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ORIGINAL ARTICLE

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On the mechanism of GABA-induced currents in cultured rat cortical neurons

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Abstract We applied the perforated-patch-clamp technique to cultured cortical neurons of the rat to characterize the ionic basis of membrane potential changes and membrane currents induced by γ-aminobutyric acid (GABA). Gramicidin was used as the membrane-perforating agent, to allow the recording of whole-cell currents without impairing the intracellular Cl^- concentration ($[Cl^-]_i$). In current-clamp experiments in the presence of 26 mM HCO_3^- the application of 50 μM GABA evoked changes in the membrane potential of neurons including depolarizations (19%), hyperpolarizations (38%) and biphasic changes in membrane potential (31%), characterized by a transient hyperpolarization followed by a sustained depolarization. Accordingly, GABA (50–200 μM) induced inward, outward or biphasic current responses under voltage-clamp. Inward and biphasic currents as well as depolarizations and biphasic membrane potential responses, respectively, occurred more frequently in the presence of 26 mM HCO_3^-. The second phase of the biphasic membrane potential or current responses was markedly reduced when the preparation was bathed in a HCO_3^--free saline, indicating a contribution from HCO_3^-. The reversal potential of the GABA-induced currents (E_{GABA}) determined with the gramicidin-perforated-patch mode and in the nominal absence of HCO_3^- was –73 mV, while it was shifted to –59 mV in the presence of HCO_3^-. Combined patch-clamp and microfluorimetric measurements using the Cl^--sensitive dye 6-methoxy-1-(3-sulphonatopropyl)quinolinium (SPQ) showed that GABA evoked an increase of $[Cl^-]_i$ in 54% (n=13) of the neurons. We conclude that this increase of $[Cl^-]_i$ in combination with the efflux of HCO_3^- results in a shift of E_{GABA} above the resting membrane potential that gives rise to GABA-mediated depolarizations.

Key words Cl^- accumulation · Depolarizing GABA response · Gramicidin · Perforated-patch clamp · SPQ-microfluorimetry

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Introduction

γ-Aminobutyric acid (GABA) is a widely used transmitter in the central nervous system of vertebrates. By activating $GABA_A$ receptors this neurotransmitter usually exerts an inhibitory action at postsynaptic neurons by opening ligand-gated ion channels permeable to Cl^-. This inhibition results in an increase in the conductance of the postsynaptic membrane, thus driving the membrane potential towards the Cl^- equilibrium potential (E_{Cl}), which is often accompanied by a hyperpolarization. However, in early developmental stages, that is from embryonic to an early postnatal age, depolarizing actions induced by GABA are frequently observed. Examples include cultures of rat hypothalamic neurons [6], cortical slice preparations of the fetal rat brain [22], or neurons from embryonic spinal cord [23, 27] and olfactory bulb regions [25]. Interestingly, in some cases these depolarizations could surpass the threshold for the firing of action potentials, leading to the generation of spike activity [6, 13].

Although depolarizing activity mediated by GABA has been recently observed in several brain areas, the underlying mechanism is still controversial. Two mechanisms have been discussed. The first mechanism is based on active Cl^- accumulation, which keeps the intracellular Cl^- concentration ($[Cl^-]_i$) higher than expected from a passive distribution [20, 24]. This results in an E_{Cl} that is more positive than the resting potential of the neurons. Hence, the activation of $GABA_A$ receptors would then lead to a depolarization [8, 24]. The second mechanism is based on the permeability of $GABA_A$ receptors to Cl^- *and* HCO_3^- [5, 15], which leads to the depolarizing phase of GABA-evoked responses [26].

In order to investigate the mechanism underlying GABA-induced depolarizations and, in particular, to investigate the contribution of HCO_3^-, we applied the gramicidin-perforated-patch configuration to cultured cortical neurons, because this allows the measurement of membrane currents without impairing physiological changes of $[Cl^-]_i$. Our data show that in the presence of HCO_3^- the majority of cortical neurons respond to GABA with an inward current or a biphasic current which is accompanied by an increase in $[Cl^-]_i$. In the absence of HCO_3^- the neurons primarily respond with an outward current, indicating that HCO_3^- efflux contributes to the GABA-induced current in cultured cortical neurons. We show that the activation of $GABA_A$ receptors leads to a shift of E_{GABA} to values more positive than the resting potential, which results in a change from a hyperpolarization to a depolarization. Some results have been communicated in abstract form [10].

