GABA- and Glycine-Mediated Fall of Intracellular pH in Rat Medullary Neurons In Situ

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LUÈCKERMANN, Mark, Stefan Trapp, and Klaus Ballanyi. GABA- and glycine-mediated fall of intracellular pH in rat medullary neurons in situ. J. Neurophysiol. 77: 1844–1852, 1997. In the region of the ventral respiratory group in brain stem slices from neonatal rats, intracellular pH (pHi) and membrane currents (Im) or potentials were measured in neurons dialyzed with the pH-sensitive dye 2′,7′-bis-carboxyethyl-5(6)-carboxyfluorescein. Currents and increases in membrane conductance (gNa) during bath application of 0.1 or 1 mM γ-aminobutyric acid (GABA) were accompanied by a delayed mean fall of pH by 0.17 and 0.25 pH units, respectively, from a pH baseline of 7.33. These effects were reversibly suppressed by 50–100 mM bicuculline. Similar effects on Im, gNa, and pHi were revealed on administration of 0.1 or 1 mM glycine. These responses were abolished by 10–100 mM strychnine. Dialysis of the cells with 15–30 mM carbonic anhydrase led to an acceleration of the kinetics and a potentiation of the GABA-induced pH decrease. GABA- and glycine-evoked pH decreases were very similar during recordings with either high- or low-Cl⁻ patch electrodes, although the reversal potential of the accompanying currents differed by ~60 mV. The GABA-induced pH decrease, but not the accompanying Im and gNa responses, was suppressed in CO₂/HCO₃⁻-free, N₂-hydroxy-ethylpiperazine-N′-2-ethane sulfonic acid pH-buffered solution. Depolarization from ~60 to +30 mV resulted in a sustained fall of pHi by maximally 0.5 pH units. In this situation, the GABA-induced fall of pHi turned into an intracellular alkalosis of 0.09–0.15 pH units. The results confirm and extend previous findings obtained in vivo that GABA- or glycine-induced intracellular acidosis of respiratory neurons is due to efflux of HCO₃⁻ via the receptor-coupled Cl⁻ channel.

INTRODUCTION

In medullary respiratory neurons of cats in vivo, γ-aminobutyric acid (GABA) and/or glycine-induced hyperpolarization (Ballantyne and Richter 1986; Richter 1996; Richter et al. 1992) is accompanied by a prominent decrease of intracellular pH (pHi) (Ballanyi et al. 1994a). It was hypothesized (Ballanyi et al. 1994a) that this intracellular acidosis is secondary because of HCO₃⁻ efflux via the receptor-coupled anion pore (Bormann et al. 1987), as was originally shown for crayfish muscle fibers and neurons (Kaila 1994; Kaila and Voipio 1987; Voipio et al. 1991). A Cl⁻-mediated inspiration-related hyperpolarization is also a characteristic feature of several types of respiratory neurons in the isolated brain stem–spinal cord preparation of neonatal rats (Ballanyi et al. 1994b; Onimaru and Homma 1992; Onimaru et al. 1990, 1996; Smith et al. 1992). This in vitro respiratory network remains functionally active in a transverse slice, containing ventral respiratory group (VRG) neurons of the pre-Bötzinger complex, which is the core of the respiratory network (Smith et al. 1991). We have further reduced this preparation to a nonrhythmic, 150-μm thin brain stem slice to measure fluorometrically pHi in voltage-clamped neurons of the region of the VRG, which were dialyzed via the patch electrode with the fluorescent indicator 2′,7′-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF) (Trapp et al. 1996a).

The results show that GABA and glycine cause a prominent and sustained fall of pHi, which depends on the presence of extracellular CO₂/HCO₃⁻ and is reversed into an intracellular alkalosis at positive membrane potential (Eₘ). This substantiates the above assumption that rhythmic fluctuations of pHi during physiological activity of respiratory neurons in vivo are due to HCO₃⁻ efflux through GABA- or glycine receptor-associated Cl⁻ channels (Ballanyi et al. 1994a). Parts of the results have been published in abstract form (LuÈckermann et al. 1995; Trapp et al. 1995).

