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Bicarbonate efflux via GABA_A receptors depolarizes membrane potential and inhibits two-pore domain potassium channels of astrocytes in rat hippocampal slices

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Abstract

Increasing evidence indicates the functional expression of ionotropic γ -aminobutyric acid receptor (GABA_A-R) in astrocytes. However, it remains controversial in regard to the intracellular Cl⁻ concentration ([Cl⁻]_i) and the functional role of anion-selective GABA_A-R in astrocytes. In gramicidin perforated-patch recordings from rat hippocampal CA1 astrocytes, GABA and GABA_A-R specific agonist THIP depolarized astrocyte membrane potential (V_m), and the THIP induced currents reversed at the voltages between -75.3 to -78.3 mV, corresponding to a [Cl⁻]_i of 3.1 – 3.9 mM that favors a passive distribution of Cl⁻ anions across astrocyte membrane. Further analysis showed that GABA_A-R induced V_m depolarization is ascribed to HCO₃⁻ efflux, while a passively distributed Cl⁻ mediates no net flux or influx of Cl⁻ that leads to an unchanged or hyperpolarized V_m . In addition to a rapidly activated GABA_A-R current component, GABA and THIP also induced a delayed inward current (DIC) in 63% of astrocytes. The DIC became manifest after agonist withdrawal and enhanced in amplitude with increasing agonist application duration or concentrations. Astrocytic two-pore domain K⁺ channels (K2Ps), especially TWIK-1, appeared to underlie the DIC, because 1) acidic intracellular pH, as a result of HCO₃⁻ efflux, inhibited TWIK-1; 2) the DIC remained in the Cs⁺ recording solutions that inhibited conventional K⁺ channels and 3) the DIC was completely inhibited by 1 mM quinine but not by blockers for other cation/anion channels. Altogether, HCO₃⁻ efflux through activated GABA_A-R depolarizes astrocyte V_m and induces a delayed inhibition of K2Ps K⁺ channels via intracellular acidification.

Keywords

Astrocytes; GABA_A receptors; bicarbonate; TWIK-1; patch clamp; hippocampus

Introduction

Ionotropic γ -aminobutyric acid receptors (GABA_A-Rs) are anion-selective channels activated by inhibitory neurotransmitter GABA. The functional GABA_A-Rs are pentamer assembling from 19 subunits (α 1-6, β 1-3, γ 1-3, δ , ϵ , θ , π and ρ 1-3) into different subtypes (Olsen and Sieghart 2008). The expression of ionotropic GABA_A-R in astrocytes has been indicated by immunocytochemistry (Bureau et al. 1995) and functional studies, including GABA_A-R currents measured from astrocytes *in situ* (Bekar et al. 1999; MacVicar et al. 1989; Meier et al. 2008; Steinhäuser et al. 1994) and in acutely isolated preparation of rat

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hippocampus (Fraser et al. 1995; Zhou and Kimelberg 2001). Activation of astrocytic GABA_A-R has been shown to elevate intracellular Ca²⁺ concentration (Bernstein et al. 1996; Fraser et al. 1995; Meier et al. 2008) and modulate outward K⁺ channel activity (Bekar and Walz 1999; Bekar and Walz 2002). GABA_A-R activation is also critical for the proliferation of astrocyte-like GFAP-expressing progenitors in postnatal subventricular zone (Liu et al. 2005).

Developmentally regulated intracellular chloride concentration ([Cl⁻]_i) from high to low underlies the functional transition of neuronal GABA_A-R from excitatory to inhibitory (Ben-Ari 2002). However, it remains controversial in regard to the [Cl⁻]_i in astrocytes; while cultured astrocytes contained a high [Cl⁻]_i of around 30-40 mM (Bekar and Walz 2002; Kimelberg 1981), glial cells identified electrophysiologically from guinea-pig olfactory cortex showed a low [Cl⁻]_i of 3.2 mM (Ballanyi et al. 1987). A high [Cl⁻]_i mainly results from an inward transport of Cl⁻ anions via Na⁺-K⁺-2Cl⁻ cotransporter (NKCC). Interestingly, the amount of mRNA of Na⁺-K⁺-2Cl⁻ cotransporter in cultured astrocytes and astrocytes isolated from postnatal day one (P1) mouse was 2.7-fold higher than that of the astrocytes isolated from P17 mouse, while the mRNAs of K⁺-Cl⁻ cotransporter KCC1 and KCC2, which function to extrude Cl⁻ from cells, developmentally increased 1.5- and 1.4-fold in isolated astrocytes from P1 to P17 mouse, respectively (Cahoy et al. 2008). This leaves the question of the actual [Cl⁻]_i in neonatal and mature astrocytes to be further investigated.

Should a low [Cl⁻]_i content exist in mature astrocytes, whether GABA_A-R activation has a discernible impact on astrocyte membrane potential is questionable, because the Cl⁻ equilibrium potential (E_{Cl}) established by a passive distribution of Cl⁻ anions equals to the K⁺ equilibrium potential (E_K) and the membrane potential. Therefore, the precise influence of GABA_A-R on astrocyte membrane potential and its downstream physiological function need to be determined.

To approach these questions, conventional and gramicidin perforated patch clamp recordings were used to study astrocytic GABA_A-R in acute rat hippocampal slices. We revealed a passively distributed [Cl⁻]_i in hippocampal mature astrocytes, and showed that GABA_A-R mediated HCO₃⁻ efflux depolarized astrocyte membrane potential and inhibited two-pore domain K⁺ channels (K2Ps) in a delayed fashion that was likely due to intracellular acidification.

