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Exogenous GABA Persistently Opens Cl^- Channels in Cultured Embryonic Rat Thalamic Neurons

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Abstract. We recorded whole-cell Cl^- currents in cultured embryonic rat thalamic neurons by brief applications of GABA or the structural analogue muscimol. In 17 of 141 neurons (12%) the Cl^- current persisted for a minute or more after the pipette was removed from the bath. Cl^- current never persisted after muscimol exposure even in those cells exhibiting persistent GABA-activated currents (PGC). The half decay times (T_{50}) of PGCs were exponentially and asymptotically related to the duration of GABA exposure and could be interrupted or completely aborted by low-pressure application of saline. PGCs were insensitive to membrane potential, to Tiagabine, a nipecotic acid analogue known to block GABA uptake, and persisted in Ca_o^{2+}-free medium. Fluctuation analysis revealed that PGCs exhibited inferred Cl^- channel properties whose kinetic components and estimated average elementary conductance showed no significant difference from those estimated during GABA exposure. The relative contribution of low frequency components was consistently reduced and that of high frequency components modestly increased during PGC compared to those recorded during GABA exposure. Taken together, the results suggest the existence of a superficial compartment in these embryonic neurons that can momentarily accumulate and release exogenous GABA.

Key words: Patch-clamp — GABA — $GABA_A$ receptor — Embryonic rat — Thalamus — Cl^- channel

Introduction

Transient conductance changes mediated by fast-acting chemical transmitters at specialized synapses are found in most, if not all multicellular organisms. Another, tonic form of signaling mediated by acetylcholine, glutamate or γ-aminobutyric acid (GABA) has been recorded at peripheral and central synapses in a variety of experimental preparations (Katz & Miledi, 1977; Vyskočil, Nikolsky & Edwards, 1983; Sun & Poo, 1985; Sah, Hestrin & Nicoll, 1989; LoTurco, Blanton & Kriegstein, 1991; Otis, Staley & Mody, 1991; Blanton & Kriegstein, 1992; Valeyev et al., 1993). Tonic transmission is attributed to nonvesicular transmitter release while transient signals are assumed to involve all-or-none discharge from vesicles following the initial hypothesis of Katz and colleagues (Fatt & Katz, 1952; del Castillo & Katz, 1954). Here we report that extracellular application of GABA to cultured embryonic rat thalamic neurons results in its time-dependent accumulation into, and release from a surface-accessible compartment, creating a long-lasting tonic signal involving random activation of Cl^- channels.

Materials and Methods

Cell Preparation

After euthanasia of pregnant Sprague-Dawley female rats (from Taconic farms, Gaithersberg, MD) by CO_2 inhalation, embryos were removed and immediately decapitated. Thalami were enzymatically dissociated with papain according to the method of Huettner and Baughman (1986). Cells were cultured at a density of $2–5 \times 10^4$ cells/cm^2 in 35 mm plates coated with poly-d-lysine. Plating medium consisted of Minimal Essential Medium (MEM, Gibco, Grand Island, New York) with 5% fetal calf serum and 5% horse serum (both from Biofluid, Rockville, Maryland). Cultures were kept at 37°C in a CO_2 incubator and after 4 days were maintained with MEM and 5% horse serum.

Electrophysiological Experiments

Cells were used in the period from the plating day to 10 days in culture. GABA (10 μM), muscimol (10 μM) or the bath medium were applied from

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a glass micropipette 20 μm away from the cell soma using ~2 psi positive pressure. Application pressure was delivered by filtered compressed air and controlled by electronic valves (General Valve, Fairfield, New Jersey). Chloride currents were recorded at room temperature (22–25°C) with a List EPC-7 patch clamp amplifier (List-Electronic, Darmstadt, Germany) using the whole-cell configuration (Hamill et al., 1981). The patch pipettes contained (in mM): CsCl (145), $CaCl_2$ (0.1), EGTA (1.1), $MgCl_2$ (2), ATP (5), phosphocreatine (5) and HEPES (10). pH was adjusted to 7.2 with CsOH and osmolarity to 290 mOsm with sucrose. The bath solution contained (in mM): NaCl (145), KCl (5.4), $CaCl_2$ (1.8), $MgCl_2$ (0.8), D-Glucose (10) and HEPES (10), adjusted to pH 7.4. Osmolarity was adjusted to 310 mOsm. 1–5 μM tetrodotoxin (Sigma, St Louis, Missouri) and Tiagabine (Novo Nordisk A/S, Novo, Denmark) were eventually added to the chamber.