METHODS

Preparation and solutions

Wistar rats (1- to 4-day-old) of either sex were anesthetized with ether and decapitated. The brain was removed and the brain stem with the cerebellum was isolated. After removal of the cerebellum, the brain stem was glued to the stage of a vibratome (FTB Vibracut, Weinheim, Germany) and three transverse slices (150 μm thick) were cut at levels of 200 μm caudal to the obex (see Smith et al. 1991). After transfer and immobilization of individual slices with a net, the recording chamber (volume 3 ml) was superfused with oxygenated standard saline (temperature 30°C, flow rate 5 ml/min) of the following composition (in mM): 118 NaCl, 3 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1 NaH₂PO₄, and 10 glucose, pH adjusted to 7.4 by gassing with 95% O₂/5% CO₂. In some experiments, a CO₂/HCO₃⁻-free superfusate of the following composition was used (in mM): 118 NaCl, 3 KCl, 1 MgCl₂, 1.5 CaCl₂, 25 N₂-hydroxy-ethylpiperazine-N′-2-ethane sulfonic acid (HEPES), and 10 glucose, pH adjusted to 7.4 with 1 N NaOH. This solution was gassed with 100% O₂. Drugs, purchased from Sigma (Deisenhofen, Germany), were added from stock solutions to the superfusion fluid.

Intracellular recordings

Patch pipettes were made from borosilicate glass capillaries (GC 150TF, Clark Electromedical Instruments, Pangbourne, UK) with the use of a horizontal electrode puller (Zeitz, Munich, Germany). The standard (‘‘high-Cl⁻’’) patch electrode solution contained (in mM) 130 KCl, 1 NaCl, 1 MgCl₂, 0.5 CaCl₂, 1 K-1,2 bis(2-aminoethoxy)ethane-N,N,N′,N′-tetraacetic acid, 10 HEPES, and 1 Na-ATP, pH adjusted to 7.4 with 1 N KOH. In some experiments, KCl was replaced by 130 mM potassium gluconate (‘‘low-Cl⁻’’).
solution”) or a solution containing the K+ channel blockers Cs+ and tetraethylammonium (TEA), with KCl replaced by 100 CsCl and 30 TEA-Cl. These solutions had an osmolality of between 270 and 290 mosmol and were adjusted to a pH of 7.4 with 1 N KOH. DC resistance of patch electrodes ranged from 3–8 MΩ, depending on the composition of the filling solution. The pH-sensitive dye BCECF (50 μM) was added to the pipette solution. In some experiments, 15 or 30 μM carbonic anhydrase (CA) was also added.

Whole cell recordings were performed on superficial neurons in the region of the VRG, located in the ventrolateral reticular formation near the nucleus ambiguus (Arata et al. 1990; Onimaru and Homma 1992; Richter 1996; Smith et al. 1991). The cells were visualized through a ×40 water immersion objective under an upright microscope (Standard-16, Zeiss, Oberkochen, Germany), with the use of an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany). Seal resistances were between 1.5 and 3 GΩ. Series resistance (8–20 MΩ) and cell capacitance (21.8 ± 9.9 pF) were compensated by >70%. Membrane conductance (gm) was measured by regular injection of hyperpolarizing or depolarizing voltage pulses (duration 500 ms, amplitude −20 mV or −10 to −100 pA in current-clamp mode). During voltage-clamp recordings, holding potential (Vh) was typically −60 mV. Steady-state current-voltage relations were determined by application of depolarizing or hyperpolarizing voltage steps (duration 300 ms) and analysis of membrane current (Im) responses. Averages of responses to three consecutive pulses were analyzed.

pH measurements

The microscope was equipped with an epifluorescence optics, a xenon lamp, and a photomultiplier system (Luigs & Neumann, Ratingen, Germany). A pinhole diaphragm limited the region from which emitted light was collected to a circular spot of 40 μm diam. The ratio of the fluorescence (F) signals was measured at 540 nm (band-pass filtering 515–565 nm) in response to alternating excitation at 440 and 490 nm (F440/F490). Once the whole cell configuration was established, steady-state loading of BCECF was achieved after a maximum of 10 min as indicated by the stable signals of the excitation fluorescence signals and a smooth ratio trace, representing pH. Signals were calibrated according to a modified (Trapp et al. 1996a) “nigericin” method (Thomas et al. 1979). For this purpose, cells were exposed to 4 μM of the K+/H+ ionophore nigericin in a high-K+ solution consisting of (in mM): 118 KCl, 3 NaCl, 1 MgCl2, 1.5 CaCl2, 25 HEPES, and 10 glucose, pH varied between 6 and 8 with 1 N KOH. For further details, see Trapp et al. (1996a).

Fluorescence signals were sampled at 2 Hz by a Mega STE-4 computer (Atari Corporation, Sunnyvale, CA) with the use of the Fura-2 Data Acquisition System (Luigs & Neumann). Electrophysiological data were sampled via an additional ITC-16 interface (Instrutech, Elmont, NY) and into a second Mega STE-4 computer with the use of the EPC-9 software (HEKA). Analysis of data was performed with Review (HEKA) and the Fura-2 Data Acquisition System. For production of figures, pH traces were low-pass filtered at 0.5 Hz. Values are means ± SD.

