \pm 0.3 \text{ Hz}$ ,  $n = 8$ ,  $P <$

$0.05$ ) was increased (by 2.3-fold). Despite the higher frequency of spontaneous IPSCs, the increase in charge transfer associated with tonic currents by midazolam and propofol was, nevertheless, still considerably more than that associated with the synaptic current. Consistent with our previous results, midazolam ( $0.04 \mu\text{M}$ ) and propofol ( $0.2 \mu\text{M}$ ) caused a 11-fold ( $n = 4$ ) and 32-fold ( $n = 4$ ) ( $P < 0.05$ ) greater increase, respectively, in the charge transfer mediated by the tonic current compared with the spontaneous IPSCs.

**Midazolam and Propofol Interact to Cause a Supra-Additive Increase in the Tonic Current.** To further define the conditions of GABA<sub>A</sub> receptor activation that underlie the tonic current, we investigated the interaction between midazolam and propofol. Isobolographic analysis indicated that midazolam and propofol interact synergistically to increase GABA<sub>A</sub> receptor function when receptors are activated by low ( $<3 \mu\text{M}$ ) but not high concentrations of GABA (McAdam et al., 1998). In contrast, the interaction between these drugs is nonsynergistic when receptors are activated by higher or near-saturating concentrations of GABA. We reasoned that if the tonic current were activated by a low concentration of GABA, then the combination of midazolam and propofol would produce an effect greater than the predicted sum of the effects of each drug alone. We observed that midazolam ( $40 \text{ nM}$ ) and propofol ( $1 \mu\text{M}$ ) caused a supra-additive increase in the tonic current that was greater than that predicted from linear summation (Fig. 7). When the benzodiazepine antagonist, flumazenil, was applied together with propofol and midazolam, the current returned to the amplitude observed when propofol was applied in the absence of midazolam. These results support the suggestion that the tonic current is activated by a low ambient concentration of transmitter.

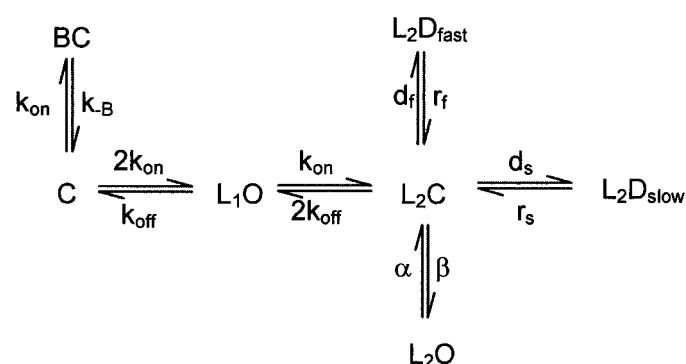
**The Tonic and Synaptic Currents Could Be Mediated by a Distinct Population of Receptors.** The differential pharmacological sensitivity of the synaptic and tonic currents to gabazine could be explained in one of two ways. Firstly, the subunit composition of a distinct population of receptors could render them particularly sensitive to background GABA levels such that they generate the tonic current (Brickley et al., 1996). Alternatively, the receptors underlying the tonic and synaptic current could contain a similar structural complement of subunits, and different states of the receptor account for the differential pharmacological sensitivity. Tonic current may be activated by low persistent concentrations of GABA whereas transient saturating concentrations of GABA activate mIPSCs. Thus, either structural or pharmacodynamic factors could contribute to the different sensitivity of the tonic and synaptic current to gabazine, midazolam, and propofol. Kinetic modeling and computer simulation was used to further explore the characteristics of the tonic current and account for the experimental findings. The apparent lack of competition between gabazine and GABA in receptors responsible for the tonic current led us to examine an allosteric model of gabazine inhibition. The single channel conductance of the tonic channels was estimated to be lower than that of the synaptic receptors activated during mIPSCs. Because low concentrations of exogenous GABA also elicited currents with a low single-channel conductance, we also considered the possibility that monoliganded GABA<sub>A</sub> receptors open to a low conductance state.