For fluctuation analysis, membrane current was stored on videotape with a VR-100 digital recorder (Instrutech, New York) and a Sharp VCR. Offline analysis was made with a IBM compatible 386 PC computer using the SPAN program (courtesy J. Dempster, Strathclyde University, Glasgow, UK) after current were lowpass-filtered at 1 kHz, highpass-filtered at 0.2 Hz and digitized at 1 kHz (12 bits LAB-PC National Instruments). The average conductance, γ, was calculated according to:

$$\gamma = \delta^2/[I_m \cdot (E_m - E_{Cl})] \quad (1)$$

where δ^2 is the average variance, I_m is the membrane current amplitude, E_m is the membrane potential and E_{Cl} is the Cl^- equilibrium potential. Kinetic components were inferred from the calculated power spectra (Neher & Stevens, 1977).

Results

GABA Persistently Activates Cl^- Currents

Embryonic thalamic neurons, either acutely dissociated or cultured for up to 10 days, were voltage-clamped at −80 mV in the whole-cell configuration. The majority (88%) of the 141 neurons responding to GABA exhibited Cl^- current responses typical of most, if not virtually all of those published throughout the existing literature in that the time course of recovery to baseline was quite rapid (Fig. 1*A* and *D*). The average time for the GABA-evoked Cl^- current to decay by 50% (T_{50}) was 1.3 ± 0.7 S for 13 of these cells expressing ≥10 pA responses to a 60-sec application of 10 μM GABA. In marked contrast, 12% (17 neurons) exhibited clearly protracted recoveries following GABA exposure. These persistent GABA-activated Cl^- currents (PGCs) lasted for up to 4 min (Fig. 1*B, C, D2*). There was no significant difference between the exponential rising phases of GABA-activated Cl^- current that were (161.2 ± 34.8 msec, n = 9) or were not (177.4 ± 16.2 msec, n = 13) followed by PGCs ($P > 0.05$) (Fig. 1*D1*). Although the time courses of current recoveries in these cells were quite variable, they were all manifestly longer than was characteristic of the vast majority of recoveries following GABA-activated Cl^- current responses of comparable amplitude. These persisting effects of GABA were recorded at early and later