RESULTS

Hyperpolarizing inhibitory postsynaptic potentials (IPSPs) are a characteristic feature of VRG neurons in the isolated brain stem–spinal cord preparation of neonatal rats (Ballanyi et al. 1994b; Onimaru et al. 1996; Smith et al. 1992; see also Feldman and Smith 1989). The contribution of GABA, and glycine receptors to these responses and their pharmacology has, however, not been analyzed in detail yet. Therefore in an initial series of experiments we tested the electrical response of voltage-clamped neurons in the region of the VRG to bath-applied GABA and glycine and to the specific antagonists bicuculline and strychnine.

Effects of GABA and glycine on Im and gm

AGONIST-EVOKED CURRENTS. For these measurements, either the standard high-Cl− electrode solution or a high-Cl− solution containing Cs+ and TEA+ for improving voltage-clamp conditions by reduction of K+ currents (Onimaru et al. 1996) was used. Bath application of 0.1–1 mM GABA produced an inward current 0.1–1.3 nA in amplitude, accompanied by an increase in gm of several hundred percent in 12 cells tested. These currents with a reversal potential (Erev) of between −8 and +9 mV (n = 4) were blocked by 50–100 μM bicuculline (Fig. 1, A and B). In nine of these neurons, glycine evoked an inward current 0.05–0.4 nA in amplitude and a concomitant gm increase, which was suppressed by strychnine (Fig. 1, C and E). Similar to the GABA response, Erev of the glycine-evoked inward currents was close to 0 mV (Fig. 1D). Two cells, in which GABA evoked an inward current and gm increase, did not respond to glycine. As tested in a total of eight cells, the GABA responses were not markedly affected by 25 μM strychnine and the glycine responses persisted in the presence of 50 μM bicuculline (Fig. 1E).

SPONTANEOUS INHIBITORY POSTSYNAPTIC CURRENTS. In ~50% of recordings in which the standard high-Cl− electrode solution was used (n = 25), and, in particular, during recordings with the Cs/TEA-filled electrodes (n = 13), spontaneous synaptic inward currents were observed at a Vh of −60 mV. These currents, which had an amplitude of up to 0.5 nA and reversed polarity at ~9 mV, were substantially attenuated by either 10–50 μM bicuculline or 10–25 μM strychnine (Fig. 1E). Whereas the bicuculline block was reversible within 5 min, recovery of such spontaneous inhibitory postsynaptic currents (IPSCs) from strychnine was still incomplete after ~10 min of washout of the drug. These results demonstrate that the majority of neonatal neurons in the region of the VRG has functional GABA, and glycine receptors.

pH measurements

EFFECTS OF GABA AND GLYCINE. Subsequent to this pharmacological characterization it was examined whether exposure to GABA or glycine for 1 min affects pH. In 10 neurons, recorded at a Vh of −60 mV with the standard high-Cl− intracellular solution containing the fluorescent pH indicator BCECF, a mean pH, baseline of 7.33 ± 0.13 (SD) was revealed. The calculated equilibrium potential for H+ and, thus, bicarbonate ranged between −10 and +9 mV. In nine of these cells, the GABA-evoked inward current and gm decrease were accompanied by a delayed fall of pH (Fig. 2A) by up to 0.4 pH units in individual cells. The average values for the GABA-induced pH decrease were 0.25 ± 0.11 pH units (n = 5) for 1 mM GABA and 0.17 ± 0.07 pH units (n = 4) for 0.1 mM GABA. On washout of GABA, pH recovered from the intracellular acidosis within ~2 min. A smaller mean fall of pH, by 0.18 ± 0.1 pH units (1 mM, n = 4) and 0.12 ± 0.06 pH units (0.1 mM, n = 3) was
FIG. 1. γ-Aminobutyric acid (GABA)- and glycine-evoked membrane currents. A: bicuculline block of a GABA-induced inward current (membrane current, \( I_m \)) and membrane conductance (\( g_m \)) increase, measured by regular application of \(-20-\) 20 mV voltage steps. B: current-voltage relation of 4 cells revealing a reversal potential (\( E_{\text{rev}} \)) of the GABA-evoked current of \(-0 \) mV. C: strychnine block of a glycine-induced inward current and \( g_m \) increase. D: current-voltage relation of 3 cells shows a mean \( E_{\text{rev}} \) of the glycine response of \(-0 \) mV. E: effects of bicuculline and strychnine on spontaneous inhibitory postsynaptic currents and \( I_m \) response to bath-applied glycine. All measurements were performed with Cs-tetraethylammonium (TEA)-Cl\(^{-}\) intracellular solution, except in C, where a KCl solution was used.

