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Embryonic Rat Hippocampal Neurons and $GABA_A$ Receptor Subunit-Transfected Non-neuronal Cells Release GABA Tonically

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Abstract. We used patch-clamp recording techniques to investigate the contribution of GABA to baseline membrane properties in cultured embryonic rat hippocampal neurons. Almost all of the neurons recorded with Cl^--filled pipettes and clamped at negative potentials exhibited baselines that were noticeably noisy, with microscopic fluctuations superimposed on the macroscopic holding current. A gentle steam of saline applied to the neuronal surface rapidly and reversibly reduced the baseline current and fluctuations, both of which were completely eliminated by bicuculline. Fluctuation analysis showed that the variance in the baseline current signal was exponentially distributed with estimated kinetics comparable to those activated by submicromolar concentrations of exogenous GABA. The kinetics of Cl^- channels activated by endogenous GABA displayed a potential sensitivity comparable to those activated by exogenous GABA. Non-neuronal cells stably transfected with α_1 and γ_2 $GABA_A$ receptor subunits exhibited little baseline current variance when recorded with Cl^--filled pipettes. Addition of micromolar GABA to the extracellular saline or to the pipette solution induced a saline- and bicuculline-sensitive baseline current signal comparable to that recorded in hippocampal neurons. Thus, both intra- and extracellular sources of GABA could contribute to the baseline properties recorded in these cultured neurons.

Key words: Patch-clamp — GABA — $GABA_A$ receptor/Cl^- channels — Hippocampus — Embryonic rat

Introduction

During the late embryonic and early postnatal period of hippocampal development there is a transient and widespread expression of GABA, which can be detected in many neurons using immunocytochemical techniques (Rozenberg et al., 1989). Electrical recordings of neurons in acutely prepared slice preparations of the early postnatal hippocampus reveal that many neurons exhibit a resting membrane potential that involves continued activation of $GABA_A$ receptor/Cl^- ion channels (Ben-Ari et al., 1989; Cherubini et al., 1990). The steady-state contribution of a GABAergic component to the resting potential provides a depolarizing signal that disappears by the end of the first week postnatal, as intracellular levels of Cl^- decrease to adult values and spontaneous, fast GABAergic transients appear. The disappearance in slice preparations suggests either that the tonic GABAergic signal is developmentally regulated in a physiologically relevant manner and becomes differentiated with respect to time into intermittent tran-

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sients or that sectioning the hippocampus to create a slice has traumatic effects with different consequences at different times in development. Tonic or ''perpetual'' contributions of GABA to baseline properties have also been recorded in slice preparations of the adult hippocampus and other supraspinal CNS regions (Otis, Staley & Mody, 1991). These baseline GABAergic signals have been attributed to the traumatic effects of sectioning CNS tissue, which results in the accumulation of elevated extracellular GABA levels derived both from high frequency GABAergic transients and a compromised rate of clearance and uptake into intracellular sites.

Electrical recordings of late embryonic hippocampal neurons cultured for hours to days have suggested that these cells also exhibit a steady GABAergic component that contributes to their baseline properties (Valeyev et al., 1993). The presence of a steady-state GABAergic contribution to baseline properties recorded in many well-recovered neurons differentiating over time in culture supports the idea that tonic GABAergic signals may not only involve the trauma necessary in preparation of slices or cultures. The monolayer growth of dissociated hippocampal neurons in culture permitted access to the surface of embryonic neurons and revealed that GABA's contribution involved a surface-accessible compartment, since it could easily be flushed away by directing a gentle stream of saline onto the cells (Valeyev et al., 1993). Fluctuation analysis of the GABAergic baseline showed that the deviating peaks of the microscopic fluctuations in Cl^- current were normally distributed about a mean level, which is characteristic of a random, time-invariant process, and exhibited estimated unitary properties characteristic of $GABA_A$ receptor/Cl^- channels activated by GABA (Valeyev et al., 1993; Vautrin, Schaffner & Barker, 1993). The contribution of $GABA_A$ receptor/Cl^- channels activated by endogenous GABA released from a surface-accessible compartment was quickly transformed by extracellular Zn^{2+} into a nonrandom, time-variant transient signal whose exponential time course(s) of decay were fitted by the same open-state kinetics describing the random activation of $GABA_A$ receptor/Cl^- channels (Vautrin et al., 1993). Together, these results indicate that embryonic hippocampal neurons exhibit a wide dynamic range in GABAergic signaling at $GABA_A$ receptor/Cl^- channels. In the present study, we have confirmed previous results and extended them to show that (i) unloading of endogenous GABA from a surface accessible compartment consistently occurs more rapidly than spontaneous reloading to generate a steady-state baseline, (ii) submicromolar levels of exogenous GABA approximate the steady-state baseline signal involving endogenous GABA, (iii) >95% of the 126 neurons studied exhibit microscopic fluctuations superimposed on the macroscopic baseline, which have exponential kinetics compared to those activated by GABA, and (iv) non-neuronal WSS-1 cells stably transfected with α_1 and γ_2 $GABA_A$ receptor subunit genes express functional $GABA_A$ receptor/Cl^- channels, which can be tonically activated by exogenous GABA, from either extracellular or intracellular sources, to generate a GABAergic baseline comparable to that recorded in hippocampal neurons. Some of these results have been presented in a short communication (Valeyev et al., 1993).

Materials and Methods

Dissociation and Primary Cell Culture of Embryonic Hippocampal Neurons

Hippocampi from 17- and 19-day old rat embryos (E17, E19) were enzymatically dissociated with papain (Worthington, Freehold, NJ; Huettner & Baughman, 1986), then gently triturated into a single-cell suspension and plated onto 35-mm plates (Corning Glass Works, Corning, NY), which had been coated with 20 μg/ml high molecular weight poly-D-lysine. The culture medium consisted of minimal essential medium (MEM) with 3.7 g/l sodium bicarbonate, 6 g/l glucose (Gibco), 5% fetal calf serum and 5% horse serum (Segal, 1983). Cultures were kept for 1–15 days at 36°C in a CO_2 incubator with medium partly exchanged with fresh medium twice each week.

Cell Culture of WSS-1 Cells

WSS-1 cells (CRL 2059, American Type Culture Collection, Rockville, MD) stably expressing rat α_1 and γ_2 $GABA_A$ receptor subunits were cultured as previously described (Wong, Sei & Skolnick, 1992). Cells were grown in DMEM (Quality Biologicals, Gaithersburg, MD) with 4.5 g/l glucose, 2 mM glutamine, 100 μg/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and maintained at 37°C in 5% CO_2 for several days.

Electrophysiological Recording

Recording electrodes were fashioned from 1.5 mm borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) using a three-stage pull on a computer-controlled BB-CH-PC horizontal pipette puller (Mecanex S.A., Switzerland). Whole-cell recordings were carried out at room temperature (22–24°C) using patch pipettes that contained (in mM): 130 CsCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1.1 ethyleneglycol-bis(β-amino-ethylether) = N,N,N′,N tetraacetic acid (EGTA), 5 ATP, 5 N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES), titrated to pH 7.15 with CsOH. Osmolarity was adjusted to 290 mosmol/kg with sucrose. Prior to recording, the culture medium was completely exchanged three times with 2-ml volumes of recording saline containing (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 10 $MgCl_2$ and 5 HEPES, (titrated to pH 7.35 with NaOH) and 0.001 tetrodotoxin (TTX). TTX and elevated $MgCl_2$ (10 mM) were included to eliminate voltage-dependent Na^+ action potentials and to reduce or eliminate spontaneous network-related synaptic activity. The medium was not exchanged during the experiment, but remained stagnant. The osmolarity was adjusted to 310 mosmol/kg with sucrose. This combination of extracellular and intracellular recording salines containing sym-

metrical Cl^- concentrations resulted in a Cl^- equilibrium potential (E_{Cl}) of approximately 0 mV. The holding current delivered by the patch-clamp amplifier L/M EPC-7 (List-Medical-Electronic, Darmstadt, Germany) to control the membrane potential was bandpass-filtered at 0.1 Hz–1 kHz, and stored on a PCM VCR system (VR100, Instrutech, Elmont, NY) using wide bandwidth recording (0–9 kHz).