**Fig. 6.** Midazolam and propofol produce a greater increase in the charge transfer associated with the tonic current compared with mIPSCs. **A**, the average of 100 to 150 individual mIPSCs is shown before and after an application of  $0.2 \mu\text{M}$  midazolam (MDZ) or  $1 \mu\text{M}$  propofol (Prop). The smooth solid lines indicate the fit of a single exponential function obtained using an iterative nonlinear Levenberg-Marquadt algorithm. The time constants ( $\tau$ ) of the decay phase were consistently increased by midazolam ( $\tau_{\text{MDZ}}$ ) and propofol ( $\tau_{\text{Prop}}$ ). The peak amplitude of mIPSCs was not significantly increased by midazolam ( $0.08$ – $1 \mu\text{M}$ ) or propofol ( $0.04$ – $0.2 \mu\text{M}$ ; see Table 1). However,  $1 \mu\text{M}$  propofol increased the peak current by  $12 \pm 4\%$  ( $n = 7$ ;  $P < 0.05$ ). **B**, the schematic drawings and equations illustrate the methods used to calculate charge transfer per unit of time where  $\Delta Q_{\text{mIPSC}}$  is the increase in charge transfer associated with mIPSCs per second;  $f_{\text{con}}$  and  $f_{\text{drug}}$  are the frequencies of mIPSC under control conditions and during drug application;  $Q_{\text{con}}$  and  $Q_{\text{drug}}$  are the average values for charge transfer per mIPSC under control conditions and during drug applications, respectively;  $\Delta Q_{\text{TC}}$  is the increase in charge transfer associated with the tonic current (represented by the shaded area under the steady-state current amplitude).  $I_{\text{TC}}$  represents the amplitude of the steady state current. **C**, the relationship of midazolam concentration and the charge transfers associated with mIPSCs (●) and with tonic current (○) are shown (left). Midazolam produced a 7- to 21-fold greater increase in charge transfer for the tonic current compared with mIPSCs ( $P < 0.05$ ). Similar to midazolam, propofol produced a 6- to 33-fold greater increase in charge transfer associated with the tonic current compared with mIPSCs (left) and tonic current (right) for propofol (●) and midazolam (○) are shown.

The detailed model used here is not the only explanation for our results but accounts for our findings.

We used a variant of the simple parallel model (Scheme 1) that was previously used to describe the response in these cells to saturating concentrations of GABA (Bai et al., 1999). The model was designed to minimize the number of states, whereas preserving some of the complexity of the system. The rate constants in the scheme, under both control and propofol conditions, are provided in Table 3. In the model presented here, the mono-liganded state was allowed to open to a low conductance state (25% of the doubly liganded state). The background concentration of GABA was selected to produce a response that was 0.4% of the maximal current. At this concentration, the GABA response was primarily attributable to low conductance, mono-liganded receptors, and <10% of the available receptors were in the slow desensitization state.



Bai et al. (1999) concluded that propofol slowed many of the rate constants of the reaction scheme, including the rate of agonist dissociation and the rate of entry into the two desensitized states. This model predicts that propofol causes

a greater increase in charge transfer for the tonic current compared with mIPSCs (2 fold versus 1.5 fold, respectively). For the receptors underlying the tonic current, bicuculline was assumed to bind to the GABA<sub>A</sub> receptor and prevent both GABA and gabazine binding. However, because gabazine did not interfere with activation of the tonic receptor by GABA, it was assumed to interfere with bicuculline binding by an allosteric mechanism. In Scheme 1, all receptor states bind gabazine equally well except for the bicuculline-bound state (BC), which excludes gabazine binding. For clarity, the parallel set of gabazine bound states are not shown. In this model, the addition of a high dose of bicuculline (10  $\mu$ M) causes a substantial inhibition of the tonic current, as shown experimentally. The addition of 10  $\mu$ M or 1  $\mu$ M gabazine has no effect on the tonic current. When the concentration of bicuculline is increased 10-fold to 100  $\mu$ M, bicuculline could overcome the reciprocal allosteric effect of gabazine to compete with GABA and reduce the tonic current. In Fig. 8B, we show the concentration-response relationship predicted for gabazine reversal of bicuculline blockade and its rightward shift caused by increasing bicuculline concentrations. The experimental observations are superimposed on the simulated curves (Fig. 8B, ●). Note the good agreement between the electrophysiological data and predictions of the reciprocal allosteric competition model.