observed on administration of glycine. The GABA-induced inward current and \( g_m \) increase as well as the accompanying fall of \( \text{pH}_i \) were reversibly blocked by 100 \( \mu \)M bicuculline, whereas 10 \( \mu \)M strychnine effectively suppressed the \( \text{pH}_i \) responses to glycine (Fig. 3). In 11 different cells tested for glycine (\( n = 10 \)) and GABA (\( n = 1 \)), \( \text{pH}_i \) remained unaffected despite a profound effect on \( I_m \) and \( g_m \) (not illustrated).

EFFECTS OF NH\(_4\)\(^+\) AND DEPOLARIZATION. To test whether this lack of a GABA- or glycine-evoked acidification was due to impaired \( \text{pH} \) sensitivity of BCECF (see DISCUSSION), alternative procedures to displace \( \text{pH}_i \) were used. For this purpose, the cells were either exposed to \( \text{NH}_4\)\(^+\) or depolarized via current injection. Bath application of 10 mM \( \text{NH}_4\)\(^+\), which is an established tool to perturb \( \text{pH}_i \) for an analysis of \( \text{pH} \) regulatory mechanisms (Thomas 1984), led to an inward current up to 0.3 nA in amplitude. The inward current was accompanied by an initial intracellular alkalinization by maximally 0.3 pH units, followed by a delayed acidification of 0.15–0.4 pH units in three cells tested. These effects of \( \text{NH}_4\)\(^+\) are illustrated for a cell in which GABA led to a prominent \( \text{pH}_i \) decrease (Fig. 2A). In a total of five neurons, depolarization to 0 or +30 mV for 1 min evoked an intracellular acidosis by 0.12–0.5 pH units (Fig. 2B, see also Fig. 5).

EFFECTS OF CARBONIC ACID. A second possible explanation for the lack of the GABA- or glycine-related intracellular acidosis in a subpopulation of these medullary neurons would be washout of carbonic acid (CA), which is essential for the generation of GABA-evoked decreases of \( \text{pH}_i \) (Pasternack et al. 1993); 15–30 \( \mu \)M of this enzyme was added to the patch pipette solution. In this series of experiments, administration of 1 mM GABA for only 30 s produced a fall of \( \text{pH}_i \) by 0.18 ± 0.05 pH units in 15 neurons loaded with 15 \( \mu \)M CA, whereas in 6 control cells, the mean GABA-related \( \text{pH}_i \) fall was 0.09 ± 0.03 pH units. In addition to this significant (\( P < 0.05 \)) potentiation of the amplitude of the \( \text{pH}_i \) response in the cells dialyzed with CA, the initial rate (0.39 ± 0.14 pH units/min) of the GABA-induced fall of \( \text{pH}_i \), which was linear.
FIG. 2. Effects of GABA, NH₄⁺, and membrane depolarization on intracellular pH (pHᵢ). A: GABA evoked an inward current and a concomitant delayed fall of pHᵢ, whereas an inward current in response to NH₄⁺ was accompanied by an initial intracellular alkalosis, turning into a fall of pHᵢ after washout of the drug. Recording was performed with a high-Cl⁻ intracellular solution. B and C: whereas GABA (B) or glycine (C) did not lead to a fall of pHᵢ in this cell, depolarization to 0 mV led to a progressive profound intracellular acidosis. Recording was performed with a low-Cl⁻ intracellular solution.

during the first 20 s of occurrence, was also significantly (P < 0.05) faster than in the control neurons (0.14 ± 0.05 pH units/min). In this set of experiments, pHᵢ remained unaffected by GABA in two of the CA-dialyzed neurons and in four cells of the control.