Materials and methods

Cell culture

The cortices of three to four rat embryos (embryonic day 15–17) were removed and maintained at approximately 0°C in a solution that contained (in mM): NaCl 120; KCl 5.4; $MgCl_2$ 0.8; Tris-HCl 25; glucose 15. After the preparation the cortices were incubated for 10 min at room temperature (approximately 25°C) in a solution containing 2.4 U/ml dispase (Boehringer, Mannheim, Germany) and then mechanically dissociated by triturating with Pasteur pipettes in a solution that contained 0.05% DNase (Boehringer). The cell suspension was centrifuged and the DNase solution was replaced by the culture medium (DMEM, Sigma, Deisenhofen; complemented with 10% horse serum, Gibco BRL; Eggenstein, Germany). The neurons were then plated on glass coverslips coated with poly-D-lysine and incubated at 7% CO_2/37°C. The experiments were done at room temperature (approximately 22–24°C) between 5 and 21 days after plating of the neurons.

Experimental set-up

For perforated-patch-clamp recordings the culture dish was mounted on the stage of an inverted microscope (Zeiss, Jena, Germany). Recording patch pipettes were pulled from borosilicate glass and the tips were fire polished (resistances 4–6 MΩ, when filled with the pipette solution). Gramicidin-perforated-patch recordings were performed using, in general, the method of Kyrozis and Reichling [18]. For perforated-patch-clamp recordings, the electrode tip was filled for 1–40 s with the gramicidin-free pipette solution (see below) to avoid problems with seal formation and then back-filled with the gramicidin-containing pipette solution. The reference potential for all measurements was the zero-current potential of the pipette in the bath before establishment of the giga-seal. An agar-salt bridge (3 M KCl) was used as the reference electrode. After forming a giga-seal [14], short steps in holding potential (V_h) were continuously applied at 2-min intervals to monitor the gradual decrease in series resistance. All experiments were performed with bipolar, non-pyramidal neurons. Drug application was not started until the series resistance decreased below 30 MΩ which usually lasted 20–30 min. Currents were amplified by an EPC-7 (List, Darmstadt, Germany) patch-clamp amplifier. Before digitization (sampling rate 0.5 or 1 kHz) currents were filtered at 3 kHz with a 3-pole low-pass Bessel filter. Data were stored and evaluated with the aid of the TIDA hardware and software package (List) for a personal computer. To determine the agonist-induced current amplitudes, the maximal deflection from the baseline was used.

Microfluorimetry

Experiments were performed using a Deltascan dual excitation spectrofluorometer (PTI, Wedel, Germany) in which shutters, monochromator settings and the data acquisition were controlled by software and interfaces from the PTI. Microfluorimetric measurement of $[Cl^-]_i$ was performed using the fluorescent dye 6-methoxy-1-(3-sulphonatopropyl)quinolinium (SPQ). Cells were loaded with SPQ (1–2 mM; pipette solution see below) via the patch pipette. The cells were excited at 356 nm, while fluorescence intensity (at wavelengths >420 nm) was measured using a photon-counting photomultiplier tube. Measurements were limited to a field of view slightly larger than the cell body of the injected neuron by a rectangular diaphragm. A decrease in the fluorescence intensity emitted by SPQ indicated a relative increase in the free Cl^- concentration. However, an estimation of the absolute changes in $[Cl^-]_i$ could not be performed, because a simple determination of $[Cl^-]_i$ using ratio measurements is not possible with SPQ. The background fluorescence for the excitation wavelength was obtained from each cell before establishing the whole-cell clamp and subtracted from the raw data.