Materials and Methods

Hippocampal slice preparation

Rat hippocampal slices were prepared from postnatal day (P) 21 to 29 Sprague-Dawley rats. The procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee, The Ohio State University and Wadsworth Center, New York State Department of Health. Rats were anesthetized with 8% chloral hydrate in 0.9% NaCl followed by decapitation. The brain was rapidly removed from skull and placed in ice-cold oxygenated (95% O₂ /5% CO₂) slice preparation solution containing (in mM): 125 NaCl, 3.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 0.1 CaCl₂, 3 MgCl₂ and 10 glucose. Coronal hippocampal slices at 300 μm thickness were cut at 4°C with a Pelco 1500 Vibratome (Ted Pella, Inc., Redding, CA) and transferred to a nylon slice holder basket immersed in artificial cerebral spinal fluid (aCSF) containing (in mM): 125 NaCl, 3.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂ and 10 glucose (osmolality, 295 ± 5 mOsm) at room temperature (20-22°C). Slices were kept in aCSF with continuous oxygenation for at least 60 min before recording.

Electrophysiology

Individual slice was transferred into a recording chamber (RC-22, Warner Instruments, Holliston, MA) with constant perfusion of oxygenated aCSF at 2.5 ml/min rate. Astrocytes in the CA1 *stratum radiatum* were identified by differential interference contrast (DIC) video microscopy (Olympus BX51), and a cooling LED camera (WAT-902H Ultimate) to display the slice image on a monitor using a 4× or 40× water-immersion objectives. Recording electrodes were fabricated from borosilicate capillaries (1.5/0.86 mm outer/inner diameter, Warner Instruments, Holliston, MA) using a Flaming/Brown Micropipette Puller (Model P-87, Sutter Instrument). The electrodes had the open tip resistance of 2.5–5.0 MΩ when filled with the electrode solution contained (in mM): 140 KCl, 0.5 CaCl₂, 1 MgCl₂, 5 EDTA, 10 HEPES, 3 Mg²⁺-ATP and 0.3 2Na⁺-GTP that was titrated with KOH to pH 7.25–7.27. The final osmolality was 280.0 ± 5.0 mOsm.

Whole-cell membrane current or membrane potential was amplified by MultiClamp 700A or 700B amplifiers and the data acquisition was controlled by PClamp 9.2 or 10.2 software (Molecular Devices, Sunnyvale, CA). DIGIDATA 1322A interface was used to convert digital-analog signals between amplifier and computer. Membrane capacitance (C_M), access resistances (R_a), membrane resistance (R_m) and input resistance (R_{in}) were measured by using the “Membrane test” protocol built into the pClampex. The voltage error in voltage clamp recording was calculated from $R_a/(R_a+R_m)*100\%$. The membrane potential (V_m) was read either in “I = 0” mode or measured directly in current clamp mode with no holding currents. Astrocytes with a resting membrane potential more positive than −75 mV were discarded. All the experiments were conducted at room temperature of 20–22°C.

Gramicidin stock solution was made by adding 5 mg gramicidin into 1 ml dimethylsulfoxide (DMSO). Working solution was made by adding 3.6 μl stock solution into 1 ml electrode solution (18 μg gramicidin/ml) and used for two hours. The electrode was first dipped in gramicidin-free electrode solution for ~10 seconds, and followed by backfilling with 2 μl gramicidin-free and 8 μl gramicidin-containing electrode solutions sequentially (final gramicidin concentration: 14.5 μg/ml). A +10 mV/10 ms voltage step was delivered every 20 s from the holding potential of −70 mV to monitor the perforation process. From the step induced current ($I_{10\text{ mV}}$), the input resistance (R_{in}) was calculated from $R_{in} = 10\text{ mV}/I_{10\text{ mV}}$ (Sup. Fig. 1). A successful perforation process typically took ~30 min to reach a stable and acceptable R_{in} of ~50 MΩ. Change in membrane conductance during recording was monitored by a periodically delivered voltage ramp command; the membrane was first stepped from −70 mV down to −160 mV and followed by a voltage ramp from −160 mV to +20 mV at the rate of 180 mV/sec. The net GABA_A-R currents induced by THIP were resolved by subtraction of voltage ramp induced currents in control from THIP.

For focal application, a drug-containing electrode with < 1 μm opening diameter was advanced to the recorded cell 20–30 μm away from soma (Fig. 3B). For GABA EC₅₀ analysis, two drug application electrodes were used (inset in Fig. 5A); one electrode contained 1 mM GABA and the second contained a lower concentration of GABA. Two doses of GABA were applied sequentially from low to high and the current induced by low concentration GABA was normalized to the one induced by 1 mM GABA. Thereafter, the second electrode was replaced with another concentration of GABA for the next test. Typically 3–5 GABA concentrations could be tested from each recorded astrocyte (Fig. 5A).

Solutions and reagents

TTX, D-AP5, NBQX (sodium salt), THIP, bicuculline, thapsigargin, SR95531, GABA, gramicidin, nipecotic acid and meclofenamic acid (MFA) were purchased from Tocris

Bioscience (Ellisville, Missouri, USA). Other chemicals were purchase from Sigma (St. Louis, MO, USA). They were dissolved in either DMSO or H₂O as stock solutions and stored in -20 °C freezer. 0.5 μM tetrodotoxin (TTX), 10 μM AP-5 and 30 μM NBQX were included in bath solution to inhibit neuronal synaptic transmission (Xie et al. 2008).

In some experiments, the intracellular Cl⁻ was reduced or totally replaced by gluconate, or K⁺ both in bath and electrode solutions was substituted with equimolar Cs⁺ for inhibition of conventional K⁺ channels. The 0 mM [Na⁺]_e focal application solution was achieved by equimolar replacement of NaCl with Choline-Cl and NaHCO₃ with Choline-HCO₃.