EFFECTS OF Cl⁻. In a different series of experiments, it was investigated whether the intracellular acidosis on activation of the receptor-associated Cl⁻ channels was affected by the Cl⁻ gradient, determining chloride equilibrium potential (E_Cl) and, thus, the direction of Cl⁻ fluxes through the receptor-coupled Cl⁻ channel. In eight cells, dialyzed with a low-Cl⁻ electrode solution, resting pHᵢ (7.37 ± 0.15) did not significantly differ from pHᵢ baseline in cells recorded with the standard high-Cl⁻ solution (see above). Figure 4A illustrates that GABA evoked a substantial g_m increase, but no major inward current, at a V_h of −60 mV in these cells. After switch to current clamp, GABA induced a g_m increase, which was accompanied by a small depolarization. However, neither the amplitude nor the kinetics of the GABA-associated intracellular acidosis was changed with respect to the response under voltage clamp. The mean E_rev of the E_m response to GABA was −64 ± 5.1 mV (n = 4). The average values of the pHᵢ decreases accompanying the GABA-induced currents in voltage clamp (which had an amplitude of ±30 pA) in low-Cl⁻ cells were 0.25 ± 0.07 pH units (1 mM, 1 min, n = 10) and 0.18 ± 0.09 pH units (0.1 mM, n = 5), and thus were almost identical to those observed with high-Cl⁻ electrodes. However, pHᵢ remained unaffected during the action of GABA on I_m and g_m in seven different neurons (Fig. 2B). As tested in three of these cells, depolarization to 0 or +30 mV for 1 min evoked an intracellular acidosis by 0.1–0.4 pH units (Fig. 2, B and C), similar to that revealed in cells recorded with the high-Cl⁻ electrodes (see above). A comparable depolarization-induced acidosis was also revealed in cells in which GABA or glycine led to the typical fall of pHᵢ.

EFFECTS OF CO₂/HCO₃⁻-FREE SUPERFUSATE. Furthermore, the effects on GABA-induced pHᵢ decreases of CO₂/HCO₃⁻-free HEPES pH-buffered solution were tested. As exemplified in Fig. 4B for a recording with a low-Cl⁻ patch electrode, superfusion of the HEPES solution produced a stable rise of pHᵢ baseline by 0.24 ± 0.16 pH units (n = 5). In three cells, recorded with high-Cl⁻ electrodes, HEPES increased steady-state pHᵢ by 0.15 ± 0.04 pH units. The absence of CO₂/HCO₃⁻ did not substantially affect resting E_m, I_m, or g_m, or the membrane response to GABA. However, the pHᵢ decreases accompanying
FIG. 3. Pharmacological blockade of GABA- and glycine-induced decreases of pH. A: bicuculline suppressed the current response and the intracellular acidosis evoked by bath application of GABA. B: strychnine led to a diminution of the inward current and to a full blockade of the accompanying pH fall on exposure to glycine, whereas the corresponding responses to GABA were not affected. Recordings were made with a KCl intracellular solution (which also contained 30 μM carbonic anhydrase in B).

the GABA-induced $I_m$ ($n = 3$) or $E_m$ ($n = 2$) responses were completely blocked (Fig. 4B). The HEPES-induced alkalinization and the suppression of the GABA-induced pH decrease were fully reversible after reintroduction of the standard CO$_2$/HCO$_3$-containing solution (not shown).

**REVERSAL OF THE GABA RESPONSE.** In a final set of experiments, the voltage-dependence of the GABA-induced intracellular acidosis was analyzed. In five cells, recorded with high-Cl$^-$ electrodes, the GABA-induced inward current and $E_m$ increase were accompanied by a pH fall by 0.1–0.22 pH units. Subsequent change of $V_h$ to either 0 or $+30$ mV led to a steady outward current and a sustained fall of pH by up to 0.5 pH units. Under these conditions, the GABA-induced current was decreased in amplitude ($0$ mV) or reversed polarity ($+30$ mV). At $+30$ mV, GABA now led to an intracellular alkalinization by between 0.09 and 0.15 pH units in three neurons (Fig. 5), whereas pH did not change in two cells. In the example of Fig. 5, a minor alkalinization was also observed during the action of GABA at 0 mV.

**DISCUSSION**

**GABA$_A$ and glycine receptors in VRG neurons**

**RHYTHMICALLY ACTIVE PREPARATIONS.** Respiratory activity in mammals in vivo critically depends on mutual Cl$^-$-mediated inhibition within the neuronal network of the VRG (Richter 1996). In isolated preparations from rodents, however, GABA$_A$ergic or glycinergic inhibition does not appear to be essential for generation of the primary rhythm, because respiratory activity is not suppressed by bicuculline or strychnine (Feldman and Smith 1989; Onimaru et al. 1990; Richter et al. 1992, 1997). It is assumed that conditional burster neurons of the VRG mediate rhythmic $E_m$ fluctuations in such reduced respiratory networks, which are devoid of afferent (peripheral) synaptic inputs (Feldman and Smith 1989; Onimaru et al. 1990; Richter et al. 1997; Smith et al. 1991).