Solutions and drug application

Two different pipette solutions, containing a low (PS1) or a high (PS2) Cl^- concentration, were used for gramicidin-perforated-patch recordings. PS1 contained (in mM): K_2SO_4 77; NaCl 5; $CaCl_2$ 0.5; ethylene glycol-bis (β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 5; *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulphonic acid) (Hepes) 10; pH adjusted to 7.2 with KOH. PS2 contained (in mM): KCl 140; NaCl 5; $CaCl_2$ 0.5; EGTA 5; Hepes 10; pH was adjusted to 7.2 with KOH. To determine E_{GABA} with the voltage-ramp method (see below) KCl in PS2 was substituted by an equimolar amount of CsCl.

Gramicidin (Sigma, St. Louis, Mo., USA; 5 mg/ml) was dissolved in dimethylsulphoxide, vortexed for 1 min, sonicated for 20 s, and then added to the pipette solution to give a final concentration of 25–50 μg/ml. In neurons in which the conventional whole-cell configuration was established after recording in the gramicidin-perforated-patch configuration, PS2 was used. Dialysis of the neurons resulted in a shift of E_{Cl} to 2 mV. GABA-induced currents were then exclusively inwardly directed, and their amplitudes increased up to 27-fold. Therefore, the use of PS2 allowed us to recognize if, during the experiments in the perforated patch-clamp mode, the conventional whole-cell-clamp configuration had occurred inadvertently. In some conventional whole-cell-clamp experiments designed to test the contribution of Cl^- to GABA-induced currents a nominally Cl^--free pipette solution (PS3) was used that contained (in mM): K^+-gluconate 140; EGTA 11; Ca^{2+}-gluconate 1; Mg^{2+}-gluconate 1; Hepes 10; pH was adjusted to 7.2 with gluconic acid.

The intracellular solution used for all SQP-fluorescence measurements contained (in mM): K^+-gluconate 150, $MgCl_2$ 2, HEPES 5, EGTA 1.1, pH was adjusted to 7.2 with gluconic acid.

During experiments the cell cultures were continuously superfused with a HCO_3^-/CO_2-buffered saline containing (in mM): NaCl 125; KCl 2.5; $NaHCO_3$ 26; NaH_2PO_4 1.25; glucose 10; $CaCl_2$ 2; $MgCl_2$ 1; glycine 0.001; pH adjusted to 7.4 with NaOH. In order to test the contribution of Cl^- and HCO_3^- to GABA-induced responses the following additional solutions were used:

1. A Hepes-buffered saline containing (in mM): NaCl 125; Na^+-gluconate 26; KCl 2.5; NaH_2PO_4 1.25; $CaCl_2$ 2; $MgCl_2$ 1; glucose 10; Hepes 10; glycine 0.001; pH adjusted to 7.4 with NaOH.
2. A Hepes-buffered, nominally Cl^--free saline containing (in mM): Na^+-gluconate 151; K^+-gluconate 2.5; Hepes 10; NaH_2PO_4 1.25; glucose 10; Ca^{2+}-gluconate 2; Mg^{2+}-gluconate 1; glycine 0.001; pH adjusted to 7.4 with gluconic acid.
3. A HCO_3^-/CO_2-buffered, nominally Cl^--free saline containing (in mM): Na^+-gluconate 125; $NaHCO_3$ 26; K^+-gluconate 2.5; NaH_2PO_4 1.25; glucose 10; Ca^{2+}-gluconate 2; Mg^{2+}-gluconate 1; glycine 0.001; pH adjusted to 7.4 with gluconic acid.

Stock solutions of GABA (50 mM in aqua bidest) were prepared and added to the saline in defined concentrations shortly before the experiment.

An application pipette positioned at approximately 0.1 mm from the cell was used for rapid focal drug delivery. Drugs were applied from a multibarrelled pipette made from eight Teflon tubes (diameter 0.1 mm) that ended in a common tip (mouth diameter approximately 0.1 mm, dead volume <1 µl) [17]. Seven of the tubes were used for delivery of the drugs at different concentrations, whereas gentle suction at the eighth tube prevented accumulation of the compounds in the tip and leakage of the substances from it. The tubes delivering the solutions were connected to magnetic valves turning the gravity-driven solution flow on and off. The valves were controlled by a personal computer using the TIDA program package (Heka, Lambrecht, Germany). Measurements of the time course of junction potentials that occurred after applying bidistilled water to the tip of an open patch pipette filled with NaCl (150 mM) indicated a time to peak in the range of 10 ms or less. The estimated rate of solution change surrounding a cell may, however, be considerably slower.