Pharmacology

Bicuculline and picrotoxin (Sigma, St. Louis, MO) and ryanodine (RBI, Natick, MA) were prepared as concentrated stock solutions in methanol. SKF-89976-A was prepared in ethanol while thapsigargin (RBI, Natick, MA) and BAPTA-AM (Molecular Probes, Eugene, OR) were prepared in dimethyl sulfoxide (DMSO). Each stock solution was diluted with extracellular recording saline at the time of experiment. The final ethanol or DMSO concentration was never greater, and usually less than 0.2% (v/v) and neither vehicle altered steady-state baseline properties when used at the same dilutions as employed for the ligands. Concentrated GABA was stored in distilled water and all concentrated stock solutions were kept frozen. Diluted stock solutions were prepared fresh in extra- or intracellular salines for each experiment. Saline was applied to the recorded neuron by pressure (<10 psi), which generated a gentle stream, from patch-type pipettes with tips of ~2–3 μm in diameter. The saline pipettes were positioned within 5–7 μm of the recorded neuron; GABA and picrotoxin were also applied by less pressure for short periods. Some ligands were introduced to the recorded neuron by slow diffusion to a final concentration in the 1-ml volume of the recording saline.

Fluctuation Analysis

We employed established techniques in fluctuation analysis to estimate the unitary properties of Cl^- channel activity contributing to the baseline (Neher & Stevens, 1977). Briefly, membrane currents were recorded at low gain as a DC signal, and then amplified and filtered to allow computer-assisted quantitative analysis of membrane current fluctuations. The stored signal was played back, then bandpass-filtered using filter model 3322R (Krohn-Hite, Cambridge, MA) for high-pass at 0.1 Hz and filter model 901F (Frequency Devices, Haverhill, MA) for low-pass filtering at 1 kHz. The data were analyzed using Strathclyde Electrophysiological Software (Dr. John Dempster, University of Strathclyde, Glasgow, Scotland). The variance of filtered membrane current, σ^2, was integrated every second, converted to voltage, and stored on a PC computer in noncontiguous records of 2048 points. σ^2 was calculated from $\sigma^2 = \int_0^\infty S(f)df$ where f is the frequency and $S(f)$ is the spectral density. Spectra were fitted with a $1/f$ function or with multiple Lorentzian functions of the form $S(f) = S(0)/[1 + (f/f_c)^2]$ where $S(0)$ is the value of S at zero frequency and f_c is the corner frequency at which power in a component falls by one-half of its asymptotic value. Spectra were fitted using the software Kaleidagraph (Synergy Software, Reading, PA) on a Macintosh computer. The number of Lorentzian components was always increased until no further statistical improvement in the fit could be obtained.

Assuming that Lorentzian kinetics approximate the activity of each population of two-state (open-closed) ion channels, then the exponential distribution of the open, conducting state, τ, of each component can be estimated from $\tau = (2\pi f_c)^{-1}$ (Neher & Stevens, 1977). When single channels in excised outside-out patches of embryonic hippocampal neurons have been recorded directly during sustained exposure to GABA transitions to the open, conducting state exhibit briefly interrupted closings that lead to a ''burst'' (Liu et al., 1996) similar to the activity of Cl^- channels opened in patches by GABA and initially reported by Bormann, Hamill & Sakmann (1987) and by most, if not all investigators since. Hence, long-lasting τ values derived from low-frequency components likely reflect ''burst-length durations'' composed of channel openings having many brief interruptions. The average conductance, γ, can be estimated from the relationship $\gamma = \sigma^2/(\Delta I \cdot V_D)$, where σ^2 is the membrane current variance ΔI is the membrane current change induced by experimental perturbations (saline, bicuculline, picrotoxin or GABA) and V_D is the driving force underlying the current signal, or potential difference between the holding potential and the Cl^- ion equilibrium potential (E_{Cl}), which is set at 0 mV with symmetrical Cl^- concentrations. Transitions in current in response to GABA recorded at the single-channel level can exhibit preferred levels and subconductance states (Bormann et al., 1987). Thus, an estimate of γ could be the algebraic sum of all conductance states. However, thus far only a single level of elementary current activated by GABA has been recorded in membrane patches excised from cultured embryonic hippocampal neurons (Liu et al., 1996).

Results

Most Embryonic Hippocampal Neurons Exhibit Surface-accessible GABAergic Baselines

We consistently recorded microscopic fluctuations in the macroscopic baseline current signal at negative potentials in Cl^--loaded cells dissociated from the late embryonic hippocampus and cultured in serum-containing medium for hours to weeks (Fig. 1*a* in left-hand pair of traces). Noticeably noisy baselines were recorded in completely isolated cells, all of which exhibited processes and morphologies characteristic of neurons. The fluctuating baseline current signal was recorded in TTX or elevated $MgCl_2$ and completely transformed within about a minute into synaptic-like transients after exposure to extracellular Zn^{2+} (Vautrin et al., 1993). These results led to the conclusion that the fluctuating baseline current signals represent a continuous form of signaling involving random activation of Cl^- ion channels by GABA, which can be rapidly transformed into fast GABAergic transients. Almost all of the 126 embryonic hippocampal neurons included in the present study exhibited such baseline signal fluctuations with varying degrees of intensity.

The fluctuating baseline current was sensitive to extracellular saline applied using low pressure (<10 psi), which decreased the DC signal and coincidentally reduced the intensity and amplitude of the associated microscopic fluctuations apparent in the high-gain, AC-coupled trace (Fig. 1*b* in left-hand pair of traces; *also see* Fig. 4*A*). All neurons tested with noticeably noisy baselines at negative clamp potentials were saline-sensitive, with the DC current signal decreasing to a variable extent (~10–40 pA; $n = 37$). The significant changes in low-gain, DC and high-gain, AC-coupled current traces typically persisted for many seconds after the saline was

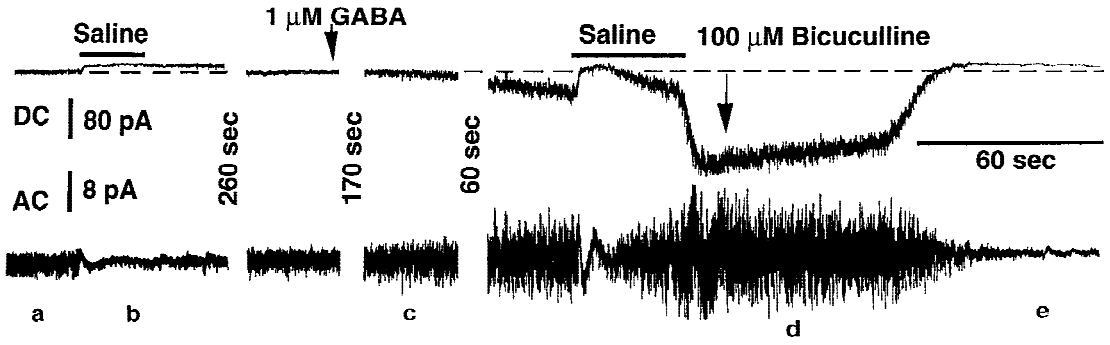

Fig. 1. Saline- and bicuculline-sensitive baseline current signal in a cultured embryonic hippocampal neuron. The neuron was clamped at −60 mV with a CsCl-filled patch pipette, and E_{Cl} set at ~0 mV. Low-gain DC and high-gain AC-coupled traces reveal a baseline current signal superimposed with microscopic fluctuations (pair of traces marked *a*). Close application of a gentle stream of saline to the cell surface for the period indicated rapidly decreases the clamp current ~20 pA and markedly reduces the ambient fluctuations (traces marked *b*). Following cessation of the saline stream the baseline current signal and associated fluctuations both gradually recover to their control levels over a four-minute period. Introduction of GABA into the medium followed by several minutes slow diffusion to a final concentration of 1 μM results, after a delay, in a progressive increase in inwardly directed current together with fluctuations of greater intensity and amplitude (traces marked *c*). A gentle stream of saline at the time indicated momentarily reduces the current signal induced by slowly diffusing GABA. After the GABA-induced current signal peaks and plateaus (traces marked *d*) slow diffusion of bicuculline (to a final concentration of 100 μM) blocks all of the current signal after a delay, leading to a baseline comparable to that recorded during the initial stream of saline (compare traces marked *e* with those marked *b*).