Nevertheless, GABA$_A$ and glycine receptors are functional in these in vitro preparations. During the inspiratory phase, different types of rhythmically active VRG neurons are hyperpolarized by synchronized Cl$^-$-mediated IPSPs (Ballanyi et al. 1994b; Onimaru and Homma 1992; Onimaru et al. 1996; Ramirez et al. 1996; Smith et al. 1992). Furthermore, bicuculline and strychnine selectively block spontaneous IPSPs of VRG neurons in rhythmically active, tilted sagittal (Paton and Richter 1995; Paton et al. 1994) or transverse (Ramirez et al. 1996) slices. In functionally identified VRG cells of the brain stem–spinal cord preparation of neonatal rats, bath application of GABA and glycine evoked
FIG. 4. Effects of CO$_2$/HCO$_3$-free solution on GABA-induced pH$_i$ changes. A: in a neuron recorded with a potassium gluconate patch electrode, GABA evoked a fall of pH$_i$ and a major $g_m$ increase but no major change in $I_m$. An almost identical GABA-induced intracellular acidosis was observed after switch to current clamp. B: continuation of the recording in A shows that introduction of a CO$_2$/HCO$_3$-free, N-2-hydroxy-ethylpiperazine-N'-2-ethane sulphonic acid (HEPES) pH-buffered saline caused a sustained intracellular alkalosis. In this situation, the GABA-evoked pH$_i$ fall, but not the $g_m$ increase, was suppressed.

prominent bicuculline- and strychnine-sensitive membrane hyperpolarizations (Brockhaus and Ballanyi 1995). In individual respiratory neurons of the latter study, the responses to both GABA and glycine were not affected by addition of tetrodotoxin. This almost excludes the possibility that these responses are due to indirect effects of the agonists, caused by modulation of spontaneous activity of presynaptic cells within the network (e.g., Khazipov et al. 1993).

THIN SLICES. For fluorometric measurements of GABA- and glycine-mediated changes of pH$_i$, it was necessary to use brain stem slices with a rostrocaudal thickness of not more than 200 $\mu$m. These slices contained only subregions of the pre-Bötzinger complex and, thus, not the entire core ( $\sim$350 $\mu$m diam) of the respiratory network, which is necessary to produce respiration-like activity in transverse slices (Richter 1996; Richter et al. 1992; Smith et al. 1991). Accordingly, functional identification of neurons in the region of the VRG was not possible in the present study. However, in the vast majority of cells in the nonrhythmic thin slices the responses to GABA and/or glycine were very similar to those of the rhythmically active VRG neurons described above. In future studies, a more precise determination of the localization and properties of pre-, post-, or extrasynaptic (Brown 1979) components of Cl$^-$/mediated inhibitory responses in these cells deserves immunohistochemical analy-
sis in combination with synaptic stimulation of identified afferents rather than agonist application.

$pH_i$ measurements

METHODOLOGICAL CONSIDERATIONS. The pH$_i$ measurements showed that bath application of GABA or glycine as well as membrane depolarization or administration of NH$_4^+$ produces a major intracellular acidosis of the VRG neurons. For monitoring of pH$_i$, we have used the rather novel technique of intracellular application of the fluorescent dye BCECF via the patch electrode (Trapp et al. 1996a). Thus some methodological aspects, which are discussed in detail in the latter study, should be considered before discussion of the mechanisms of the observed pH$_i$ changes. The steady increase of both the $F_{440}$ and $F_{490}$ fluorescence signals within $\sim$10 min after establishment of the whole cell configuration indicates ongoing loading with BCECF. However, the ratio trace, representing pH$_i$, was typically stable after $<2$ min of intracellular recording (compare Fig. 2 of Trapp et al. 1996a). Therefore HEPES, which should enter the cells similarly to BCECF during this phase, does not appear to affect pH$_i$ baseline. Nevertheless, 10 mM intracellular HEPES should, at physiological levels of pH$_i$, contribute by $\sim$5 mM/pH unit to intrinsic buffering power, which was 18
FIG. 5. Voltage dependence of the GABA-induced pHi transient. At a holding potential ($V_h$) of −60 mV, GABA led to the typical inward current, $g_m$ increase, and pHi fall in a cell recorded with a KCl electrode. After depolarization to 0 mV and, subsequently, to +30 mV, the GABA current was reduced and changed polarity, and also the pHi fall was reversed into an intracellular alkalosis. Note the prominent and sustained decrease of pHi baseline on membrane depolarization.