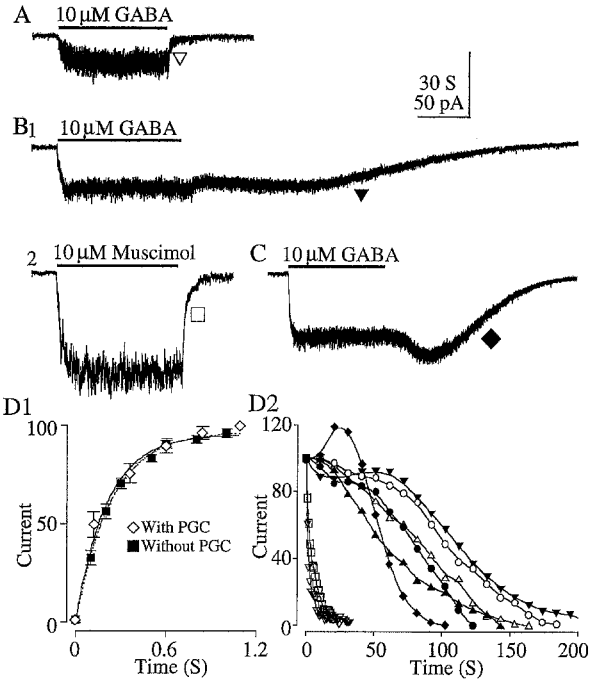

Fig. 1. Persistent GABA-activated Cl^- current (PGC) in cultured embryonic thalamic neurons. (*A*) GABA activates a Cl^- current that exhibits ~seconds-long recovery typical of the majority of neurons recorded. (*B1*) GABA induces a PGC lasting for about 4 min after 60 sec GABA exposure, while in the same neuron (*B2*) muscimol evokes Cl^- current that decays within seconds after the end of application. (*C*) Example of PGC that exhibits a transient phase whose amplitude exceeds the Cl^- current evoked during GABA exposure. (*D1*) Normalized rising phases of GABA-induced Cl^- current in 13 cells without (■) and 9 cells with (◇) PGCs have an identical exponential growth. (*D2*) Plots of current recoveries (normalized to the amplitude recorded at the end of the agonist application) of 8 different thalamic cells. ▽: GABA application displayed in *A*. ▼: GABA application displayed in *B1*. □: Muscimol application displayed in *B2*. ♦: GABA application displayed in *C*. ▲, ○, ●, △: GABA exposure of four other neurons. Each symbol represents the mean current amplitude calculated for 10-sec epochs in cells with PGC and for 0.5-sec intervals in cells without PGC.

days in vitro and in cells maintained under the same culture conditions as those recovering rapidly following GABA exposure.

The initial amplitudes of the persisting current was either similar to, or slightly less than that of the Cl^- current recorded during applications (Fig. 1*B1, D*). However, in three cells, the initial PGC amplitude became noticeably but transiently greater than that recorded during agonist application (e.g., Fig. 1*C*). Muscimol, a structural analogue and well-established agonist of GABA at Cl^- channels, evoked Cl^- current in all GABA-responsive cells tested (n = 24), including five cells exhibiting PGCs, but these were never followed by a prolonged recovery to baseline (Fig. 1*B2*). T_{50} values for recoveries following 60-sec exposure to muscimol averaged 1.3 0.5 S (n = 24). The protracted recovery

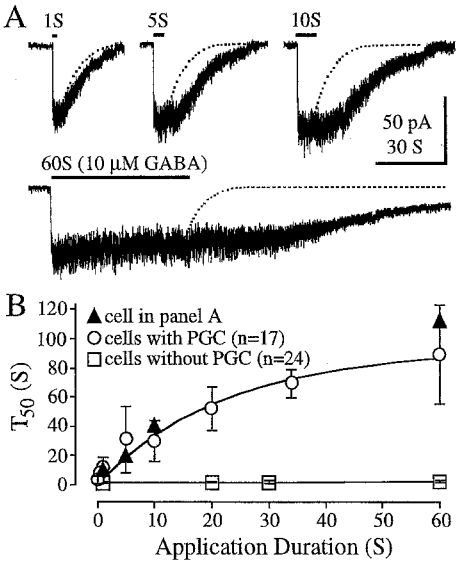

Fig. 2. PGC duration is related in an exponential and asymptotic manner to the duration of GABA exposure. (*A*) Cl^- current induced by GABA applications of increasing duration. Dotted lines represent rapid recovery typical of most neurons. (*B*) T_{50} of the PGC is plotted as a function of the application duration. ○: means of 3–5 cells expressing PGCs. ▲: results from *A*. □: means of 10–21 cells without PGC. The results were fitted by a simple exponential function (solid line): $T_{50} = T_{max} \cdot (1\text{-}\exp(-T/\tau))$ where T_{max} (92.2 S) is the maximum T_{50}, T is the application duration and τ (22.5 S) is the application time constant. Bars are standard deviations.