Determination of current-voltage relations; data evaluation and statistics

GABA was applied to the bath solution when the holding current was stable for at least 3 min. The current-voltage (*I-V*) relationships of GABA-evoked currents were determined by applying voltage ramps (from –20 mV to –100 mV; duration 100 ms) both in the absence and presence of GABA. In order to eliminate the contamination by leakage and voltage-activated currents, the digitized response to a ramp before the GABA application was subtracted from that during a GABA response. The fast slope of the voltage ramps (0.8 mV/ms) induced small shifts of E_{GABA} of at most –2 mV, thus causing some differences in the amplitude of GABA-activated currents at –60 mV compared to the change in holding currents activated by GABA at a V_h of –60 mV. In voltage-ramp experiments, the Cl^- concentration of the pipette solution (146 mM) was relatively close to that of the external saline (133.5 mM). Liquid junction potentials were adjusted shortly before the seal formation, and the external Cl^- concentration remained constant throughout the experiments. Additional problems could have come from changes in the $[Cl^-]_i$. However, because gramicidin forms pores of negligible Cl^- permeability [18, 21], GABA-activated changes in $[Cl^-]_i$ were not recorded by the electrode inside the patch pipette and thus could not cause a voltage error. Data are given as mean ± standard error of mean (SEM). The significance of the difference between mean values of two samples was determined using the Student's *t*-test. All correlations were determined using the product moment correlation coefficient *r*.

Results

Effect of GABA in the presence of HCO_3^-

Membrane potential responses evoked by GABA were investigated in bipolar, non-pyramidal cultured cortical neurons in the presence of 26 mM HCO_3^- using the gramicidin-perforated patch-clamp technique with 6 mM Cl^- in the pipette solution (PS1). Since gramicidin forms pores of negligible Cl^- permeability [18, 21], an impairment of physiological changes in $[Cl^-]_i$ was not expected. The effects of GABA on the membrane voltage were recorded in the current-clamp mode. The mean resting potential before GABA applications was –68±2 mV (*n*=16). The application of 50 µM GABA resulted in three distinct response patterns (Fig. 1). In 31% of the neurons

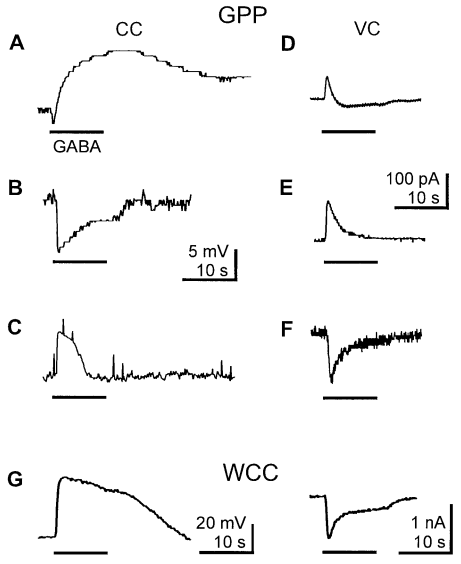

Fig. 1A–G Effect of γ-amino-butyric acid (GABA) in cultured cortical neurons. **A–F** The gramicidin-perforated-patch configuration (*GPP*) was used to record from cultured cortical neurons. GABA (50 µM; applied as indicated by the *bars*) evoked biphasic (**A**, **D**) and monophasic (**B**, **C**, **E**, **F**) responses. Membrane potential changes were measured using the current-clamp mode (*CC*; *left column*; resting potential is –65 mV in **A**, –57 mV in **B** and –66 mV in **C**); changes in membrane current in the voltage-clamp mode (*VC*; V_h =–60 mV; *right column*). **G** In the conventional whole-cell-clamp configuration (*WCC*) GABA induced exclusively depolarizations (*CC*; *left column*) or strong inward currents (*VC*; V_h=–60 mV; *right column*), with the neuronal cytoplasm dialysed with a 146 mM Cl^--containing pipette solution (E_{Cl}=2 mV)