GABA- AND GLYCINE-INDUCED INTRACELLULAR ACIDOSIS. Physiological relevance. The observed GABA- and glycine-induced pHi decreases are not caused by a nonspecific effect of these inhibitory amino acids, because bicuculline or strychnine completely abolished the agonist-evoked current, the $g_m$ increase, and also the accompanying fall of pHi. These findings are consistent with previous measurements in mammalian brain slices, which showed that GABA-induced increases of extracellular pH are blocked by the GABA$\alpha$ receptor antagonist picrotoxin (Chen and Chesler 1992; Kaila et al. 1992a; Taira et al. 1995; see also Chen and Chesler 1991). The blocking effects of bicuculline in the present study almost exclude a contribution of activation of GABA$\alpha$ receptors to the observed intracellular acidosis.

The magnitude of the intracellular acidosis on activation of receptor-coupled Cl$^-$ channels could exceed 0.3 pH units in individual cells. Even larger pHi decreases will possibly be detected in future studies with the use of imaging techniques, allowing for a higher spatial resolution and thus analysis of pHi changes in the vicinity of the GABAergic and glycinerenic synapses (see also Trapp et al. 1996a). It might be argued that receptor activation for periods of ~1 min by bath application of the agonists does not refer to the physiological situation. It should, however, be considered that maximum currents, conductance changes, and thus pHi decreases might not be observed at all because of partial desensitization of the GABA and glycine receptors during such a slow rate of agonist exposure (Kaila 1994). In expiratory neurons of the in vivo cat, trains of synchronized IPSPs are responsible for inspiratory inhibition, which persists for periods of up to several seconds. Such hyperpolarizations can reach amplitudes of >20 mV within several hundred milliseconds (Ballantyne and Richter 1986; Richter 1996). As measured with pH-sensitive microelectrodes in these cells, these hyperpolarizations are accompanied by rapid periodic decreases of pHi by >0.1 pH units (Ballanyi et al. 1994a). However, the pHi of mammalian neurons is not only perturbed during such massive, albeit physiological activity. It was recently demonstrated that tonic spike activity with a frequency of ~1–6 Hz produces ongoing acidification of medullary dorsal vagal neurons (Trapp et al. 1996a) by a mechanism that is discussed below. It remains to be determined whether spontaneous repetitive activity in these cells, as revealed in the VRG neurons on recording with (Cs/TEA-containing) high-Cl$^-$ electrodes, also affect pHi.

$\text{HCO}_3^-$ permeability. The observation that the GABA-induced pHi decrease, but not the current response and $g_m$ increase, was blocked in CO$_2$/HCO$_3^-$-free solution suggests efflux of the base equivalent HCO$_3^-$ through the anion pore as the ionic mechanism of this acidosis. This assumption is supported by the observation that the pHi fall turned into an intracellular alkalosis when the cells were depolarized. Because the equilibrium potentials for H$^+$ and bicarbonate were close to 0 mV in the present study (see also Chesler 1990; Chesler and Kaila 1992; Kaila 1994), an influx of bicarbonate was expected at positive $E_m$. It is, indeed, established that GABA$\alpha$ receptor-coupled anion channels have a substantial permeability to HCO$_3^-$ (Bormann et al. 1987; Chesler and Kaila 1992; Kaila 1994). In a series of elegant experiments, it was originally demonstrated in crayfish muscle fibers and neurons (Kaila 1994; Kaila and Voipio 1987; Voipio et al. 1991) that HCO$_3^-$ efflux through the GABA$\alpha$ receptor-coupled anion pore can produce a fall of pHi by several tenths of a pH unit in the presence of extracellular CO$_2$ and bicar-
bonate. Similar GABA-induced intracellular acidifications have currently also been shown for mammalian neurons (Pasternack et al. 1993; Trapp et al. 1996a) or glial cells (Kaila et al. 1991).

A major contribution of a HCO$_3^-$ permeability could theoretically also explain that the $E_{\text{rev}}$ of the GABA or glycine response was $\sim 25$ mV more positive than the $E_{\text{Cl}}$, which is typically close to $-90$ mV during recording with low-Cl$^-$ electrodes (Kaila 1994; Kaila et al. 1993). However, as suggested by preliminary findings, the average $E_{\text{rev}}$ of the GABA response is not considerably more negative in HEPES-buffered solutions, in which the HCO$_3^-$-mediated acidifications were fully blocked. It could well be that the depolarizing action of a putative GABA uptake counteracts the receptor-mediated hyperpolarization (for references, see Kaila et al. 1992b). The presence of an electrogenic GABA uptake is, indeed, suggested by the observation that GABA still produced an inward current, but no conductance increase, after blockade of GABA$_A$ receptors with bicuculline (Fig. 3A). A further explanation for the discrepancy of $E_{\text{rev}}$ and calculated $E_{\text{Cl}}$ could be incomplete equilibration of cellular Cl$^-$ with that of the patch electrode, possibly combined with ongoing activity of an inwardly directed Cl$^-$ pump leading to a depolarizing shift of $E_{\text{Cl}}$ (Ballanyi and Grafe 1985).