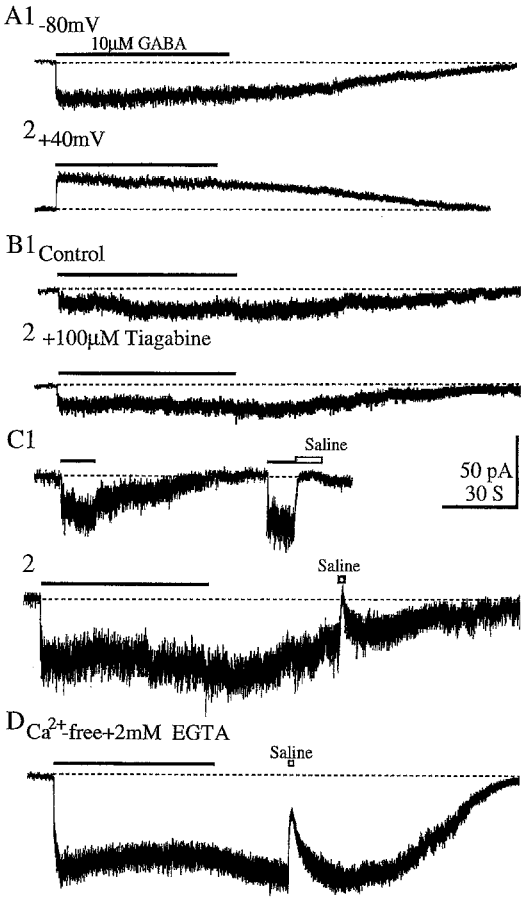

Fig. 3. PGC properties are surface-accessible. (*A*) PGC generation is not affected by membrane potential as illustrated for Cl^- current evoked by GABA at +40 mV (*A1*) and −80 mV (*A2*) in the same neuron. At each potential the recovery after GABA exposure is similarly protracted and the recovery profiles are approximately symmetrical. (*B*) Addition of the GABA uptake inhibitor Tiagabine (100 μM) to the bath medium has no obvious effect on PGC expression. (C) PGC expression can be abruptly interrupted simply by gently blowing bath solution onto the recorded neuron. A long (~10 sec) blow coincident with the end of the GABA application effectively aborts PGC expression (*C1*). Short blows (~1 sec) only transiently interrupt PGC expression either completely (*C2*) or partially (*D*). PGC can be recorded in the absence of added Ca^{2+} and presence of 2 mM EGTA (*D*).

period remained even when the pipette containing 10 μM GABA was immediately and completely removed from the recording medium. These latter results eliminate continued GABA leakage from the pipette as a trivial explanation for the persistent current response.

PGC Duration Depends on the Duration of GABA Exposure

The relationship between the duration of exposure to GABA and the T_{50} value of recovery was evaluated by varying the period of application from 0.5 to 60 sec, then measuring the time required to recover halfway to the baseline. T_{50} was directly related to the duration of GABA application in an exponential and asymptotic manner (Fig. 2*A*). The T_{50} value for recovery asymptoted at ~90 seconds GABA exposure; about 22–23 seconds of GABA exposure was sufficient to reach about two-thirds of the asymptotic value. There was no evidence of an abrupt threshold in exposure time that triggered PGCs or of discontinuities in the relationship (Fig. 2*B*). These results indicate that PGC duration depends directly on the period of GABA exposure.

PGC Properties

Physiological and pharmacological properties associated with PGCs were studied in order to characterize their relationship to the Cl^- conductance activated during GABA exposure. Clamping cells at positive potentials reversed the polarity of Cl^- current occurring during GABA exposure as well as PGCs (Fig. 3*A*). In each of the 5 cells tested, PGC reversed polarity at the same potential as the Cl^- current response to GABA, which was about 0 mV under these experimental conditions (with symmetrical Cl^- and the expected equilibrium potential for Cl^- ions, E_{Cl}, being ~0 mV). The long-lasting time course in the decline of the PGC appeared similar at positive and negative potentials (Fig. 3*A*). These results indicate that PGCs are virtually insensitive to membrane potential and reverse polarity near or at E_{Cl}, which is

consistent with the notion that it is the Cl^- conductance that persists.

Neither the duration nor the time course of PGCs was altered by addition to the bath of 100 *M* Tiagabine, a potent nipecotic acid analogue that inhibits GABA uptake (Braestrup et al., 1990) (Fig. 3*B*). Thus, PGCs do not involve a Tiagabine-sensitive cellular compartment loaded with GABA. Somewhat surprisingly, PGCs could be virtually eliminated when a gentle puff of saline was applied to the cell surface coincident with the end of the GABA application (Fig. 3*C1*). Furthermore, a brief pulse of saline could transiently depress or eliminate it (Fig. 3*C2, D*). PGCs were also recorded in Ca_o^{2+}-free saline containing 2 mM EGTA (Fig. 3*D*), indicating that they did not require physiological levels of Ca_o^{2+}. These results imply that PGCs are not related to previously reported uptake and/or release processes.