Data Analysis

(1) Intracellular Cl concentration—Intracellular Cl⁻ concentration was resolved from the reversal potential of GABA_A-R currents from the equation bellow:

$$E_{\text{GABA-A}} = (-RT/F) \ln \left(\frac{([Cl^-]_e + 0.2[HCO_3^-]_e)}{([Cl^-]_i + 0.2[HCO_3^-]_i)} \right) \quad (1)$$

Where T is temperature (295.15 K, corresponding to 22°C), R and F are universal gas constant and Faraday constant, respectively. $[Cl^-]_i$ and $[Cl^-]_e$ are intra- and extracellular Cl⁻ concentrations respectively. The intracellular HCO₃⁻ ($[HCO_3^-]_i$) used in this calculation was 16 mM and the relative permeability of HCO₃⁻ to Cl⁻ (P_{HCO_3}/P_{Cl}) was 0.2 (Kaila and Voipio 1987; Staley et al. 1995).

(2) Correction of voltage error in the reversal potential of GABA_A-R currents—

Voltage error in astrocyte voltage clamp recording was corrected to resolve the actual reversal potential of GABA_A-R currents ($E_{\text{GABA-A}}$).

$$E_{\text{GABA-A}} = V_r - (V_r - V_{\text{THIP}}) * R_m / (R_m + R_a) \quad (2)$$

Where the V_r is the reversal potential of ramp voltage command induced currents in control aCSF solution or in the presence of GABA_A-R specific agonist THIP (Fig. 2C). V_{THIP} is the measured reversal potential resulting from the subtraction of voltage ramp induced currents between control and THIP (Fig. 2C).

(3) Astrocyte membrane potential (V_m) established by transmembrane K⁺ ions

$$V_m = E_K = (-RT/F) \ln \left(\frac{[K^+]_i}{[K^+]_e} \right) \quad (3)$$

This assumption is made upon a close approximation of E_K with V_m in perforated patch recording (see Results).

(4) Anion driving force of GABA_A-R—In perforated patch recording, astrocyte V_m closely matched E_K (see Results). Because passive Cl⁻ distribution means $E_{Cl} = E_K = V_m$, therefore, astrocyte $[Cl^-]_i$ in passive distribution can be calculated from the following equation:

$$[Cl^-]_i = [Cl^-]_e [K^+]_e / [K^+]_i \quad (\text{Johnson and Wu 1995}) \quad (4)$$

Thus, the equation (1) can be altered to:

$$E_{\text{GABA}} - A = (-RT/F) \ln \left[\frac{([Cl^-]_e + 0.2[HCO_3^-]_e)}{([Cl^-]_e [K^+]_e / [K^+]_i + 0.2[HCO_3^-]_i)} \right] \quad (5)$$

Based on (3) and (5), the total anion driving force via GABA_A-R is:

$$\begin{aligned} V_m - E_{\text{GABA}} - A &= (-RT/F) \ln \left(\frac{[K^+]_i}{[K^+]_e} \right) \\ &- (RT/F) / \ln \left[\frac{([Cl^-]_e + 0.2[HCO_3^-]_e)}{([Cl^-]_e [K^+]_e / [K^+]_i + 0.2[HCO_3^-]_i)} \right] \\ &= (-RT/F) \ln \left[\frac{([Cl^-]_e [K^+]_e + 0.2[HCO_3^-]_i [K^+]_i)}{([Cl^-]_e [K^+]_e + 0.2[HCO_3^-]_e [K^+]_e)} \right] \end{aligned} \quad (6)$$

Because $[Cl^-]_e [K^+]_e$ is present in both numerator and denominator in the logarithm, the sign of anion driving force is determined by:

$$[HCO_3^-]_i [K^+]_i - [HCO_3^-]_e [K^+]_e \quad (7)$$

This relationship indicates that the direction of anion flux is determined by HCO_3^- and K^+ , but not by Cl^- .

(5) The GABA dose-response curve was fitted by the Hill equation below—

$$I = I_{\max} \{1 / [1 - (EC_{50} / [GABA])^n]\} \quad (8)$$

Data are presented as means $\pm$ SEM, unless indicated otherwise. *Chi* square test and Student's *t* test were performed to determine the statistical significance of the differences between the two groups. The level of significance was set at $P < 0.05$.

Results

Activation of GABA_A-R depolarizes astrocyte membrane potential under intact $[Cl^-]_i$

Astrocytes showing a linear voltage-current (*I-V*) relationship and low membrane resistance (R_m) have been reported since 1990's (Berger et al. 1991; D'Ambrosio et al. 1998; Jabs et al. 1997; Kang et al. 1998; Steinhauser et al. 1992). Mature astrocytes in rat hippocampal CA1 stratum radiatum identically show the same electrophysiological characteristics after P21 (Zhou et al. 2006), therefore, these criteria were used to identify mature astrocytes in the present study. GABA has been shown to induce GABA_A-R currents in hippocampal astrocytes (Fraser et al. 1995; Meier et al. 2008; Steinhauser et al. 1994; Zhou and Kimelberg 2001). To determine the role of GABA_A-R on membrane potential (V_m) and $[Cl^-]_i$ in intact astrocytes, gramicidin perforated patch recording (PPR) was used (see Methods and Sup. Fig. 1 for details). Stably established PPR recordings had a membrane potential (V_m), R_a and R_m of -88.0 ± 0.4 mV, 42 ± 7.8 M Ω and 13.8 ± 2.0 M Ω , respectively ($n = 8$).

In current clamp recording, 0.3 mM GABA and a specific GABA_A-R agonist 0.1 mM THIP depolarized astrocytic V_m by 2.4 ± 0.4 mV ($n = 6$) and 3.8 ± 0.3 mV ($n = 5$), respectively (Fig. 1 C, E), and the depolarization was almost inhibited by 0.01 mM SR95531, a specific GABA_A-R antagonist (Fig. 1 D, F), indicating that under the condition of intact $[Cl^-]_i$, GABA_A-R activation led to V_m depolarization in hippocampal astrocytes.