(Fig. 1A; *n*=16) GABA application resulted in a biphasic membrane potential change, characterized by a transient initial hyperpolarization of –2±1 mV (range –1 to –6 mV; *n*=5) that was succeeded by a longer lasting depolarization of 6±1 mV (range: 4–12 mV; *n*=5). GABA hyperpolarized 38% of the neurons by –7±3 mV (range –1 to –23 mV; *n*=6; Fig. 1B), and 19% of the cells were depolarized by 5±2 mV (range 2–22 mV; *n*=3; Fig. 1C). In one neuron the first response evoked in a series of GABA applications was biphasic while the following responses were depolarizing, while another neuron first responded with depolarizations, but later with a hyperpolarization.

When using the voltage-clamp mode (V_h=–80 and –60 mV) in the gramicidin-perforated-patch configuration with a 146 mM Cl^--containing pipette solution (PS2) to record membrane currents, GABA (50–200 µM) also evoked three response types which correspond to the GABA-activated changes in the membrane potential. In 63% of the neurons (*n*=10) GABA induced biphasic currents characterized by a transient initial outward component (amplitude: 38±10 pA) which was followed by an inward current (–27±6 pA; Fig. 1D). In one neuron only outward currents were observed (16 pA at V_h=–60 mV and 8 pA at V_h=–80 mV; Fig. 1E), while in another five (31%) exclusively inward currents (–65 ±12 pA at V_h=–60 mV; Fig. 1F) were observed after the application of GABA. Biphasic currents activated by

GABA were also observed when K^+ currents were inhibited by using a Cs^+-containing pipette solution. Therefore, we suggest that a negligible contribution is made by currents mediated by the activation of $GABA_B$ receptors.

The conventional whole-cell configuration was established in 12 neurons by rupturing the cell membrane under the tip of the patch-pipette after measuring in the gramicidin-perforated-patch configuration from the same cells. Within 2–3 min, possibly because of the dialysis of the cytosol with the 146 mM Cl^--containing pipette solution (PS2), these neurons responded to GABA application with a large depolarization or a strong inward current, when recorded in the current-clamp or voltage-clamp mode, respectively (Fig. 1G). The mean amplitude of the inward current recorded in the conventional whole-cell-clamp configuration was –2±1 nA (n=5), about 30-fold larger than recorded in the gramicidin-perforated-patch configuration. This distinct difference in absolute current amplitude was taken as evidence to distinguish between both configurations and to discard experiments in which an unintended spontaneous rupture of the cell membrane had occurred.

GABA evokes an increase in $[Cl^-]_i$

In order to test whether GABA could evoke a significant change of the Cl^- gradient, cultured cortical neurons were loaded via the patch-pipette with the Cl^--sensitive dye SPQ (1–2 µM), and the whole-cell-clamp configuration was used to measure changes in membrane current or voltage simultaneously. When using a 4 mM Cl^--containing pipette solution, GABA (100 µM, 30 s) evoked outward (three of six cells) or inward currents (three of six cells) at a V_h of –60 mV, while at more positive holding potentials (–40 mV or –20 mV) outwardly directed GABA-activated currents were observed more often (six of seven cells, Fig. 3A). In all neurons these currents were accompanied by changes in SPQ fluorescence, indicating changes of $[Cl^-]_i$. At more positive holding potentials (Fig. 2A–C) the changes of $[Cl^-]_i$ increased. In three out of five neurons an increase of $[Cl^-]_i$ was also observed when the membrane potential was recorded in the current-clamp mode (Fig. 2D). A typical time course of dye loading and of the changes in SPQ fluorescence before and after the establishment of the whole-cell-clamp configuration in a cultured cortical neuron is shown in Fig. 2E.