terminated, as can be seen in Figs. 1 and 4. Complete recovery to the initial baseline current levels together with intense fluctuations usually required more than one minute (Fig. 1, second pair of traces between *b* and *c*). During whole-cell recordings lasting ~30 min the baseline signals remained relatively stable and gentle puffs of saline induced similar, reversible changes of similar magnitude and time course ($n = 4$ cells; *see* Fig. 3). Thus, these cultured hippocampal neurons exhibit baseline properties, which are dominated by a steady-state process that can be interrupted by a gentle stream of saline.

Previous research has shown that the surface-accessible process associated with a saline-sensitive baseline could be completely eliminated by introducing bicuculline into the medium at concentrations sufficient to block GABA's activation of $GABA_A$ receptor/Cl^- channels, which are expressed by virtually all cultured embryonic rat hippocampal neurons (Valeyev et al., 1993). These initial results implied that activation of $GABA_A$ receptor/Cl^- channels by endogenous GABA generated the surface-accessible baseline properties. To compare the putative endogenous GABAergic activity with that induced by a known concentration of exogenous GABA, a high concentration of GABA in a small volume was discretely introduced into the recording medium. All 5 neurons exposed to slow diffusion of GABA responded in the same manner. Typically, after several minutes delay, baseline fluctuations began to intensify accompanied by modest (~10–20 pA) changes in the DC current level (Fig. 1, pair of traces marked *c*). Over the next 4–5 min, the baseline current signal gradually increased in a smooth and progressive manner in parallel with ever-intensifying microscopic fluctuations, reflecting the activation of Cl^- channels by exogenous GABA (Fig. 1, final pair of traces). The progressive changes could be momentarily interrupted by a gentle stream of saline. Following cessation of the saline stream the current response induced by GABA rapidly recovered and eventually asymptoted at levels several hundred pA greater than that of the original baseline. Slow diffusion of bicuculline in the bath completely abolished the asymptotic current response to exogenous GABA and reduced the macroscopic baseline current level and accompanying microscopic fluctuations to levels comparable to those recorded during the gentle stream of saline (Fig. 1, final pair of traces marked *d* and *e*).

Collectively, these results support the notion of a steady-state equilibrium between endogenous GABA released from a surface-accessible compartment and tonic, random activation of $GABA_A$ receptor/Cl^- channels. Further, submicromolar concentrations of exogenous GABA approximate the endogenous equilibrium.

$GABA_A$ Receptor/Cl^- Channels Activated by Endogenous and Exogenous GABA Have Comparable Properties

We used fluctuation analysis to infer the unitary properties of $GABA_A$ receptors/Cl^- channels activated by endogenous and exogenous GABA in five neurons using the same experimental paradigm outlined in Fig. 1. Results similar to those illustrated in Fig. 2 were found in all five neurons. Power density spectra calculated for the baseline current variance resulting from the equilibrium between endogenous GABA and $GABA_A$ receptor/Cl^-

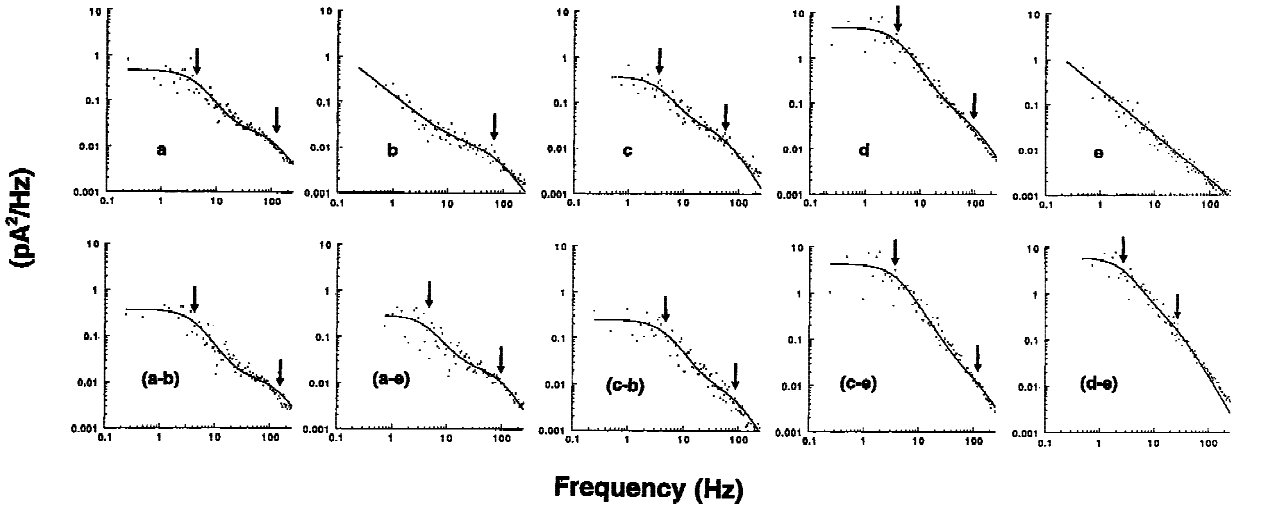

Fig. 2. Endogenous baseline noise exhibits kinetic components similar to those induced by submicromolar GABA. Power density spectra were calculated for the membrane current fluctuations associated with epochs *a-e* illustrated in Fig. 1. Corner frequencies (f_c) derived from statistical fitting of calculated spectra are identified by downward arrows. (*a*) f_c values of 4.3 Hz and 150.2 Hz correspond to τ_{Long} of 38.1 msec and τ_{Short} of 1.3 msec, respectively; (*b*) Low frequency power is lost during saline application, leaving some power at the higher frequencies whose f_c yields a τ_{Short} = 2.3 msec; (*a-b*) saline-sensitive ''difference'' spectrum: τ_{Long} = 37 msec, τ_{Short} = 1.1 msec; (*c*) initial increase in current variance induced by submicromolar GABA: τ_{Long} = 44.2 msec, τ_{Short} = 2.8 msec; (*c-b*) GABA and saline-sensitive ''difference'' spectrum: τ_{Long} = 35.4 msec, τ_{Short} = 1.8 msec; (*d*) asymptotic level of GABA-induced current variance is distributed with τ_{Long} = 43 msec and τ_{Short} = 1.7 msec; (*e*) after slow diffusion of bicuculline, all of the exponentially distributed power is eliminated, leaving a 1/*f* spectrum; (*a-e*) bicuculline-sensitive ''difference'' spectrum reflecting both initial [GABA] and underlying baseline signals: τ_{Long} = 41.9 msec, τ_{Short} = 1.4 msec; (*d-e*) bicuculline-sensitive ''difference'' spectrum at asymptotic [GABA]: τ_{Long} = 58.9 msec and τ_{Long} = 6.3 msec.

channels were composed primarily of two (or three) Lorentzian components, reflecting exponentially distributed channel openings or burst-durations (Fig. 2*a*). In the cell illustrated, saline preferentially eliminated more of the power associated with the low-frequency component (Fig. 2*b*). Therefore, ''difference'' spectra derived from subtraction of the baseline current signal variance remaining in saline (Fig. 2*b*) from that recorded initially (Fig. 2*a*) revealed a two-component spectrum (Fig. 2*a* and *b*) whose inferred kinetics closely paralleled those calculated under control conditions. This indicates that the steady-state equilibrium recorded under these experimental conditions is largely dominated by continuing activation of $GABA_A$ receptor/Cl^- channels many of which exhibit distinct, exponentially distributed kinetics. After exposure to exogenous GABA, fluctuation analysis consistently revealed two-component spectra with kinetics similar to those calculated under control conditions for Cl^- channels activated by endogenous GABA (Fig. 2*c*). The difference spectra (Fig. 2*c* and *b*) also exhibited two components, which were comparable to those calculated for saline-sensitive spectra (Fig. 2*a* and *b*). Spectra calculated after complete equilibration with exogenous GABA, likewise exhibited two kinetic components similar to those calculated both for Cl^- channel activity generated by endogenous GABA and for submicromolar GABA (Fig. 2*d*).