Properties of Persistently-activated Cl^- Ion Channels

The macroscopic Cl^- current evoked by GABA always exhibited microscopic fluctuations reflecting the moment-to-moment changes in the number of open Cl^- ion channels. Inspection of the PGCs revealed that they too exhibited similar fluctuations. The fluctuations in both GABA-evoked current and PGCs are the results of random activities of Cl^- channels as revealed by the low degree of skewness in the amplitude distributions (0.26 ± 0.05 for GABA-evoked current and 0.22 ± 0.05 for PGC; n = 8, $P > 0.05$). We estimated the electrical properties of the Cl^- channels using fluctuation analysis. γ was not significantly different for estimates calculated during and after GABA applications in those cells exhibiting PGCs (15.1 ± 0.7 pS for GABA and 13.5 ± 1.1 pS for PGC, n = 8, $P > 0.05$). The spectra of the current fluctuations could be fitted by three Lorentzian components, reflecting the contributions of different exponentially-distributed channel openings (Fig. 4*A*). In 8 cells, the open times of Cl^- channels activated during exposure to GABA averaged 3.4 ± 0.7, 15.4 ± 3.1 and 69.8 ± 15.6 msec. The shortest and the longest open times for persistently-activated channels opening during PGCs were similar (2.8 ± 1.4 and 68.3 ± 8.4 msec, $P > 0.05$) while the intermediate open time was significantly shorter (9.2, 2.0 msec, $P < 0.05$, Fig. 4*B1*). However, during PGCs power consistently shifted from low to higher frequency components (Fig. 4*B2*). The relative contributions of the short, intermediate and long components were $22.6 \pm 4.8\%$, $47.2 \pm 5.1\%$ and $30.2 \pm 7.0\%$ during GABA exposure (n = 8) and became $33.9 \pm 8.2\%$ (+50.0%, $P < 0.05$), $49.0 \pm 5.6\%$ (+3.8%, $P > 0.05$) and $17.1 \pm 4.6\%$ (–43.4%, $P < 0.05$) during PGCs. These results reveal that PGCs involve Cl^- channels whose elementary properties are similar to those activated during GABA exposure.