## Discussion

The principal findings of this study are that the tonic and quantal synaptic currents exhibit distinct pharmacological sensitivities to gabazine and bicuculline as well as to two therapeutically important neurodepressive drugs. Simulation studies indicate that our electrophysiological data are consistent with the tonic being mediated by a population of receptors that bind gabazine in a manner that does not

TABLE 1

Dose-dependent actions of propofol (Prop) and midazolam (MDZ) on features of mIPSCs

All data was obtained during the drug application. Relative changes were presented as percentage of control (%).

	Dose $\mu$ M	n	Decay time constant		Amplitude		10–90% rise time		Area	
			ms	%	pA	%	ms	%	pA ms	%
Prop	0.04	5	35.4 $\pm$ 1.8	100 $\pm$ 3	–34.5 $\pm$ 2.8	103 $\pm$ 4	3.9 $\pm$ 0.3	108 $\pm$ 7	1151 $\pm$ 108	99 $\pm$ 10
	0.2	9	39.8 $\pm$ 1.8**	119 $\pm$ 4	–34.6 $\pm$ 3.1	104 $\pm$ 3	3.4 $\pm$ 0.1	112 $\pm$ 11	1423 $\pm$ 138**	137 $\pm$ 10
	1	7	64.3 $\pm$ 4.4**	219 $\pm$ 35	–36.5 $\pm$ 3.8*	112 $\pm$ 4	3.1 $\pm$ 0.1**	128 $\pm$ 5	2427 $\pm$ 305**	247 $\pm$ 17
MDZ	0.008	5	40.7 $\pm$ 3.1	124 $\pm$ 13	–49.6 $\pm$ 3.3	115 $\pm$ 11	2.5 $\pm$ 0.2	99 $\pm$ 6	2092 $\pm$ 105	138 $\pm$ 17
	0.04	5	42.1 $\pm$ 3.0*	130 $\pm$ 9	–50.6 $\pm$ 3.9	110 $\pm$ 6	2.4 $\pm$ 0.2	102 $\pm$ 5	2155 $\pm$ 153	146 $\pm$ 19
	0.2	7	41.5 $\pm$ 2.2*	129 $\pm$ 6	–61.9 $\pm$ 6	113 $\pm$ 7	2.4 $\pm$ 0.3	112 $\pm$ 9	2483 $\pm$ 315	140 $\pm$ 9
	1.0	7	45.5 $\pm$ 4.5*	144 $\pm$ 10	–56.8 $\pm$ 5.1	122 $\pm$ 9	2.2 $\pm$ 0.2	112 $\pm$ 5	2442 $\pm$ 515	157 $\pm$ 19

\*  $P < 0.05$ ; \*\*  $P < 0.01$  (with paired T test).

TABLE 2

Actions of propofol and midazolam on the frequency of mIPSCs

	Dose $\mu$ M	n	Frequency			
			Control	Drug	Wash	%
Prop	0.04	5	0.75 $\pm$ 0.25	0.76 $\pm$ 0.26	N.D.	103 $\pm$ 4
	0.2	7	0.75 $\pm$ 0.31	0.84 $\pm$ 0.30*	N.D.	137 $\pm$ 13
	1	7	0.81 $\pm$ 0.31	0.81 $\pm$ 0.36	0.67 $\pm$ 0.19	109 $\pm$ 15
MDZ	0.008	5	0.66 $\pm$ 0.44	0.82 $\pm$ 0.53	0.72 $\pm$ 0.53	131 $\pm$ 15
	0.04	5	0.23 $\pm$ 0.02	0.64 $\pm$ 0.11*	0.30 $\pm$ 0.04	297 $\pm$ 63
	0.2	5	0.47 $\pm$ 0.08	0.80 $\pm$ 0.15*	0.66 $\pm$ 0.1	170 $\pm$ 21
	1.0	5	0.68 $\pm$ 0.12	1.41 $\pm$ 0.42	1.35 $\pm$ 0.51	196 $\pm$ 37