To determine $[Cl^-]_i$ of astrocytes, a voltage ramp command was applied to the established PPRs (Fig. 2A), first in aCSF solution, then in addition of 0.1 mM THIP (Fig. 2A). The net GABA_A-R currents were resolved by subtracting currents induced in control aCSF from the

currents in the presence of THIP (Fig. 2C) that resulted in a GABA_A-R reversal potential ($E_{\text{GABA-A}}$) of -38.5 ± 7.1 mV ($n = 5$). As the reversal potentials were resolved from current subtraction that carried 75% of voltage error, the error in each recording was corrected as exemplified in Fig. 2C. The corrected $E_{\text{GABA-A}}$ values were -78.3 ± 2.2 mV to -75.3 ± 2.3 mV ($n = 5$), corresponding to a total anion driving force ($V_m - E_{\text{GABA-A}}$) of -5.4 mV to -9.7 mV. The $[\text{Cl}^-]_i$ was calculated according to the Equ. 1. The resulted $[\text{Cl}^-]_i$, 3.1 ± 0.6 to 3.9 ± 0.7 mM ($n = 5$), was comparable to a passively distributed $[\text{Cl}^-]_i$ of 3.4 mM as calculated from the Equ. 4.

Bicarbonate efflux underlies GABA_A-R induced astrocyte V_m depolarization

To explore the mechanism underlying a low $[\text{Cl}^-]_i$ mediated V_m depolarization, we first calculated the Cl^- driving force (CDF) below.

$$\text{CDF} = V_m - E_{\text{Cl}} = -88.0 \text{ mV} - (-95.0 \text{ or } -89.2 \text{ mV}) = 7.0 \text{ or } 1.2 \text{ mV}$$

Where the V_m is the membrane potential of PPRs, and E_{Cl} is the Cl^- equilibrium potential calculated based on $[\text{Cl}^-]_e = 134.5$ mM in bath and $[\text{Cl}^-]_i = 3.1 - 3.9$ mM in PPRs. A positive CDF value indicates Cl^- influx that should not underlie GABA_A-R activation induced V_m depolarization.

GABA_A-R is also permeable to HCO_3^- with a relative permeability of $P_{\text{HCO}_3^-}/P_{\text{Cl}} = 0.2$ (Kaila and Voipio 1987; Staley et al. 1995). According to the used $[\text{HCO}_3^-]_e$ of 25 mM in bath and a $[\text{HCO}_3^-]_i$ of 16 mM (Staley et al. 1995), the $E_{\text{HCO}_3^-}$ was -12.2 mV and the Bicarbonate Driving Force (BDF) was:

$$\text{BDF} = V_m - E_{\text{HCO}_3^-} = -88 \text{ mV} - (-12.2 \text{ mV}) = -75.8 \text{ mV}$$

An outward BDF indicates efflux of $[\text{HCO}_3^-]_i$ via activated GABA_A-R that must be responsible for the observed V_m depolarization in astrocytes.

Characteristics of GABA_A-R currents in astrocytes

To further characterize astrocyte GABA_A-R currents, symmetric Cl^- gradient was used in the recording solutions. The V_m and R_{in} were -78.4 ± 0.2 mV ($n = 84$) and 18.4 ± 1.3 M Ω ($n = 89$) (Sup. Fig. 2). Astrocytes were held at their resting potentials for bath application of 0.3 mM GABA for 1 min, which induced an inward current of -162 ± 43 pA ($n = 10$) (Fig. 3 A). Noticeably, withdrawal of GABA was followed by a delayed inward current (DIC) in 70% of astrocytes ($n = 7/10$).

To segregate DIC better from the initial GABA_A-R currents, focal GABA application was used to synchronize receptor activation (Fig. 3B). A short pulse (< 1 s) focal application of 0.3 mM GABA increased peak receptor current amplitude considerably compared to bath GABA application, i.e., -321 ± 56 pA ($n = 36$), so was a faster time-to-peak kinetic of receptor activation (Fig. 3C). However, a short pulse focal GABA application was insufficient to induce DIC in the vast majority of cells (98%) ($n = 91/93$).

The induction of DIC was clearly depended on the time of GABA application. A longer 5-second GABA induced a salient DIC in 63.4% of cells (59/93) (Fig. 3C-E), and this application time was adopted in the following experiments unless indicated otherwise.

While the initial GABA receptor current reached the peak rapidly within 0.2-1 s ($n = 50$), the DICs became evident only after GABA/THIP withdrawal. In current clamp recording,

focal GABA application also induced a delayed V_m depolarization (DVD). Next, the time-to-peak of DIC and DVD was measured from the same astrocytes for comparison. The values were matched closely one another, i.e., 6.6 ± 0.5 s for DIC vs. 6.8 ± 0.3 s for DVD ($n = 4$; $P = 0.75$, Fig. 3E-F), which confirmed that the DIC and DVD represent the same event subsequent to astrocytic GABA_A-R activation. Contrary to the DIC that was induced at a clamped voltage of -80 mV (Fig. 3E), DVD appeared at the resting membrane potential without voltage control (Fig. 3F). We next varied the voltages from -110 mV to -60 mV and the results clearly showed that DVD was independent of the applied voltages (Fig. 7 A), suggesting that inhibition of voltage-independent K^+ channels, such as two-pore domain K^+ channels (K2Ps), could be accountable for DVD/DIC.

The amplitude of DICs varied considerably between astrocytes; three representative DICs with different current amplitudes, compared to their preceding receptor currents, are shown in Fig. 4A-C. It took 1-2 min for the induced DIC to recover fully and DIC could be re-evoked multiple times from the same cell.