The GABA-activated current amplitudes were plotted as a function of the holding potential and fitted by a linear regression (r=0.77) resulting in a current reversal at –56 mV (Fig. 3A). The relative changes in SPQ fluorescence induced by GABA were plotted against the holding potential and fitted by a line (r=–0.77). The potential at which no changes in fluorescence occurred was estimated to be –57 mV (Fig. 3A). We also found a significant correlation between the GABA-activated changes in SPQ fluorescence and the corresponding currents (r=–0.91; n=13; P<0.01; Fig. 3B), indicating that the change of $[Cl^-]_i$ was directly caused by the GABA-induced current. We conclude that the activation of $GABA_A$ receptors frequently evokes a change of the Cl^- gradient which in most cases leads to an acute increase of $[Cl^-]_i$ in cultured cortical neurons.

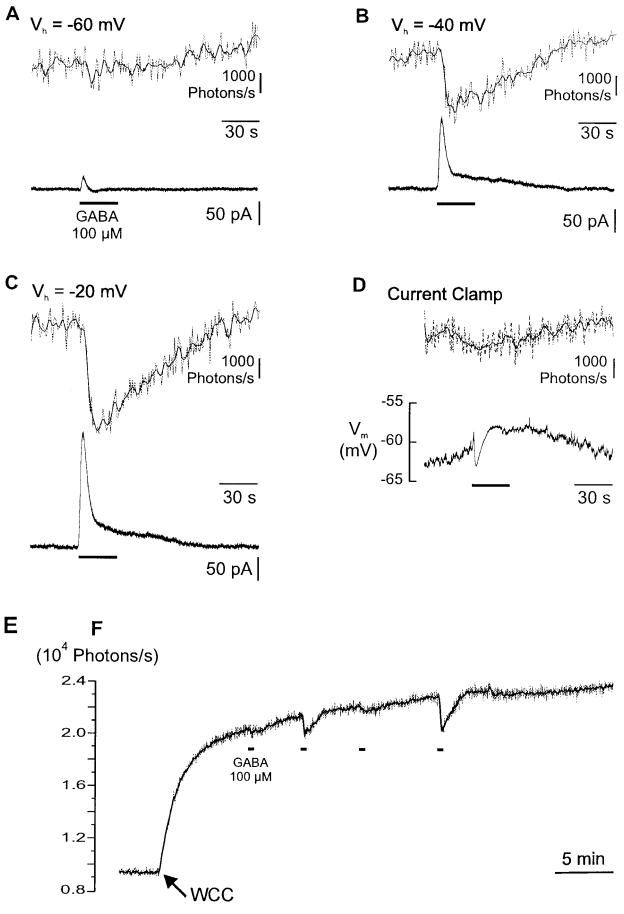

Fig. 2A–E Effect of GABA on the fluorescence intensity of the Cl^--sensitive dye SPQ and measurements of membrane responses. **A–C** GABA (100 µM) was applied as indicated by the *bars*, while currents were recorded (*lower traces*) in the whole-cell configuration at three different holding potentials in combination with simultaneous SPQ-fluorescence measurements (*upper traces*). A decrease in the fluorescence intensity indicates an increase in intracellular Cl^--activity. **D** GABA-induced change of the membrane potential (*lower trace*) and SPQ fluorescence (*upper trace*). **E** The change of the Cl^--sensitive fluorescence intensity (F_{356}) during a complete experiment is displayed: constant intensity during the first few minutes is due to background fluorescence, which is not influenced by Cl^-. After establishment of the conventional whole-cell configuration (*WCC*; indicated by the *arrow*), SPQ diffused into the cell as indicated by the rapid increase of fluorescence. Within 5–10 min the intensity reached a relatively constant plateau. Then, GABA (100 µM; 30 s) was repetitively applied for 30 s at different holding potentials or in the current-clamp mode. **A–D** Enlarged parts of the *trace* in **E**. In **E** they are displayed in the sequence they were recorded: **A**, **B**, **D**, **C**. The *lighter points* in the fluorescence traces are original data points, while the *fat lines* were created by a smoothing algorithm