Spectra calculated after bicuculline were completely devoid of Lorentzian components, since bicuculline blocked the tonic activation of $GABA_A$ receptor/Cl channels whose summed activities underlie the exponentially distributed power in the signal. All of these could then fit by a 1/f term (Fig. 2*e*). Subtraction of this baseline from those generated by endogenous and exogenous GABA revealed bicuculline-sensitive contributions to each that were generally comparable (Fig. 2*a-e*, *c-e* and *d-e*). Hence, exogenous GABA stimulates $GABA_A$ receptor/Cl^- channels whose open-state kinetics are generally quite comparable to those activated by endogenous GABA.

We estimated the elementary conductance of $GABA_A$ receptor/Cl^- channels, γ, from the relationship $\gamma = \sigma^2/\Delta\,(I \cdot V_D)$. The decrease in baseline current (ΔI) at the holding potential (−60 mV) induced by a stream of saline, by diffusion of bicuculline in the bath or by close application of bicuculline together with the corresponding changes in associated membrane current variance (σ^2) provided the denominator and numerator, respectively, to estimate γ values of channels activated by endogenous GABA. The difference between the current activated by exogenous GABA and that remaining after bicuculline together with the corresponding variance difference provided the denominator and numerator for estimating γ values of channels activated by exogenous GABA. A representative account of σ^2 changes resulting from a stream of saline and from diffusion of bicu-

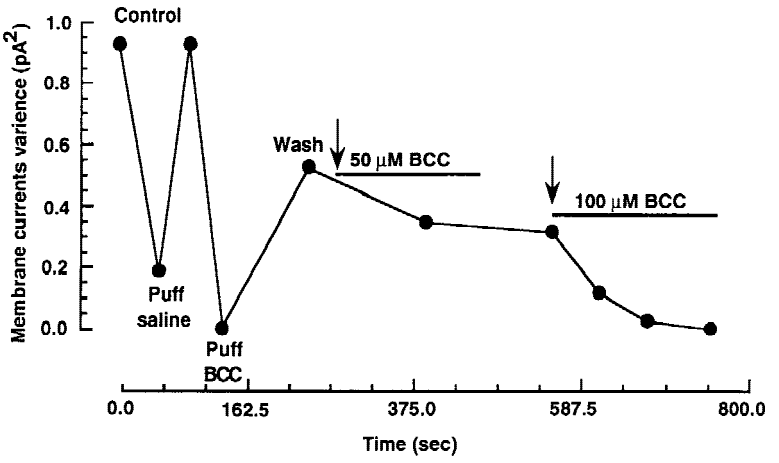

Fig. 3. Saline- and bicuculline-sensitive baseline current variance. The neuron was clamped at −60 mV as in Fig. 1. Average values of baseline variance are plotted at various times during a typical experiment. Gentle application of saline (''puff saline'') reduces variance by ~80% in a completely reversible manner. Close application of 100 μM bicuculline (''puff BCC'') eliminates virtually all of the ambient variance and this is followed by partial recovery, since the medium is not perfused continuously. Slow diffusion of bicuculline first to a final concentration of 50 μM and then to 100 μM leads to stepwise loss and virtual elimination in the variance of the current signal.

culline is shown in Fig. 3. There was some variability in the estimated γ values, which ranged from 10 to 18 pS for saline-sensitive ($n = 37$ cells) and 12 to 20 pS for bicuculline-sensitive signals activated by endogenous GABA ($n = 33$ cells). Comparable γ values were also obtained when the effects of saline and bicuculline were measured directly on the same cell together with estimated γ values associated with Cl^- channels activated by submicromolar GABA. These results indicate that exogenous GABA stimulates $GABA_A$ receptor/Cl^- channels with unitary properties comparable to those activated by endogenous GABA. The estimated unitary conductance range for activated Cl^- channels indicates that the baseline current signal is derived from the summed activities of ~10–40 channels.

Picrotoxin also Blocks Endogenous $GABA_A$ Receptor/Cl^- Channel Activity

Since picrotoxin is well established to block $GABA_A$ receptor/Cl^- channel activity in cultured hippocampal neurons (Segal & Barker, 1984*a*), we compared the effects of saline and picrotoxin ($n = 3$). In the cell illustrated, which exhibited a single-component spectrum, saline eliminated almost all traces of low frequency Lorentzian contributions, thus resulting in a ~1/f spectrum (Fig. 4*A* and *B*) and the ''difference'' spectrum was well-fitted by a single Lorentzian term (Fig. 4*B*). When the cells were depolarized and clamped at E_{Cl} (~0 mV) there was no evidence of microscopic fluctuations (Fig. 4*A c*), as expected, and close application of saline had no effect, eliminating purely technical considerations from explaining the effects of saline at negative potentials. The resulting spectra calculated at 0 mV were all ~1/f in form (Fig. 4*B*). Return to a negative clamp potential restored the driving force acting on Cl^- channels and thus the baseline current fluctuations. Close application of 100 μM picrotoxin decreased the baseline signals (Fig. 4*A-D*) and eliminated all of the power distributed over the lower frequency range (Fig. 4*B*). In the cell shown, the resulting ''difference'' spectrum was also better fitted by two, rather than one component. The effects of picrotoxin to alter the endogenous signal outlasted the effects of saline by many seconds (Fig. 4*A*) and were also reproduced by slowly diffusing picrotoxin into the medium (*not shown*). These pharmacological effects of picrotoxin to alter the baseline signal provide further evidence that the endogenous signal involves GABA acting at $GABA_A$ receptor/Cl^- channels.

Potential Sensitivity of Cl^- Channel Kinetics Tonically Activated by Endogenous GABA Resembles that of Channels Activated by Exogenous GABA or Occurring Physiologically During Fast GABAergic Transmission

We evaluated the possibility that the endogenous $GABA_A$ receptor/Cl^- channel activity might exhibit potential sensitivity, since depolarization of cultured embryonic rat hippocampal neurons to positive potentials is associated with increases both in burst-length duration of pharmacologically activated Cl^- channels (Segal & Barker, 1984*a*) and in the exponential time constant of physiologically occurring GABAergic Cl^- transients at synapses (Segal & Barker, 1984*b*). Stepwise depolarization of four neurons over a 120 mV range of potential revealed changes in the overall variance of the signal and in the estimated values of the two Lorentzian components that describe the exponentially distributed channel openings (Fig. 5). Low- and high-frequency Lorentzian components in the baseline signal were readily apparent at both negative and positive potentials, but always dis-