The DIC appeared more frequently in slices prepared from the rostral than that of the caudal side of hippocampus (79.2% , $n = 38/48$ vs. 39.3% , $n = 11/28$, $P < 0.01$; Fig. 3H). No significant difference in DIC frequency was found between recordings obtained from superficial (or close to pyramidal layer) and deep half of *stratum radiatum* slices (58.3% , $n = 14 / 24$, vs. 57.1% , $n = 16 / 28$). Interestingly, a higher percentage of DIC in rostral slices coincided with a higher level of TWIK-1 K^+ channel mRNA expression in this region (Talley et al. 2001).

NG2 glia also express functional GABA_A-Rs and could be identified based on their voltage-gated K^+ and Na^+ conductance expression and lack of intercellular coupling (Xie et al. 2007). However, DIC was never inducible from NG2 glia ($n = 16$; Sup. Fig. 3A), nor the interneurons ($n = 5$; Sup. Fig. 3A), suggesting an astrocytic specific of DIC.

Although neuronal GABA_A-R activation has been shown to induce K^+ transient in *stratum pyramidale* resulting from outward transport of K^+ via neuronal K^+ - Cl^- cotransporter KCC2 (Grover et al. 1993; Viitanen et al. 2010), we found that the induction of neuronal K^+ transient required a much excessive exposure of pyramidal neurons to GABA (Sup. Fig. 4). Thus, DIC and K^+ transient are separate events subsequent to astrocytic and neuronal GABA_A-R activation, respectively.

A preceding GABA_A-R activation appeared to be required for the induction of DIC, because DIC was largely inhibited in the presence of SR95531 (Fig. 4, $n = 5$).

The amplitude of DIC also depended on applied GABA concentration (Fig. 5A), and an apparent threshold of GABA concentration for DIC induction was around 0.1 mM. We here also examined the dose-response relationship between GABA concentration and the amplitude of the initial peak receptor currents (see Methods for details). The resultant EC_{50} and Hill coefficient were 0.3 mM and 0.75 , respectively (Fig. 5B). This EC_{50} value indicated a rather low affinity of astrocytic GABA_A-R to GABA compared to the recombinant neuronal subtypes of GABA_A-R (Esmaeili et al. 2009). The EC_{50} was also 6-fold higher than the one examined from the freshly isolated hippocampal astrocytes (Fraser et al. 1995).

GABA_A-R mediated inhibition of Cs^+ permeable K2P likely underlies DIC

Activation of astrocytic GABA_A-R has been shown to transiently elevate intracellular Ca^{2+} (Fraser et al. 1995; Meier et al. 2008). A potential contribution of Ca^{2+} to the DIC was tested by inclusion of Ca^{2+} channel blockers, 0.1 mM Ni^{2+} , 0.1 mM Cd^{2+} and 0.15 mM

verapamil, in the bath solution. Ca^{2+} release from intracellular stores was inhibited by 0.1 mM ruthenium red. In a separate experiment, the Ca^{2+} stores were depleted by bath application of 5 μM thapsigargin. These conditions together did not inhibit or modify DIC (Sup. Fig. 5A-D), and the DIC was not affected by Ca^{2+} -free bath solution either (data not shown).

Pharmacological concentration of GABA could over-activate Na^+ dependent electrogenic GABA transporters. However, GABA uptake inhibitor, 0.1 mM nipecotic acid, induced only a small current (-11.0 ± 4.5 pA, $n = 4$; Sup. Fig. 6A), and did not affect DIC (Sup. Fig. 6B-C).

GABA_A-R activation induced DIC could also be ascribed to activation of some unknown type of anion channel. However, 0.1 mM NPPB, a broad spectrum anion channel inhibitor (Zhang et al. 2011), did not affect DIC (Sup. Fig. 5A). A potential involvement of anion channel to DIC was explored further by reducing or total replacement of KCl with K^+ -gluconate in electrode solution. However, 5 mM or 0 mM $[\text{Cl}^-]_i$ did not affect DIC (Sup. Fig. 5E).

We also explored a possible contribution of $[\text{Na}^+]_e$ to the DIC. As shown in Sup. Fig. 5F, when the DIC was induced, focal application of 0 mM $[\text{Na}^+]_e$ did produce a partial reduction of DIC. However, 0 mM $[\text{Na}^+]_e$ alone also induced a outward current with a comparable size in the same cell. Therefore, it was unlikely that 0 mM $[\text{Na}^+]_e$ has a significant contribution to the DIC. Na^+ channels blockers 0.5 μM TTX and 0.05 mM amiloride also did not affect the appearance of the DIC (data not shown). A potential impact of gap junction coupling on the induction of DIC was tested by bath application of gap junction inhibitor, 0.1 mM meclofenamic acid (MFA) (Xu et al. 2010). DIC was not affected by MFA (data not shown).

HCO_3^- efflux via activated GABA_A-R has been shown to acidify intracellular pH (Kaila and Voipio 1987) that is inhibitory for TWIK-1 K^+ channels (Lesage et al. 1996) expressed abundantly in astrocytes (Zhou et al. 2009). We hypothesized that HCO_3^- efflux induced intracellular acidification and TWIK-1 inhibition underlies DIC.

Activation of GABA_A receptor is known to decrease membrane resistance (R_m). However, GABA induced a progressive increase in input resistance (R_{in}) in astrocytes that amounted to 5.6 ± 0.8 M Ω ($n = 4$, Fig. 6A). The results indicated that astrocytic GABA_A-R activation is associated with a strong suppression of background open channels and the latter overwhelmed the receptor activation mediated R_m decrease. This effect was astrocytic specific, because GABA_A-R activation decreased R_m markedly in NG2 glia (Fig. 6B), pyramidal neurons and interneuron (Fig. 6C) that accounted to 76 ± 12 M Ω ($n = 2$), 107 ± 17 M Ω ($n = 4$) and 300 M Ω ($n = 1$), respectively.