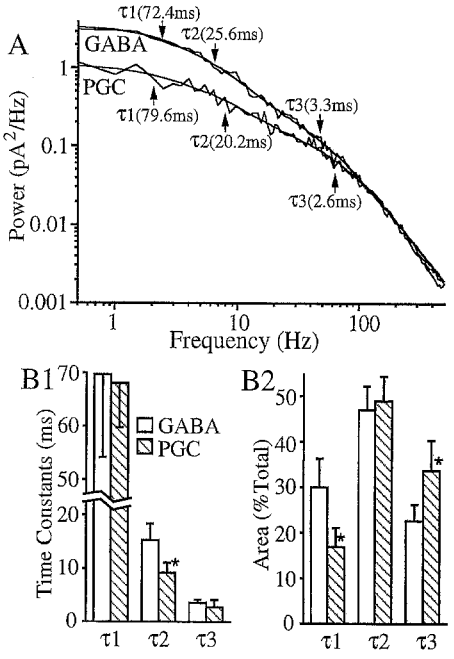

Fig. 4. Spectral properties of GABA-activated current and PGCs reveal three components whose kinetics overlap, but whose relative contributions change. (*A*) Typical power density spectra calculated during GABA-activated Cl^- currents and plateau phase of PGCs are well-fitted by triple Lorentzian terms. Arrowheads mark the corner frequencies (f_c) of the three components. Open-time constants (τ) were obtained from $\tau = (2\pi f_c)^{-1}$. (*B1*) $\tau 1$ and $\tau 3$ are not significantly different between GABA exposure and PGC groups ($P > 0.05$) when results from 8 cells are averaged while $\tau 2$ is significantly shorter (*, $P < 0.05$) during PGC. (*B2*) The relative power in each component was calculated by $S_{o(n)}f_{c(n)}/\Sigma S_o f_c$, where $S_{o(n)}$ and $f_{c(n)}$ are the asymptote in power and the corner frequency of the n^{th} Lorentzian respectively, and $\Sigma S_o f_c$ is the sum of the product of S_o and f_c of each lorentzian component. The low-frequency, long-lasting open-time component ($\tau 1$) contributes significantly less (*, $P < 0.05$), while the shortest component ($\tau 3$) contributes significantly more (*, $P < 0.05$) in PGC relative to their respective contributions during GABA exposure. The intermediate component ($\tau 2$) is not significantly different.

Discussion

PGCs are a Biological Phenomenon

PGCs are unlikely to result from inadvertent leakage of GABA from the pipette since the phenomenon always persisted after the pipette was entirely removed from the recording medium. Inadvertent leakage is also eliminated by recording cells with and without PGCs in the same culture with the same pipette. Activation of Cl^- channels with the well-established GABAmimetic muscimol never led to persistent current. This result implies a structure-activity requirement that is independent of Cl^- channel activation. Physicochemical properties of GABA that involve the distribution of charge and flexibility of molecular shape not shared by muscimol may

account for the difference. A more complete structure-activity study with other GABAmimetics and naturally-occurring amino acids that activate Cl^- channels should further reveal the structural requirements for this phenomenon.

PGCs Involve Random Activation of Cl^- Channels

Like GABA-activated Cl^- current, PGCs reversed polarity at E_{Cl} and were sensitive to bicuculline (*data not shown*) suggesting that PGCs involve Cl^- conductance persistently activated by GABA. The lack of any apparent voltage sensitivity eliminates the need for physiologically negative membrane potential in the phenomenon, or any mechanisms derived from, or dependent on a physiologically negative potential. Insensitivity to Tiagabine, the potent blocker of GABA uptake (Braestrup et al., 1990), indicates that a different "uptake" process is involved in PGCs. The sensitivity of PGCs to local flow at the cell surface with discrete, gentle puffs of saline indicates that the GABA molecules responsible for persistently activating the Cl^- channels are accessible at the cell surface. The duration of PGCs was exponentially related to the time of GABA exposure in an asymptotic manner, suggesting that PGCs are due to time-dependent accumulation of GABA in a surface-accessible compartment. Storage in, and release of GABA from such a putative compartment was not dependent on physiological levels of extracellular Ca^{2+}, eliminating the evolvement of this ion in the phenomenon.

Spectral analysis of PGCs could not differentiate Cl^- channel conductance and kinetics during PGCs from those during GABA application. This indicates that the persistent channel activity involves the same population of receptor channels activated during the response. The relative power in low- and high-frequency components changed in a quite noticeable manner so that less power was supported by long-lasting openings and more power by short-lasting events. Concentration-dependent changes in the relative contribution of short- and long-lasting components in single-channel recordings of GABA-gated Cl^- channels in outside-out patches excised from cultured embryonic mouse spinal cord neurons have been reported (Macdonald, Rogers & Twyman, 1989). Increasing the GABA concentration from 0.5 to 5.0 M promoted the proportion of the longest-lasting openings. The ~1 second time course of recovery in the great majority of cells not expressing PGCs indicates that GABA rapidly becomes ineffective, presumably reflecting the diffusion-limited decrease of GABA at the cell surface. Hence, Cl^- channel activity persists even when the GABA concentration at the cell surface is below that necessary to activate Cl^- channels. As GABA putatively accumulated in the surface-accessible compartment dissipates its efficacy in activating Cl^- channels presumably decreases. This might explain the switch in the relative contributions of different spectral components during PGCs. At equilibrium during exposure to GABA the probability of saturating receptors with agonist may promote the longest-lasting, most stabilized openings, as was reported in single-channel recordings (Macdonald et al., 1989). Alternatively, there may be several independent classes of receptor-channels with different properties distributed on the cell surface.