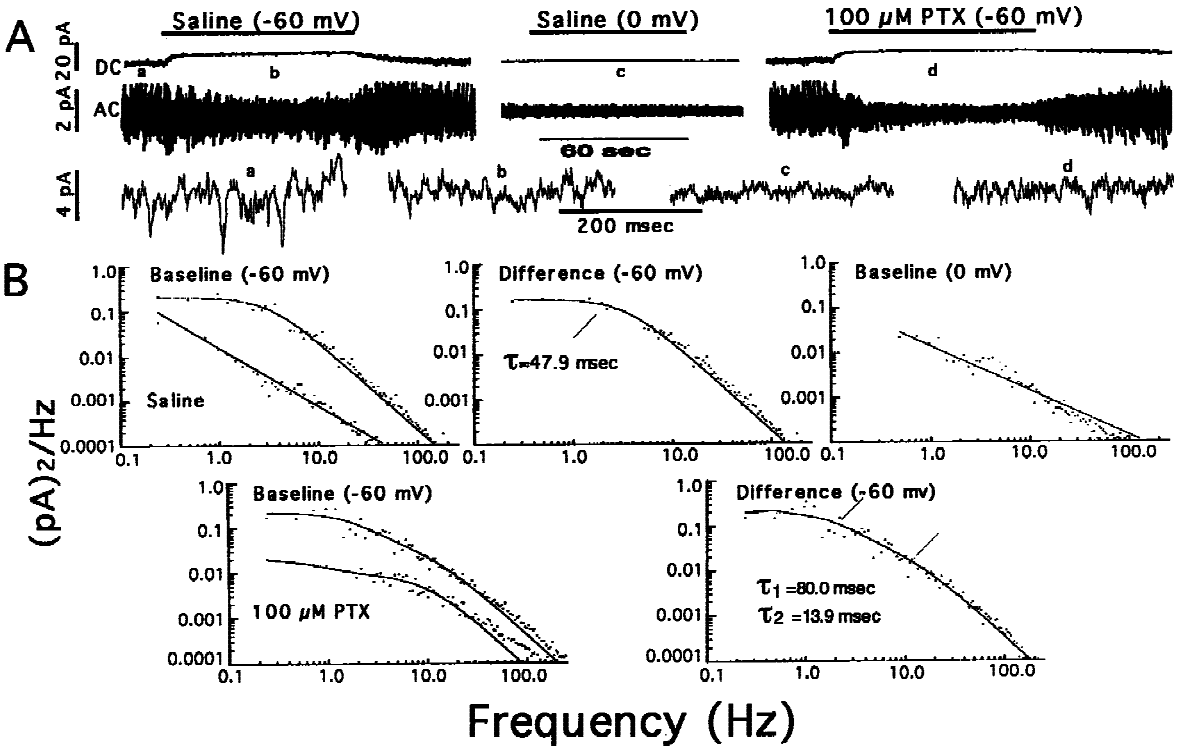

Fig. 4. Picrotoxin also blocks baseline membrane current signals in embryonic hippocampal neurons. The neuron was clamped at −60 mV and patch pipettes with saline or saline containing 100 μM picrotoxin (PTX) positioned near the cell. (*A*) Gentle application of saline rapidly reduces the baseline current signal, which slowly recovers after cessation of the stream. Polarization of the cell to 0 mV ($\sim E_{Cl}$) eliminates all of the most intense fluctuations in the current signal. Return to −60 mV recovers the fluctuating baseline, which is blocked by close application of PTX, with recovery requiring significantly longer than after saline. (*B*) Spectral plots of the baseline current fluctuations (*a* in panel *A*), the residual signal remaining in the gentle stream of saline (*b*), the current signal at 0 mV (*c*), the return to baseline at −60 mV and the residual signal in locally applied PTX (*d*). Spectra of baseline currents at −60 mV exhibit Lorentzian components, while during saline or at 0 mV the spectrum becomes 1/*f*. Picrotoxin blocks most of the power in the low frequency range.

appeared completely at ~0 mV, as expected for a Cl^--dependent conductance recorded in symmetrical [Cl^-] (Fig. 5*A; see also* Fig. 4*A*). Plots of membrane current variance as a function of potential were U-shaped with a nadir at 0 mV (Fig. 5*B*), with consistently more variance in each cell at positive potentials and driving forces (Fig. 5*A*). When quantified, we found that variance markedly increased in a nonlinear manner at positive potentials (Fig. 5*B*). Spectral analysis revealed characteristic two-component spectra at negative and positive potentials and a 1/f distribution at 0 mV (Fig. 5*C*). The two Lorentzian components calculated for baseline fluctuations had significantly lower corner frequencies at positive potentials than those at negative potentials, indicating both longer duration short and long Cl^- channel openings. These results show that the kinetics of Cl^- channels randomly activated by endogenous GABA exhibit potential sensitivity comparable to that previously reported for channel kinetics activated by exogenous GABA on hippocampal neurons and to that reported for the physiological time course of decay of GABAergic transients at synapses between cultured hippocampal neurons.

Heterogeneity of Cl^- Channel Kinetics Activated by Endogenous GABA

We studied the variability in Cl^- channel kinetics activated by endogenous GABA in cultured embryonic hippocampal neurons clamped at a putative resting membrane potential (e.g., −60 mV). We found that ~96% of the spectra calculated for fluctuating baseline currents were fitted best by either two or three Lorentzian components (Fig. 6). In the remaining population, the spectra were either best fitted by a single Lorentzian or a 1/*f* function. We found a wide range in the estimated τ values derived from the multicomponent spectra. τ_{short} ranged from 1.1 msec to 48 msec, while τ_{long} ranged from 16.4 msec to 353 msec for two-component spectra. τ_{short} ranged from 0.4 msec to 13.1 msec, τ_{medium} from 6.2 msec to 90 msec and τ_{long} from 42.3 msec to 6.2

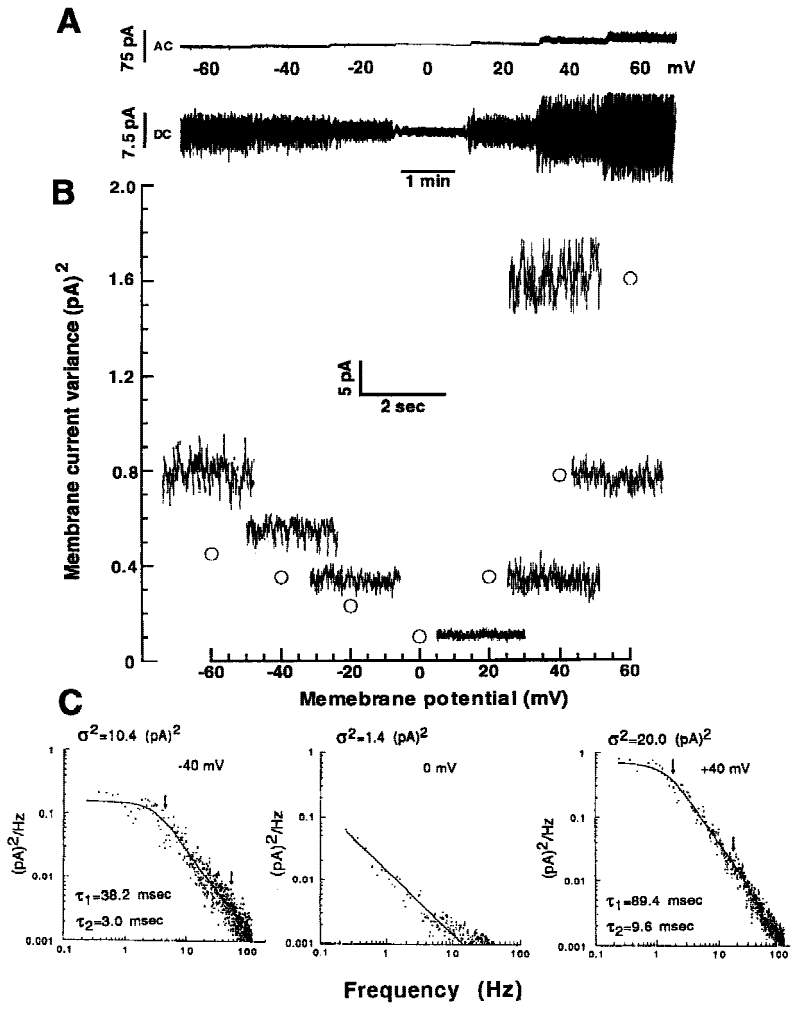

Fig. 5. Potential sensitivity of baseline current variance. The cell was initially clamped at −60 mV, then commanded over a 120-mV range to more depolarized potentials. (*A*) Low- and high-gain traces show the stepwise disappearance of the fluctuating signal intensity as the cell is depolarized, with a nadir at 0 mV (E_{Cl}) and the progressive reappearance of fluctuations with even greater intensity at positive potentials. (*B*) Quantitative averages of variance at each potential (open circles) are plotted as a function of membrane potential. High-gain traces illustrate the frequency and intensity of fluctuations near each data point. There is significantly more variance at positive potentials even at comparable driving forces. (*C*) Spectral plots of current signals show two Lorentzian components (arrowheads identify corner frequencies) at −40 mV and +40 mV and a 1/*f* spectrum at 0 mV. The estimated τ values for the two components are increased at the positive potential.