To test the dependence of DVD on the HCO_3^- efflux, we altered the HCO_3^- driving force from manipulation of V_m . At the holding V_m more negative than the resting V_m to drive HCO_3^- efflux, the DVD was readily inducible (Fig. 7A), while the DVD was abolished at a holding V_m near the $E_{\text{HCO}_3^-}$ of -12 mV ($n = 5$) (Fig. 7B). These results indicated that HCO_3^- efflux and the following intracellular acidification should be required for DVD induction.

We next sought to determine if TWIK-1 blocker quinine could inhibit DIC. Quinine is a commonly used TWIK-1 channel inhibitor (Lesage et al. 1996; Ma et al. 2011; Zhou et al. 2009). Bath application of 1 mM quinine resulted in a progressive V_m depolarization (Fig. 8C). Following to V_m depolarization, quinine inhibited DIC in a time-dependent fashion (Fig. 8 A-B). The inhibitory effect of quinine on DIC was quantitatively calculated by using the ratio of the amplitude of DIC (I_{DIC}) over its preceding GABA_A-R currents ($I_{\text{GABA-R}}$),

i.e., $I_{\text{DIC}}/I_{\text{GABA-R}}$. In the end of 40 min of quinine treatment, the $I_{\text{DIC}}/I_{\text{GABA-R}}$ ratio reduced to $25.5 \pm 4.0\%$ of the control level ($n = 3$, $P < 0.01$; Fig. 8 A-B and inset below B). Note that the remaining 25.5% of $I_{\text{DIC}}/I_{\text{GABA-R}}$ (green traces) was indistinguishable from the nondesensitizing GABA_A-R currents, or the quinine produced actual inhibition should be considerably greater than 74.5%. The results indicated that quinine sensitive K⁺ channels, most likely K2Ps, were required for the induction of DIC. The quinine produced inhibition was reversible and repeatable in the same recording (data not shown).

Astrocytic K2Ps are highly permeable to Cs⁺, a commonly used cation substitute for inhibition of conventional K⁺ channels (Hille 2001). Had K2P inhibition truly underlied the DIC, an intact DIC would remain in the recording solutions without K⁺ and quinine should still be effective. In the recordings, where the K⁺ ions were totally replaced by Cs⁺ in the solutions, GABA and THIP still induced DIC and the currents were effectively inhibited by 1 mM quinine ($n = 4$, $P < 0.05$) (Fig. 8D).

Collectively, the evidences indicated strongly that inhibition of Cs⁺ permeable K2Ps, especially TWIK-1, most likely was accountable for the GABA_A-R induced DIC in astrocytes.

The DIC in our experimental condition require prolonged exposure of nonphysiological concentration of GABA, therefore it is necessary to determine to what extent this could occur *in situ*. In dual interneuron recording in CA1 striatum radiatum, over stimulation of neuron with positive current injection induced neither astrocytic GABA_A-R currents nor DIC (Sup. Fig. 7). The functional implication might be that synchronized GABA release from multiple GABAergic neurons are required to activate astrocytic GABA_A-R and induce the following DIC.

Discussion

The [Cl⁻]_i follows a passive distribution in mature hippocampal astrocytes

In the present study, we have revealed, for the first time, a rather low [Cl⁻]_i of 3.1 to 3.9 mM in mature rat hippocampal astrocytes that favors a passive distribution of Cl⁻ anion. Such a low [Cl⁻]_i differs strikingly from the high [Cl⁻]_i of 30 to 40 mM high in cultured astrocytes (Bekar and Walz 2002; Kimelberg 1981). In an early study by Ballanyi and colleagues, a low [Cl⁻]_i of 3.2 mM was reported from glial cells of guinea-pig olfactory cortex measured with Cl⁻ sensitive microelectrode (Ballanyi et al. 1987). In the same study, the glial cells were identified to have a membrane potential more negative than -75 mV and lack of synaptic potentials, therefore, they most likely represented astrocytes, although some oligodendrocytes might also be included.

High intracellular Cl⁻ content is mainly ascribed to active accumulation of Cl⁻ from Na⁺-K⁺-2Cl⁻ cotransporter. According to a transcriptome study, the high [Cl⁻]_i in cultured astrocytes may well be resulted from a 2.7-fold higher Na⁺-K⁺-2Cl⁻ cotransporter expression compared to astrocytes isolated from young mature P17 mouse (Cahoy et al. 2008). Interestingly, the same study also revealed a 2.7 fold higher Na⁺-K⁺-2Cl⁻ cotransporter expression in astrocytes isolated from neonatal P1 mouse compared to P17 mouse, implying a similarity between cultured astrocytes and immature astrocytes. It would be interesting to know in the future whether an age-dependent down regulation in Na⁺-K⁺-2Cl⁻ cotransporter expression would correlate to a decreasing [Cl⁻]_i during astrocyte maturation (Cahoy et al. 2008).

Similar to the mature neurons (Ben-Ari 2002; Owens et al. 1996), our results revealed a low [Cl⁻]_i in mature hippocampal astrocytes. In contrast, other mitotically active cell types in the

brain, such as NG2 glia and subventricular zone neuronal progenitors maintain a high $[Cl^-]_i$ (Lin and Bergles 2004; Wang et al. 2003), so is the cultured astrocytes and glioma (Sontheimer 2008; Walz 2002). Increasing evidence indicates that cultured astrocytes are similar to reactive astrocytes in various pathological states, and reactive astrocytes appear to have a up-regulated K^+ uptake ability that is coupled with Cl^- influx, suggesting a high Cl^- content in reactive astrocytes (MacVicar et al. 1989; Walz and Wuttke 1999). These data together favors a notion that a low $[Cl^-]$ is a characteristic of differentiated cells in the central nervous system.