\*  $P < 0.05$  (paired  $t$  test); N.D., not determined.



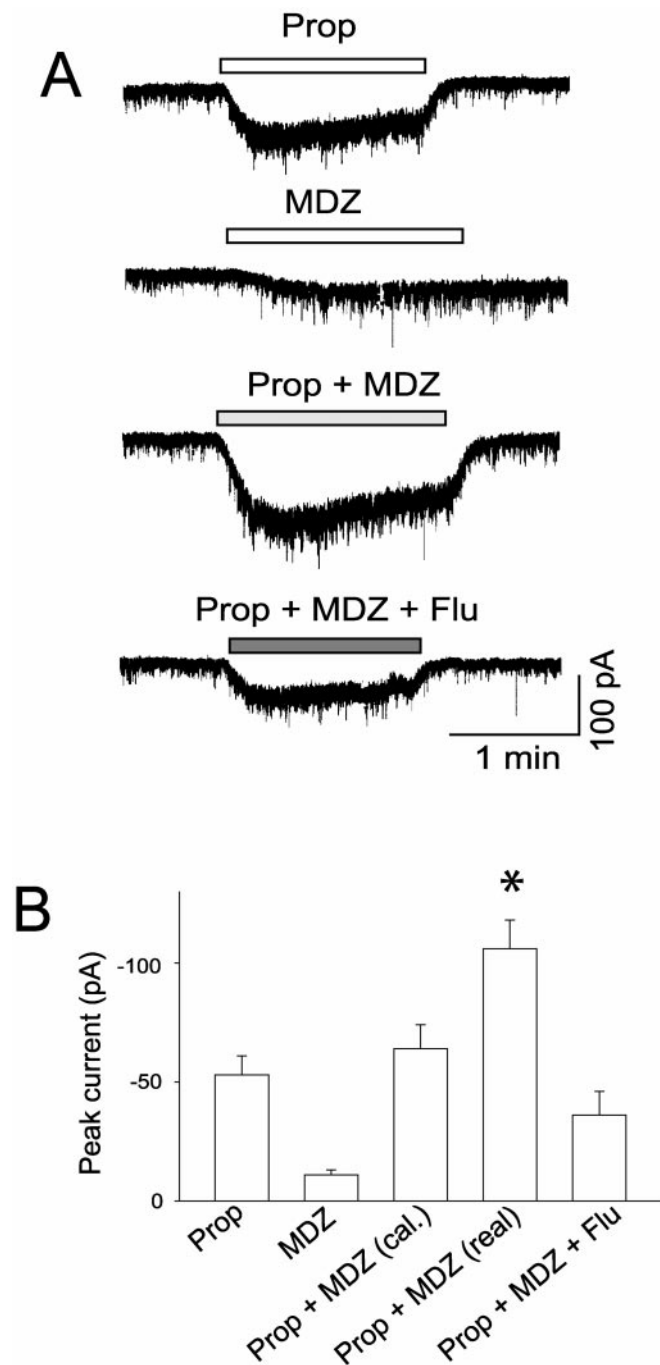
prevent channel opening by GABA. Most importantly, both midazolam and propofol evoked a greater increase in the total charge transfer of the tonic current compared with that associated with the prolongation of synaptic currents. These findings suggest a potential therapeutic role for the population of receptors responsible for the tonic current. Further-

more, we speculate that differences in the effects of midazolam and propofol on the tonic current may account for the differences between sedative and anesthetic compounds that act at the GABA<sub>A</sub> receptor.