Role of CA. A proportion of cells in our study did not show a major acidification on exposure to GABA or glycine despite a considerable current and/or conductance response. The observation that administration of NH$_4^+$ or membrane depolarization led to a substantial change of pH$_i$ in these cells excludes the possibility of loss of pH sensitivity of the dye, e.g., by interference of intracellular constituents in these recordings (see also Trapp et al. 1996a). In isolated hippocampal neurons, it was found that acetazolamide, a blocker of CA, strongly attenuated the GABA-induced decreases of pH$_i$. In hippocampal neurons (Pasternack et al. 1993). The importance of CA for the generation of these HCO$_3^-$-related neuronal acidifications is also suggested by our observation of significantly faster and also larger GABA- and glycine-induced decreases of pH$_i$ in cells dialyzed with the enzyme. Although no information is available at present on the content of soluble CA in neurons, an intracellular concentration of 15–30 $\mu$M as provided by the patch pipette might be considerably higher than in the intact cell (Chesler 1990; Kaila 1994). It remains to be determined whether those neurons in which GABA or glycine did not change pH$_i$ even after dialysis of CA express receptors of a specific subunit composition (McKernan and Whitting 1996) that might not be permeable to HCO$_3^-$.

Role of Cl$^-$. With the use of high-Cl$^-$ electrodes, the average $E_{\text{rev}}$ of the GABA- and glycine-induced currents was $\sim 0$ mV. In this situation, a prominent efflux of Cl$^-$ is likely to accompany the HCO$_3^-$ efflux at a $V_h$ of $-60$ mV. Such Cl$^-$ efflux, which was shown to decrease intracellular Cl$^-$ by $>10$ mM in rat sympathetic neurons (Ballanyi and Grafe 1985), does not seem to hamper the efflux of HCO$_3^-$, because GABA- and glycine-evoked acidifications of very similar magnitude were revealed with low-Cl$^-$ patch electrodes. Because the apparent $E_{\text{Cl}}$ was close to $-60$ mV under these conditions (see previous paragraphs), only a minor trans-

membrane flux of Cl$^-$ is expected despite a prominent increase of $g_m$. The latter consideration implicates that the agonist-induced acidosis is not due to redistribution of Cl$^-$, which could potentially involve pH regulatory mechanisms like Cl$^-$/HCO$_3^-$ exchange (Ballanyi and Grafe 1985; Kaila 1994).

Depolarization-induced acidosis. Whereas the mechanism of the biphasic change of pH$_i$, on application of NH$_4^+$ is well established (Chesler 1990; Thomas 1984), the origin of the depolarization-induced intracellular acidosis needs to be discussed briefly. It was recently demonstrated in hippocampal pyramidal neurons that depolarization-induced Ca$^{2+}$ influx via voltage-gated Ca$^{2+}$ channels leads to a delayed intracellular acidosis (Trapp et al. 1996b). This depolarization-evoked fall of pH$_i$ appears to be due to activation of a vandate- and eosin-sensitive plasma membrane pump, which extrudes intracellular Ca$^{2+}$ in exchange for extracellular H$_3$O$^+$. As was originally described for snail neurons (Schwiening et al. 1993; see also Paalasmaa and Kaila 1996; Trapp et al. 1996b). Because almost identical results were recently reported for dorsal vagal neurons of medullary slices from rats (Trapp et al. 1996a), we hypothesize that activation of the plasmaemmal Ca$^{2+}$/H$_3$O$^+$ pump is responsible for the depolarization-induced neuronal acidosis in the present study.

Conclusions

Our findings support recent assumptions that decreases in pH$_i$ during periodic trains of IPSPs, as occurring in respiratory neurons in vivo (Ballanyi et al. 1994a), are due to GABA$_A$ and/or glycine receptor-mediated HCO$_3^-$ efflux. Because a variety of cellular processes, including membrane channel function, critically depends on pH (Chesler and Kaila 1992; Kaila 1994; Takahashi and Copenhagen 1996), it is a challenge of future studies to illuminate to what extent these intracellular acidifications might contribute to modulation of neuronal function.

We thank U. Strube for technical assistance and Dr. J. Brockhaus for critical reading of the manuscript.

This study was supported by the DFG (Heisenberg Program and SFB 406).

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Received 25 June 1996; accepted in final form 6 December 1996.

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