Bicarbonate efflux via activated GABA_A-R induces V_m depolarization in astrocytes

We showed in the perforated patch recordings that GABA_A-R activation mediated a V_m depolarization. As noted that passive Cl^- distribution means tied values of E_{Cl} and E_K (-93 mV) with a zero driving force for Cl^- flux. The fact that V_m in perforated patch recordings (-88 mV) is very close to E_{Cl} and E_K indicates a negligible driving force ($V_m - E_{Cl}$) for Cl^- flux during astrocytic GABA_A-R activation.

In cultured astrocytes, bicarbonate efflux via activating GABA_A-R induced astrocyte V_m depolarization (Kaila et al. 1991). The same mechanism also underlies the V_m depolarization of astrocytes in slices. We here extend the analysis further to show that the only impact of astrocytic GABA_A-R activation on V_m is depolarization. Because $[Cl^-]$ becomes irrelevant to the V_m depolarization, thus, the direction of HCO_3^- flux follows to a relation of $[HCO_3^-]_i[K^+]_i - [HCO_3^-]_e[K^+]_e$ (Equ. 7). The physiological $[HCO_3^-]$ varies between 24 mM to 26 mM in most of extracellular fluids and 10mM to 15 mM inside cells (Litvin et al. 2003; Tresguerres et al. 2010), and the $[K^+]_e$ fluctuates between 4 mM to 12 mM (Heinemann and Lux 1977; Walz 2000), thus, the $[HCO_3^-]_i[K^+]_i - [HCO_3^-]_e[K^+]_e$, should always be a negative value that drives HCO_3^- efflux and V_m depolarization. In summary, HCO_3^- efflux and V_m depolarization should be the direct functional consequences of astrocytic GABA_A-R activation, a role that has been previously demonstrated from muscle fibers (Kaila and Voipio 1987). The HCO_3^- efflux also implies a role of astrocytic GABA_A-R in pH regulation.

A critical factor that determines the amount of HCO_3^- efflux is V_m . However, the V_m measured from conventional whole-cell (-78 mV) and perforated patch (-88 mV) recordings varied by 10 mV ($P < 0.01$) and the latter was more close to the E_K (-93 mV) in our experimental conditions (Fig. 2B). At least two factors could underlie the difference between the two recording modes. First, the distribution of V_m (Sup. Fig. 2B) showed that some of the V_m values did exceed the -80 mV to close to the V_m of perforated patch recordings, thus, variation in gigohm seal quality in whole-cell recording may partially contribute to the difference. Second, perforated patch recording does not involve extensive dialysis of cytoplasmic content that may also underlie the observed difference.

Activation of astrocytic GABA_A-R induces delayed inhibition of astrocytic K2P

Our data showed that GABA_A-R activation is followed by a delayed inhibition of astrocytic K2P K^+ channels. The present study showed that the DIC remained intact when intracellular and extracellular $[Ca^{2+}]$, $[Na^+]$ and $[Cl^-]$ were altered and DIC was insensitive to a variety of cation/anion channel inhibitors. The involvement of K2P in DIC has been indicated, as DIC was intact in Cs^+ based recording solutions, a condition that inhibits conventional K^+ channels, such as Kir4.1, but remains the function of Cs^+ permeable K2Ps in astrocytes. The involvement of Cs^+ sensitive K2P in DIC was further supported by inhibition of DIC by quinine (Lesage et al. 1996; Ma et al. 2011; Zhou et al. 2009). The involvement of TWIK-1 in DIC has been proposed because 1) GABA_A-R activation leads to intracellular acidification in cultured astrocytes (Kaila et al. 1991); 2) intracellular acidification is shown

to inhibit TWIK-1 K⁺ channels (Lesage et al. 1996) and 3) TWIK-1 is highly expressed in astrocytes and sensitive to quinine with a EC₅₀ of 85 μM (Zhou et al. 2009). These together Previously, GABA_A-R activation has been shown to alter [Cl⁻]_i that in turn modulate astrocyte outwardly rectifying K⁺ channels (Bekar and Walz 2002). In addition to that, the present study show that GABA_A-R activation could modify other K⁺ channels expressed in mature hippocampal astrocytes via bicarbonate efflux and intracellular acidification.

The affinity of astrocytic GABA_A-R to GABA

By using focal GABA application to astrocytes *in situ*, we revealed a GABA EC₅₀ value of 300 μM, which was 6-fold higher than a EC₅₀ value (50 μM) analyzed from astrocytes freshly isolated from rat hippocampus of similar ages (Fraser et al. 1995). The freshly isolated astrocytes offer the advantages of being able to clearly determine the properties of astrocytes without having to control the indirect effect from the surrounding neurons or other glial cells. The isolated astrocytes also have significantly improved *R_m* for a better voltage clamping quality (Kimelberg et al. 2000). However, the poor voltage clamping quality in slice recording should mask the current amplitude induced by high concentration of GABA more severely than the ones induced by the lower concentrations. In other words, the EC₅₀ in our study should be further underestimated. The actual reasons underlying the different EC₅₀ values between the two studies are unclear.

Nevertheless, astrocytic GABA_A-R showed a much lower affinity to endogenous ligand GABA compared to neurons that have a EC₅₀ in the range of 0.2 – 0.4 μM (Wu et al. 2007). In view of the low ambient GABA in the range of 0.1 – 0.4 μM (Attwell et al. 1993), it is unlikely that neuronal GABA release could induce phasic GABA_A-R activation. Therefore, astrocytic GABA_A-R may mainly function to sense ambient GABA and this has been shown to play role in maintaining the morphology of astrocytes in developing and adult brain (Velez-Fort et al. 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. GABA_A receptor activation induced astrocyte V_m depolarization in gramicidin

A. A mature astrocyte characterized by its passive membrane conductance that was induced by the voltage commands shown in the inset below the recording; the cell was held at -80 mV at resting and stepped to command voltages of -160 to 20 mV in 10 mV increment. **B.** The current-to-voltage (I-V) relationship of this the same cell. **C, E.** A 2-min application of 0.3 mM GABA or 0.1 mM THIP induced V_m depolarization in astrocytes that could be substantially (**D**) or completely (**F**) inhibited by 0.01 mM SR95531.