The tonic current recorded here was insensitive to gabazine (SR-95531), an aryl-aminopyridazine derivative that selectively binds to low affinity GABA<sub>A</sub> receptors (Bureau and Olsen, 1990). Gabazine and bicuculline are generally considered to act as competitive antagonists of the GABA<sub>A</sub> receptor (Hamann et al., 1988; Ueno et al., 1997). However, gabazine and bicuculline may not have identical mechanisms of action. Bicuculline inhibits currents induced by both GABA and pentobarbital, whereas gabazine does not antagonize current activated by pentobarbital in rat hippocampal neurons (Uchida et al., 1996). Consistent with the notion of distinct receptor populations, gabazine binding was shown previously to coincide with the benzodiazepine 2 site, whereas bicuculline colocalized with muscimol-preferring high-affinity sites (Olsen et al., 1990).

Noise analysis indicated that "low conductance" channels mediated the tonic current. A low unitary conductance ( $\gamma = 6$  pS) was also evident in single channel recordings of GABA<sub>A</sub> receptors from rat hippocampal neurons (Eghbali et al., 1997), and neurons from the rat substantia nigra (Guyon et al., 1999). This unitary conductance is lower than that reported for receptors that mediate quantal synaptic currents in hippocampal neurons ( $\sim 24$ – $28$  pS) (De Koninck and Mody, 1994; Otis et al., 1994) and is lower than the main conductance of GABA<sub>A</sub> receptors studied using single-channel recording methods (Orser et al., 1994). The low conductance state may represent a mono-liganded form of the GABA<sub>A</sub> receptor that predominates when receptors are activated by low concentrations of ligand. Low conductance states activated by low agonist concentrations have been reported for other ligand-gated channels (Smith and Howe, 2000) although direct evidence for concentration-dependent substate gating of GABA<sub>A</sub> receptors is lacking at this time.

The source of GABA that activates the tonic current in culture and slice is not known. The tonic current could be mediated by synaptic receptors that are distant from the vesicular release sites and hence exposed to subsaturating concentrations of transmitter (Mody et al., 1994). Alternatively, spillover of vesicular released GABA could activate receptors located extra-synaptically or at other synapses at which quantal release has not occurred. It remains to be determined whether the receptors underlying the tonic current in hippocampal neurons are localized to synaptic and/or extra-synaptic regions of the cells. Regardless of location, the



**Fig. 7.** Midazolam and propofol cause a supra-additive increase in the tonic current. A, the increase in amplitude of the tonic current by propofol (1  $\mu$ M), midazolam (40 nM), and propofol plus midazolam is shown. B, the bar graph summarizes the changes in amplitude of the tonic current. The peak current amplitudes were measured in the presence of propofol 1  $\mu$ M ( $53 \pm 8$  pA;  $n = 12$ ), midazolam 40 nM ( $11 \pm 2$  pA;  $n = 12$ ), propofol plus midazolam ( $106 \pm 12$ ;  $n = 12$ ) and propofol, midazolam, and flumazenil. The amplitude of the tonic current calculated (cal.) for an additive interaction between midazolam and propofol ( $64 \pm 10$ ;  $n = 12$ ) was less than actual measured (real) response ( $P < 0.05$ ).

**TABLE 3**

The values of rate constants used in the kinetic scheme are presented below

Rates (s <sup>-1</sup> )	Control	Propofol	Control/Propofol
$k_{on}$	$1.0 \times 10^6 \text{ M}^{-1}$	$1.0 \times 10^6 \text{ M}^{-1}$	1.00
$k_{off}$	103	56	1.85
$\beta$	6000	6000	1.00
$\alpha$	400	400	1.00
$D_f$	3000	1620	1.85
$R_f$	200	120	1.70
$R_s$	0.027	0.027	1.00
$D_s$	26	14	1.85
$k_{-G}$	0.1	0.1	1.00
$K_{-B}$	0.9	0.9	1.00

differential responsiveness to nonsaturating and saturating agonist concentrations could lead to differential contributions of the tonic and quantal responses to neurodepressive compounds.