Taken together, the results reveal that during the first two weeks in culture a small subpopulation of embryonic rat thalamic neurons are capable of transiently accumulating and releasing exogenous GABA. Time-dependent exposure to GABA leads to its accumulation in, or at the surface that is readily accessible to local flow. This tenuous pool of transmitter gradually dissipates or itself no longer remains capable of activating Cl^- channels. The elementary properties of Cl^- channels activated by GABA from this compartment appear identical to those opening during GABA exposure. Presumably, GABA molecules at the surface are in some way able to access and activate the same population of receptors as GABA delivered from the pipette. Interaction of exogenous GABA with GABA receptor/Cl^- channels appeared to be a continuous process since the tonic signal returned in a gradual and progressive manner following a brief blow to the surface. Membrane current variance correlated in time with mean current (*data not shown*) and the degree of skewness in the fluctuations was low and indistinguishable from that recorded during GABA exposure. These observations imply that exogenous GABA randomly activates Cl^- channels in a continuous manner. The disappearance of the PGC presumably reflects the gradual dissipation of GABA, which could diffuse into the medium and/or into the cell.

Tonically Active Cl^- Conductance Mediated by Endogenous GABA

The present results involving persistent activation of Cl^- channels by exogenous GABA may provide some insight into tonic Cl^- conductance mediated by endogenous GABA previously reported both in acutely-prepared slices of CNS tissues and in cultured neurons. Patch-clamp records of neurons in slice preparations of supraspinal regions of the adult rat CNS reveal "perpetual" GABA-mediated Cl^- conductance composed of a continuous signal and transients characteristic of synaptic activity (Otis et al., 1991). The tonic signal may be independent of the transients or the latter may have summed to generate the Cl^- conductance component of the baseline. Trauma to the tissue, inevitable in creating the slice preparation, makes an unknown contribution to both tonic and transient signals. Hence, the GABA-

mediated Cl^- conductance that composes the baseline in these acute preparations may in part reflect summating synaptic transients whose physiological basis may be altered/promoted by tissue preparation. A tonic Cl^- conductance and synapticlike transient Cl^- current mediated by GABA have also been recorded in early postnatal slices of rat hippocampus when both are depolarizing relative to the resting potential (Cherubini, Gaiarsa & Ben-Ari, 1991; Hosokawa et al., 1994). Interestingly, this tonic depolarizing contribution of GABA to resting membrane properties disappears postnatally coincident both with E_{Cl} becoming hyperpolarizing with respect to the resting potential and with GABA mediating synapticlike inhibitory transients. These latter results would appear to eliminate summating transients and tissue trauma in generating the tonic signal in the early postnatal slice since the latter disappeared despite the trauma and the transients remained, but did not summate.

Many embryonic rat hippocampal neurons cultured for days to several weeks also exhibit tonic Cl^- conductance that is mediated by endogenous GABA (Valeyev et al., 1993). Like PGCs, the continuous signal can readily and reversibly be eliminated by gentle puffs of saline and is independent of membrane potential. Surprisingly, the tonic signal can be rapidly transformed into synapticlike transients by exogenous Zn^{2+} (Vautrin, Schaffner & Barker, 1993). The transformation of the continuous, random activation of Cl^- channels by endogenous GABA into intermittent transients by Zn^{2+} is itself seemingly continuous. There is a progressive, uninterrupted increase in the degree of skewness in the fluctuating signal until transients become visibly manifest. Many of these cultured hippocampal neurons are GABAergic and some exhibit both cis- and transmission (Vautrin, Schaffner & Barker, 1994). Cismitting cultured hippocampal neurons exhibiting interconvertible tonic and transient signals have been recorded (Vautrin, *unpublished observations*). Thus, tonic, random activation of Cl^- channels by GABA can be converted rapidly and reversibly into nonrandom openings of Cl^- channels characteristic of synapticlike transients. If this endogenous signal transmission also involves GABA that persists at the cell surface then GABAergic signal generation may initiate there rather than from intracellular sites.

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