Figure 2. Astrocytic GABA_A-R currents

A. Gramicidin perforated patch recording from an astrocyte; the cell was held at -70 mV and a 1 second long voltage ramp from -160 mV to $+20$ mV was delivered every 20 second to induce currents (inset below **A**). Addition of $100 \mu\text{M}$ THIP shifted the holding currents downwardly (dotted line). **B.** Chronological display of reversal potentials of ramp induced current traces shown in **A**. The black and red arrows in **A** and **B** pointed to the ramp induced current traces and their corresponding reversal potentials in aCSF control and THIP application, respectively. **C.** Black and red traces in **A** are plotted against the applied voltages. THIP induced GABA_A-R currents (blue) were resolved by off-line digital

subtraction of red trace from the black trace that yielded an apparent $E_{\text{GABAA-R}}$ of -30 mV. As the voltage ramp induced currents carried a 75% of voltage error, the error correction yielded the actual $E_{\text{GABAA-R}}$ in the range of -77 mV to -72 mV for this recording.

Figure 3. GABA_A-R activation induced delayed DIC

A. Bath application of 0.3 mM GABA for 1 min induced a rapid initial receptor current that was followed by a delayed inward current (DIC) after GABA withdrawal. **B.** Shown in the image was the placement of the second electrode on the top-left serving for focal drug application to the recorded cell on the right electrode. Scale bar is 10 μ m. **C-E.** Focal application of 0.3 mM GABA at variable durations; the induction of DIC depended on the duration of the GABA exposure. **F.** A 5-second application of 0.3 mM GABA induced two phases of V_m depolarization. The second phase, or delayed V_m depolarization (DVD), peaked 2 s after the withdrawal of GABA, which was consistent with the peak time of DIC in the same cell (**E**). **G.** A representative recording without GABA induced DIC. **H.** Frequency of the DIC appearance in hippocampal slices prepared from rostral (79.2%, 38/48 cells), vs. caudal side (39.3%, 11/28 cells). The difference between the frequencies in these subregions is statistically significant at $P < 0.01$. M: middle, D: dorsal, L: lateral, V: ventral.

Figure 4. Dependence of DIC on GABA_A-R activation

A, B, C. The amplitude of the DIC induced by either 0.3 mM GABA or 0.1 mM THIP varied from cell to cell (black traces), but depended on the preceding gating of GABA_A-R; the DICs were substantially abolished in the presence of 0.01 mM SR95531 for GABA_A-R inhibition.

Figure 5. Dependence of DIC on GABA concentration

A. Representative GABA_A-R currents induced by 0.01 mM, 0.3 mM and 1 mM GABA from one astrocyte. The GABA induced DIC became evident in 0.3 mM GABA and further enhanced in 1 mM GABA. The inset shows three-electrode configuration for dose-response experiments. The right electrode was for recording and the two left electrodes were used for GABA application. **B.** Dose-response relation of GABA induced initial GABA_A-R currents. GABA-induced currents at various concentrations were normalized to 1 mM in the same cell. Each data point represents the mean of 3 to 4 cells. Error bars are SEMs. The smooth line gives the best fit according to the Hill equation (see Materials and Methods), yielding an EC_{50} of 0.3 mM and n_H of 0.75.

Figure 6. Activation of astrocytic GABA_A-R is associated with a rapid increase in input resistance

Membrane input resistance (R_{in}) was measured by a pair of ± 0.1 nA/ 300 ms current pulse delivered periodically to a recorded astrocyte (**A**), NG2 glia (**B**) and SR interneuron (**C**). To compare GABA effect on R_{in} , two representative recording segments from control (shaded grey square) and GABA application (shaded red square) are superimposed in **A2**, **B2** and **C2**, where increase in R_{in} in astrocytes and decrease in R_{in} in a NG2 glia and an interneuron are shown. **A3**, **B3**, **C3**. Chronological display of R_{in} changes from control to GABA application and washout for an astrocyte, NG2 glia and interneuron, respectively.

Figure 7. HCO_3^- efflux was required for the induction of DVD

A. Representative recordings from an astrocyte, where DVD appeared when the V_m was changed from resting (**A1**) to -105 mV (**A2**) and -59 mV (**A3**). **B.** Representative V_m recordings from another astrocyte, where depolarization of V_m from resting (**B1**) to -13 mV (**B2**) abolished DVD.

Figure 8. Quinine inhibition of GABA induced DIC

A. Time-dependent inhibition of GABA induced DIC by bath application of 1 mM quinine. 0.3 mM GABA induced receptor currents and DIC at different times by focal application. After control recording, 1 mM quinine was bath applied that inhibited the DIC in a time-dependent fashion. **B.** Continued recording from the cell in **A**. After washout, the DIC appeared 78 min later with 57% recovery in current amplitude. Quinine inhibition (inset) was calculated and presented as I_{DIC}/I_{GABA_R} that decreased to 25.5% of the control level in the end of 40 min of quinine application. **C.** Change in V_m during quinine application and washout for the cell shown in **A** and **B**, where quinine produced V_m depolarization, coincided with the quinine mediated DIC inhibition. **D.** DIC remained in Cs^+ a recording solution that was inhibited in a time-dependent fashion. The holding level of the recording traces in **A**, **B** and **D** were normalized to the same level for comparison of DIC amplitude.