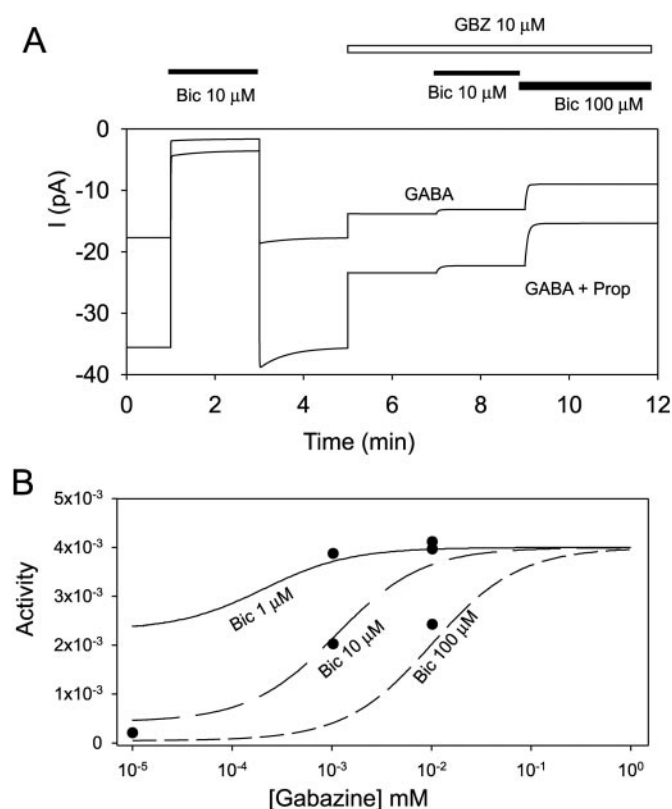
The tonic conductance is not a phenomenon unique to immature neurons. Persistent GABAergic currents have been recorded in the rat slice preparation of postnatal and adult hippocampus (Otis et al., 1991); cortex (Salin and Prince, 1996); and cerebellum (Brickley et al., 1996; Wall and Usowicz, 1997). Furthermore, the relative importance of the tonic current compared with synaptic currents may increase with neuronal maturation. Age-dependent changes in the relative importance of the tonic current and mIPSCs have been reported in postnatal granule cells from rat cerebellum. The magnitude of the tonic current increased during postna-

tal maturation, as did the ratio of charge transfer from the tonic current compared with mIPSCs (Brickley et al., 1996).

**Potential of Tonic Current and Synaptic Currents by Midazolam and Propofol.** GABA<sub>A</sub> receptors activated by persistent low concentrations of GABA are not subject to the same strict temporal and spatial constraints as postsynaptic receptors activated by vesicle-mediated quantal release. Although the amplitude of the tonic current is much less than evoked synaptic currents, the persistence of the tonic current results in a substantial integrated charge transfer. As mentioned above, pharmacological modification of GABA<sub>A</sub> receptors depends on the occupancy of the receptor by GABA and the state of receptor activation and desensitization. The greatest increase in GABA<sub>A</sub> receptor activity produced by benzodiazepines and anesthetics occurs when receptors are activated by low concentrations of transmitter (Harris et al., 1995). Consequently, GABA<sub>A</sub> receptors activated by a low concentration of GABA are likely to be more sensitive to benzodiazepines and anesthetics. Indeed, benzodiazepines and anesthetics caused a relatively greater enhancement of the tonic current compared with synaptic when measured as an absolute increase in charge transfer (Fig. 6C). It is generally assumed that the binding of GABA to the postsynaptic receptor is diffusion-limited, with the peak of the IPSC occurring when the free concentration of GABA is high. Factors that increase agonist binding are not expected to influence the peak amplitude. Accordingly, midazolam and propofol generally exerted little effect on the amplitude of mIPSCs, but instead prolonged their duration. The decay of IPSCs probably occurs during or after the clearance of GABA from the cleft (De Koninck and Mody, 1994). Thus, gating steps and the unbinding of GABA regulate the time course of IPSCs. Presumably, the prolongation of mIPSCs by midazolam and propofol results from a reduction in agonist dissociation.