msec. The heterogeneity in estimated τ values for the statistically fit spectra was so great that standard deviations of mean values was either comparable to, or a significant proportion of the mean. The variability was not obviously due to time in culture or specific neuronal morphology since similar values were recorded both at early and late times in culture and in neurons with quite different morphologies in the same field (*not shown*). Furthermore, the individual τ values estimated for exponentially distributed, short, medium and/or long channel open-times on different neurons were not significantly correlated, suggesting that the different Lorentzian components were distinct and not necessarily related to each other (*not shown*).

$GABA_A$ Receptor Subunit-Transfected Non-neuronal Cells Can Release Exogenous GABA Tonically

The source of the surface-accessible compartment of endogenous GABA underlying continuing activation of $GABA_A$ receptor/Cl^- channels in hippocampal neurons was investigated with non-neuronal (WWS-1) cells stably transfected with $GABA_A$ receptor subunit transcripts (α_1, γ_2) that encode proteins forming functional Cl^- ion channels. These cells exhibited linear *I-V* relations over a wide range of potentials (*not shown*) with low levels of ambient membrane current variance (Fig. 7, traces marked *a*), whose power was always distributed across the frequency spectrum in a monotonic, 1/*f*-type function (Fig. 8*B*). In all five cells studied, slow diffusion of micromolar levels of GABA into the medium led, after several minutes delay, to a modest, yet stable increase in inwardly directed current (~10–20 pA) at the holding potential (−60 mV) together with clearly detectable microscopic fluctuations (Fig. 7, traces marked *b*). Addition of higher concentrations of GABA resulted in substantially greater increases in the current coincident with progressively more intense fluctuations of higher amplitude (Fig. 7, traces marked *c*). Most of the GABA-induced signals could be eliminated, in a reversible manner, by a gentle stream of saline applied to the cell sur-

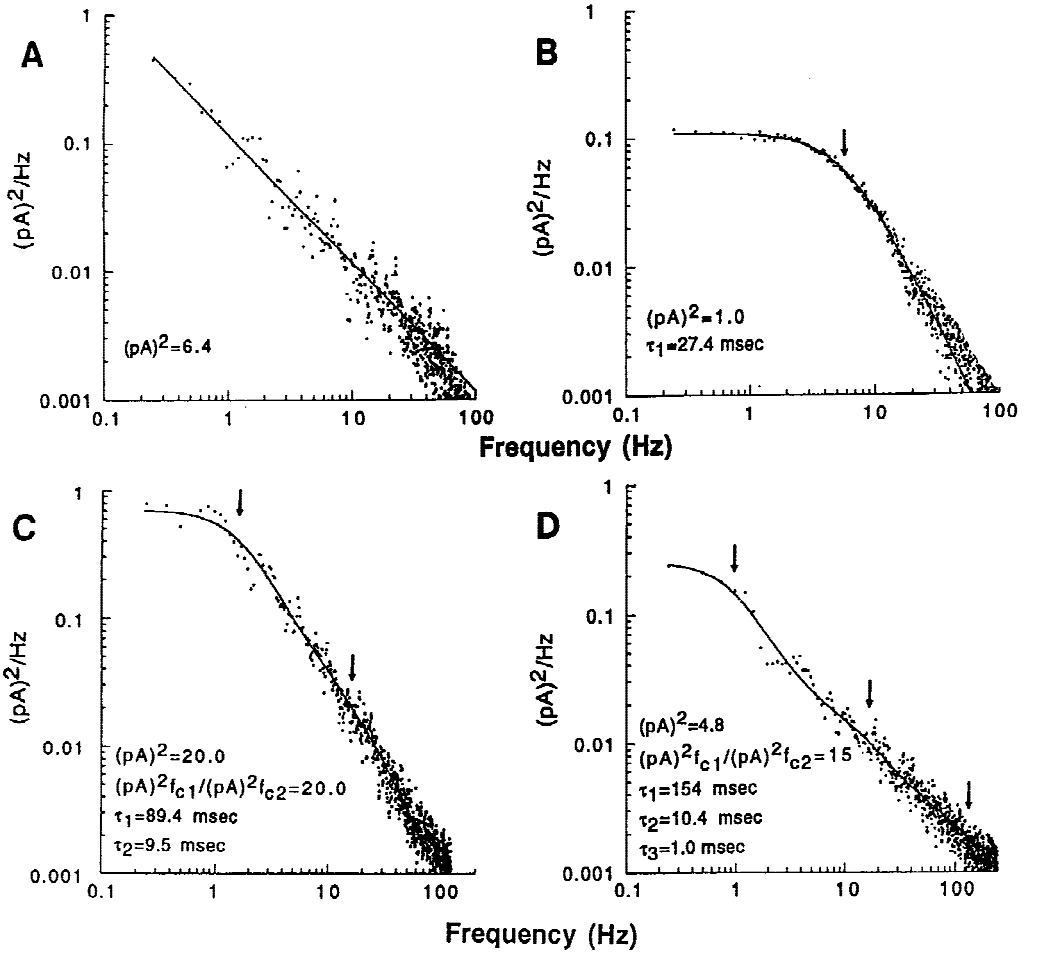

Fig. 6. Almost all cultured hippocampal neurons exhibit Lorentzian spectra. Plots of representative power density spectra calculated for fluctuations endogenous to baseline currents expressed by a sample of 126 cultured hippocampal neurons. The percentages indicate the relative proportions of cells whose fluctuations in membrane current are distributed as a function of frequency in four characteristic patterns. All but ~2% of the neurons exhibit spectra fit with one, two or three Lorentzian components. The different components are identified by downward arrowheads, which mark the corresponding corner frequencies from which the τ values have been derived. When there are multiple components, most of the power in the fluctuating signal is consistently carried by the lowest frequency, which accounts for ~95% of the signal in two-component (*C*) and ~94% of the signal in three-component spectra (*D*). There is no apparent relationship between the complexity of the spectra or lack of it, and the intensity of the fluctuations in membrane current, as measured using average variance.

face (Fig. 7, traces marked *d*). Slow diffusion of bicuculline into the medium rapidly blocked all of the signals (Fig. 7, traces marked *e*). These results show that when 5–10 μM levels of exogenous GABA are in steady-state equilibrium with $GABA_A$ receptor/Cl^- channels a stable DC signal with microscopic fluctuations comparable to the baseline current recorded in embryonic hippocampal neurons is generated, which can be readily and reversibly interrupted by a gentle stream of saline. Thus, one source of endogenous GABA capable of generating continuous activation of $GABA_A$ receptor/Cl^- channels in hippocampal neurons is extracellular.