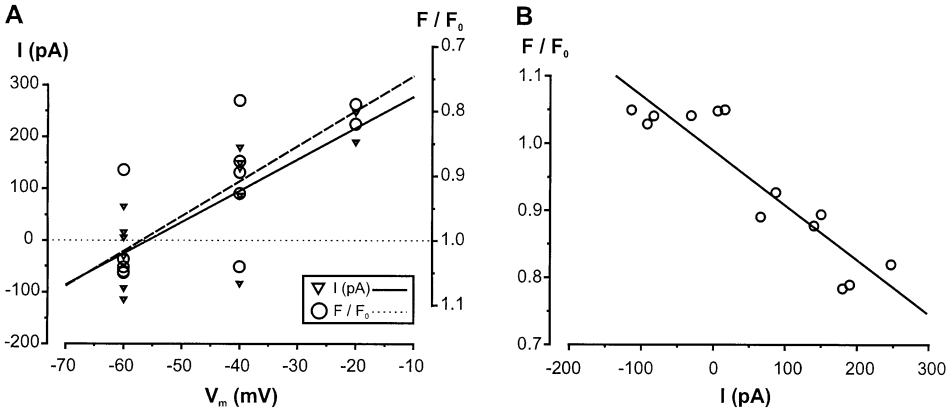

Fig. 3A, B Dependence of GABA-activated changes of SPQ-fluorescence on membrane voltage and current amplitude. **A** GABA-induced membrane currents (indicated by the *triangles*) and relative changes in SPQ-fluorescence (indicated by the *circles*) were plotted as a function of the membrane potential (n=13). *I-V* relation (*continuous line*) and fluorescence-voltage relationships (*dashed line*) were created by linear regression, resulting in r=0.77 for *I-V* correlation and r=-0.77 for fluorescence voltage correlation. **B** Relative GABA-evoked changes in fluorescence intensities (F/F_0) shown as a function of the GABA-induced current amplitude. Data points were fitted by a linear regression (r=–0.91)

Influence of HCO_3^- on GABA-activated responses

Since $GABA_A$ receptors can be permeable to HCO_3^- [5], we have investigated the possible contribution of HCO_3^- to the GABA-evoked response. Therefore, GABA (50 µM; 10 s) was also applied in a nominally HCO_3^--free/Hepes-buffered saline. In the absence of HCO_3^-, the depolarizing phase of GABA-evoked biphasic membrane potential responses continuously decreased and finally ceased (Fig. 4A). Correspondingly, the inward component of biphasic GABA-induced currents did not occur in the absence of HCO_3^- (n=4; Fig. 4B). Readdition of HCO_3^- resulted in a slow recovery of the inward current component (Fig. 4B). In a few cells GABA-activated currents showed a rundown, as indicated by the decrease in the outward current in Fig. 4B. In the nominal absence of HCO_3^- only 1 out of 11 cells showed a biphasic membrane potential response, while 36% of the applications evoked depolarizations (9±4 mV; range 3–35 mV) and 46% hyperpolarizations (–11±4 mV; range –4 to –29 mV). These data suggest that the GABA-induced depolarization in cultured cortical neurons is dependent on the presence of HCO_3^- in the extracellular saline.

The withdrawal of extracellular HCO_3^- could have influenced the regulation of $[Cl^-]_i$; for example, by inhibiting HCO_3^--dependent Cl^- transport, which could in turn increase $[Cl^-]_i$. A resulting shift of E_{Cl} in the positive direction could then mediate the delayed depolarization or inward current component evoked by GABA. To address this point we used the conventional whole-cell-clamp configuration to investigate the effect of GABA in the nominal absence of extra- and intracellular Cl^-. The application of GABA in the standard HCO_3^--buffered saline (containing 133.5 mM Cl^- and 26 mM HCO_3^-) exclusively produced outward currents