**Charge Transfer Mediated by IPSCs Compared with the Tonic Current: Clinical Implications.** Although acknowledging that general anesthetics and benzodiazepines influence a variety of neuronal receptors, overwhelming evidence has implicated the GABA<sub>A</sub> receptor as a primary target. A major neurodepressive action of benzodiazepines and anesthetics may be to enhance a tonic GABAergic inhibition as well as prolong synaptic currents. The concentrations of midazolam and propofol used in our experiments are similar to the free concentrations in the plasma, measured in patients during anesthesia. We compared the relative efficacy of propofol and midazolam in increasing the tonic current and low concentrations of propofol (>1  $\mu$ M) activated a greater increase in the tonic current compared with that of saturating concentrations of midazolam.

The relative efficacy of propofol and midazolam to enhance the tonic current, but not synaptic currents, seems to be consistent with important differences in the clinical efficacy of anesthetics and benzodiazepines. Both propofol and midazolam obtund memory and consciousness but only propofol produces a level of neurodepression sufficient to prevent movement in response to painful stimuli. Propofol has a narrow therapeutic index and causes respiratory arrest when administered in excessive doses. In contrast, an overdose of midazolam or diazepam is rarely fatal, suggesting a "ceiling effect". The "ceiling effect" with midazolam but not propofol is also observed for electroencephalogram waveform changes.



**Fig. 8.** Simulations of the effects of bicuculline and gabazine on the GABA-induced tonic and quantal synaptic currents. **A**, simulations of GABA<sub>A</sub>R-mediated activity generated by a tonic application of a low concentration of GABA. The persistent GABA signal generates a tonic current approximately 0.4% of the maximal possible response if all receptors were fully activated. Different combinations of bicuculline and gabazine concentrations on the tonic and synaptic currents, in the presence and absence of propofol were simulated. We selected a concentration of propofol that increased the area of the synaptic-like responses by 1.5-fold and increased the tonic current 2-fold. The dissociation constants of bicuculline and gabazine were set at 0.1 and 0.9  $\mu$ M, respectively. Application of bicuculline (10 and 100  $\mu$ M, solid bars), and gabazine (10  $\mu$ M, open bar) are indicated. **B**, dose-response relations for the effects of gabazine on bicuculline inhibition of GABA<sub>A</sub> receptors in the presence of 0.8  $\mu$ M GABA. At low gabazine concentrations (where GABA-occupied receptors dominate the current), 1  $\mu$ M bicuculline reduces the tonic current somewhat, although 100  $\mu$ M bicuculline causes a near-complete inhibition. ●, the experimentally measured values of tonic current in the presence of different combinations of gabazine and bicuculline. Note the good agreement between the electrophysiological data and the simulations that assumes reciprocal competitive allosteric interactions between bicuculline and gabazine.

Finally, propofol is effective for the treatment of status epilepticus that is refractory to diazepam or midazolam. The extracellular concentration of GABA is reduced in epileptic hippocampi (During et al., 1995) and under these conditions propofol may act as a surrogate agonist and activate a profound increase in tonic inhibition. We speculate that benzodiazepines are comparably less effective because they serve only to potentiate the tonic current, which is abnormally reduced because of the low concentrations of ambient GABA.

In summary, the GABA<sub>A</sub> receptors underlying the tonic current are distinct from those activated during the generation of mIPSCs and quantal synaptic transmission. The tonic channels may serve as a novel target for benzodiazepines and anesthetic drugs and we speculate that an increase in the tonic current contributes to the clinical properties of these drugs.

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