We used fluctuation analysis techniques to characterize the estimated elementary kinetics of the underlying $\alpha_1\gamma_2$ receptor/Cl^- channels. We found that the power at various frequencies of GABA-induced fluctuations could be described by either single (Fig. 8*B*) or multi-component Lorentzians (*not shown*). Saline reduced the power in the spectrum so that it became more monotonic and 1/*f*-type, while bicuculline always induced a 1/*f* distribution (*not shown*), which was identical to that recorded under control conditions (e.g., Fig. 8*B*).

Surprisingly, we found that 10 μM GABA introduced via the patch pipette in the whole-cell recording mode also generated a continuously fluctuating signal at $GABA_A$ receptor/Cl^- channels, albeit of modest (~10–20 pA) amplitude (Fig. 8*A; n* = 5 cells tested). After rupture of the high-resistance seal, development of detectable inward current superimposed with microscopic fluctuations proceeded in a progressive, continuous manner

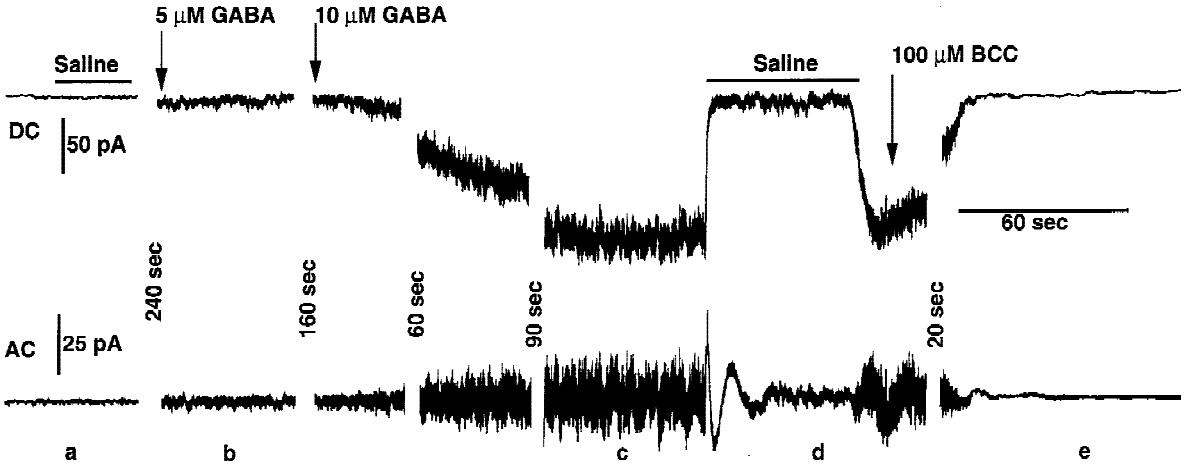

Fig. 7. Extracellular GABA tonically activates $GABA_A$ receptor/Cl^- channels in a WSS-1 cell transfected with α_1- and γ_2-$GABA_A$ receptor subunits. A non-neuronal WSS-1 cell stably transfected with α_1 γ_2 $GABA_A$ receptor subunits was recorded with a CsCl-filled patch pipette. With the cell clamped at −60 mV and E_{Cl} set at 0 mV there is a low level of fluctuation characteristic of all baselines in these cells recorded in this way (pair of traces marked *a*). GABA was added to the medium and slowly diffused to a final concentration of 5 μM, thus inducing ~10–15 pA inwardly directed current superimposed with fast and slow fluctuations (traces marked *b*). Slow diffusion of another 5 μM GABA gradually leads to substantially more current baseline signal superimposed with more intensified fluctuations (traces marked *c*). A gentle stream of saline momentarily eliminates most, but not all of the GABA-induced fluctuating current signal (traces marked *d*). Inclusion of bicuculline in the medium at a final concentration of 100 μM eliminates all of the signal induced by extracellular GABA (traces marked *e*).

over several minutes until a stable level was reached (Fig. 8*A*). Like the baseline current recorded in hippocampal neurons this steady signal could be eliminated in a reversible manner by a gentle stream of saline (*not shown*). Fluctuation analysis of the baseline current signal revealed much more power at low to moderate frequencies, which was exponentially distributed, with kinetics identical to those activated by extracellular GABA (Fig. 8*B*). Thus, both intracellular and extracellular sites could be potential sources of GABA tonically activating $GABA_A$ receptor/Cl^- channels in hippocampal neurons.

Discussion

Salient Findings

Many embryonic rat hippocampal neurons cultured for hours to days exhibit resting baseline properties at negative potentials, which involve steady-state contributions of GABA randomly activating $GABA_A$ receptor/Cl^- channels. This conclusion is supported by the transient loss of the baseline signal following a gentle stream of saline to disturb the unstirred layer at the cell surface and by the complete loss of baseline signal following slow diffusion of bicuculline to the cell surface, which is a competitive antagonist of GABA at $GABA_A$ receptor/Cl^- channels. Fluctuation analyses of the $GABA_A$ receptor/Cl^- channel activity revealed estimated properties of channels activated by endogenous GABA that were comparable to those activated by submicromolar levels of GABA. There was a wide-ranging heterogeneity in estimated $GABA_A$ receptor/Cl^- ion channel properties activated by endogenous or exogenous GABA. Endogenous $GABA_A$ receptor/Cl^- channel activity exhibited potential-sensitive properties comparable to channels activated by exogenous GABA. Exposure of non-neuronal cells expressing functional $GABA_A$ receptor/Cl^- channels showed that either extracellular or intracellular sources of micromolar GABA could generate fluctuating baseline signals identical to those recorded in hippocampal neurons.

Surface-accessible Source of GABA

The sensitivity of the baseline current to a gentle stream of saline indicates that freely diffusible GABA is in steady-state equilibrium with $GABA_A$ receptor/Cl^- channels. The rapid disappearance (~seconds) of GABAergic baseline signal is presumably due to immediate disruption of the GABA-$GABA_A$ receptor/Cl^- channel equilibrium at the surface, while the protracted recovery likely reflects the kinetics of GABA diffusing from intra- and/or extracellular sources and gradual return to an equilibrium with $GABA_A$ receptor/Cl^- channels. Hence, the baseline signal and its recovery results from a continuing supply of freely diffusible GABA, which could be derived from either extracellular and/or intracellular sources, and which equilibrates with $GABA_A$ receptors in the unstirred layer at the neuronal surface. The results obtained introducing GABA into non-neuronal cells ex-

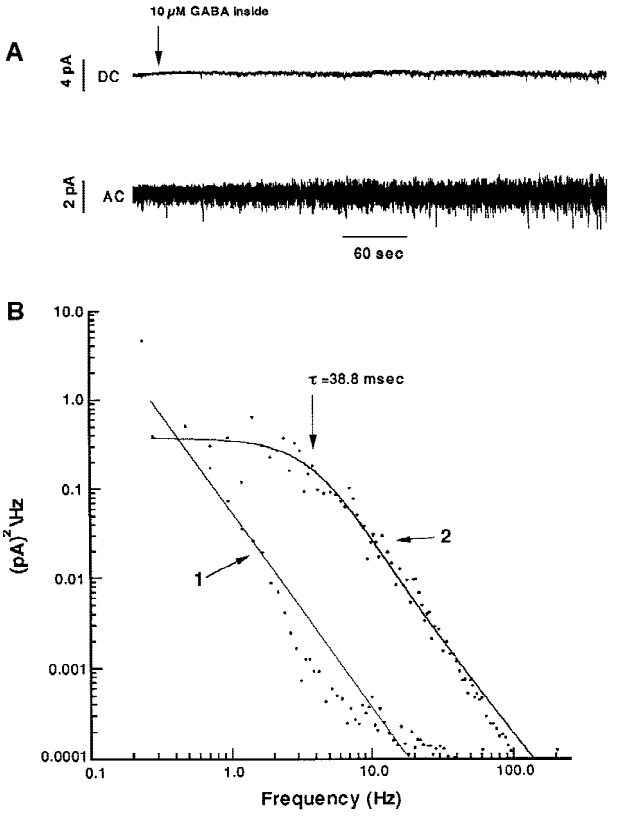

Fig. 8. Intracellular GABA tonically activates $GABA_A$ receptor/Cl^- channels. 10 μM GABA was added to the CsCl used to fill the patch pipette. (*A*) Rupture of the high resistance seal with a WSS-1 cell starts equilibration of GABA in the pipette with the cytosol (downward arrow). High- and very-high-gain current traces reveal slowly increasing inwardly directed baseline current superimposed with progressively more intense fluctuations. (*B*) Spectral analysis of baseline fluctuations within the first minute of recording (i) and several minutes later, after new levels of baseline current and fluctuations have been established (ii) reveal the development of fluctuations whose τ value is characteristic of $GABA_A$ receptor/Cl^- channel activation by extracellular GABA.

pressing functional $GABA_A$ receptor/Cl^- channels show that freely diffusible micromolar levels of GABA can readily pass through the plasma membrane to activate $GABA_A$ receptor/Cl^- channels. In fact, GABA has previously been reported to pass through simple lipid bilayer membranes (Hell et al., 1990). It is possible that uncharged species of GABA can permeate the bilayer. Therefore, the principal sources of GABA generating the baseline current signal in cultured hippocampal neurons would be either the recorded neuron, which could be GABAergic, and/or other GABAergic neurons, which could secrete GABA directly or indirectly onto the recorded neuron. In this regard, a sizable population of cultured embryonic hippocampal neurons (20–50%) are GABA-immunopositive (A.E. Schaffner, *unpublished observations*). Furthermore, some of these GABAergic neurons intermittently discharge GABA to transiently activate $GABA_A$ receptor/Cl^- channels simultaneously at presynaptic release sites having $GABA_A$ receptor/Cl^- channels and at postsynaptic receptors of a synaptically coupled pair (Vautrin et al., 1994). Thus, there are multiple sources of GABA that could contribute to the tonic GABAergic signaling at $GABA_A$ receptor/Cl^- channels.

In cultured embryonic rat thalamic neurons, which exhibit little endogenous GABAergic baseline signal, *exogenous* GABA can be loaded into the surface-accessible compartment (Liu et al., 1995). After loading, spontaneous and continuing release of GABA generates a baseline signal comparable to that recorded in hippocampal neurons, which can be rapidly and reversibly reduced by a gentle stream of saline. Recovery to an equilibrium state following a stream of saline occurs much more rapidly than in hippocampal neurons generating a GABAergic baseline from endogenous sources. Furthermore, like the endogenous signal in hippocampal neurons, the loading and subsequent release of exogenous GABA from thalamic neurons is not affected by inhibitors of GABA uptake nor by Ca^{2+}-free conditions nor by membrane potential (Liu et al., 1995).

In a preliminary series of experiments, we have not been successful in our attempts to alter the baseline GABAergic signal in hippocampal neurons using experimental manipulations to perturb intracellular Ca^{2+} homeostasis (e.g., BAPTA-AM to chelate free cytoplasmic Ca^{2+}, ryanodine to alter Ca^{2+} stores in organelles, thapsigargin to disturb Ca^{2+} pumps in various intra- and extracellular membranes and ionomycin to elevate cytoplasmic Ca from extra- and intracellular sources). Pharmacological interference with GABA uptake using nipecotic acid and SKF-89976A, a nipecotic acid derivative, both of which block GABA uptake, had little, if any effects. Therefore, the molecular mechanisms underlying the tonic GABAergic baseline signal at $GABA_A$ receptor/Cl^- channels remain to be elucidated. It is possible that the membrane is the source of GABA since in excised, outside-out patches of cultured spinal cord neurons spontaneous, random activation of $GABA_A$ receptor/Cl^- channels can be eliminated in a reversible manner by a gentle stream of saline (Barker, Harrison & Owens, 1990).

Regulation of GABAergic Signaling During Hippocampal Neuron Development

Several forms of enzymatic activity that generate GABA by decarboxylating glutamate to GABA (glutamic acid decarboxylase, GAD) have been characterized in the mammalian CNS. Both forms (65,000 MW GAD_{65} and 67,000 MW GAD_{67}) appear in neurons during the embryonic period of CNS development at all levels of the neuraxis, including the hippocampus (for review, *see*

Barker et al., 1998). After differentiation most GABA-immunopositive hippocampal neurons express both GAD forms to varying degrees (Houser & Esclapez, 1994). GAD_{65} readily associates with vesicular and plasma membranes via its palmitoylation moiety, which is lacking in GAD_{67}, resulting in a diffuse cytoplasmic distribution of the latter (Esclapez et al., 1994). Either one or both of these enzymes could be the source of the continual supply of GABA that generates the baseline signal in developing hippocampal neurons, both in acutely prepared slice preparations and in dissociated cell cultures.

In this regard, a baseline signal reflecting GABA's random activation of $GABA_A$ receptor/Cl^- channels has been reported in slices taken from the postnatal hippocampus (Ben-Ari et al., 1989), which contains numerous GABA-immunopositive neurons detected in fixed sections with immunocytochemistry (Rozenberg et al., 1989). The baseline GABAergic signal at $GABA_A$ receptor/Cl^- channels, which serves effectively to steadily polarize neurons nearer E_{Cl}, gradually disappears during postnatal development, paralleling the decrease in intracellular [Cl^-] and the appearance of hyperpolarizing GABAergic transients. In vitro, tonic GABAergic signaling can be transformed into transients in ~1 min following diffusion of the trace metal Zn (Vautrin et al., 1993). The transformation induced by Zn occurs in a continuous, uninterrupted manner. Initially, the baseline current signal exhibits amplitude fluctuations, which over time are normally distributed around an average level. The normal distribution reflects a random process, namely, random activation of $GABA_A$ receptor/Cl^- channels by endogenous GABA. During the transformation, the variance decreases coincident with a gradual appearance of a skewed rather than normal distribution, as GABA release becomes synchronized and generates all-or-none synapticlike transients (Vautrin et al., 1993; Barker et al., 1998). The GABAergic transients exhibit exponential decays identical to those estimated from fluctuation analysis of the Cl^- channels randomly activated by GABA (Vautrin et al., 1993). Thus, tonic and transient forms of GABAergic signaling at $GABA_A$ receptor/Cl^- channels appear to be derived from a common source of GABA, which can be released at rates exhibiting a wide dynamic range (continuous and tonic to intermittent and transient). The mechanisms underlying the dramatic effects of Zn remain to be elucidated. In this regard, embryonic hippocampal GABAergic neurons have been recorded, which simultaneously signal to themselves and postsynaptic neurons in a transient manner (Vautrin et al., 1994). At negative potentials, GABAergic transients occur spontaneously in these cells, while at positive potentials, the transients rapidly decompose into a tonic signal whose exponential open-time kinetics are comparable to those calculated for transient decay (J. Vautrin, *unpublished observations*). Return to negative potentials recovers the spontaneous GABAergic transients within seconds. Hence, embryonic hippocampal neurons exhibit a wide dynamic range in their ability to generate tonic and transient GABAergic signals at $GABA_A$ receptor/Cl^- channels, which can be modulated by membrane potential as well as Zn.

Conclusions

Developing hippocampal neurons exhibit baseline properties in culture and in slice preparations that are due in part, to a continuous release of GABA, which randomly activates $GABA_A$ receptor/Cl^- channels. This tonic GABAergic contribution to polarize neurons is not simply due to trauma, but is likely to have physiological relevance during development. Both extracellular and intracellular sources of GABA could steadily polarize neurons near E_{Cl}, which during early differentiation lies depolarized to the resting potential. The developmental role(s) of a tonic GABAergic signal in morphogenesis of the hippocampus and in synapse formation are a challenge for future study.

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