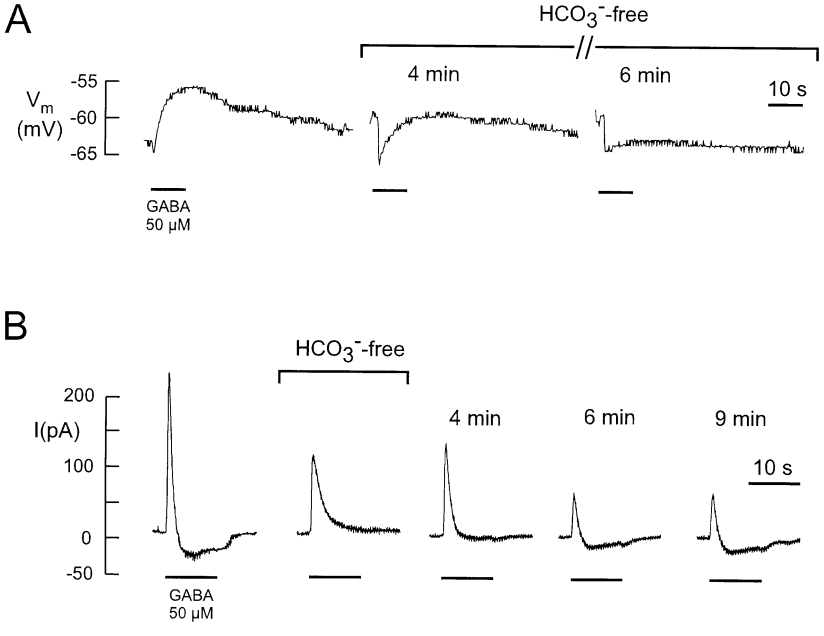

Fig. 4A, B Dependence of GABA-activated responses on HCO_3^-: gramicidin-perforated-patch recordings. **A** Current-clamp mode. In the presence of HCO_3^- (*left trace*), GABA evoked a biphasic change in membrane potential; withdrawal of HCO_3^- (*middle* and *right traces*) induced a time-dependent decrease of the GABA-induced depolarization. *Values* at the *top* of the *traces* indicate the time after removal of HCO_3^-. **B** Voltage-clamp mode. A biphasic current response changed to a monophasic outward current, after HCO_3^- was withdrawn as indicated. Readdition of HCO_3^- led to a slow recovery of the inward current component. *Values* at the *top* of the *traces* indicate the time after readdition of HCO_3^-. GABA (50 µM) applications in **A** and **B** are indicated by *solid bars*

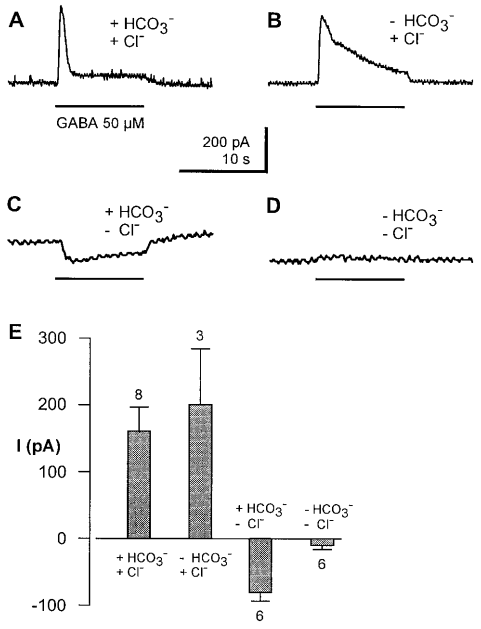

Fig. 5A–E Effect of GABA in the absence of intracellular Cl^-. Recordings in the conventional whole-cell-clamp configuration with a Cl^--free pipette solution at a holding potential of –60 mV. **A**, **B** GABA activated outward currents in the presence of extracellular Cl^- (133.5 mM) and in the presence (**A**) or absence (**B**) of 26 mM HCO_3^-. **C** In the absence of extracellular Cl^-, but in the presence of HCO_3^- GABA evoked inward currents. **D** In the absence of Cl^- and HCO_3^-, hardly any current was observed. Note that the traces in **A**, **C** and **D** are records from the same cell. **E** Mean GABA-activated current amplitudes in different saline solutions as indicated. The *vertical bars* and *numbers* at the *top* indicate SEM and number of neurons, respectively

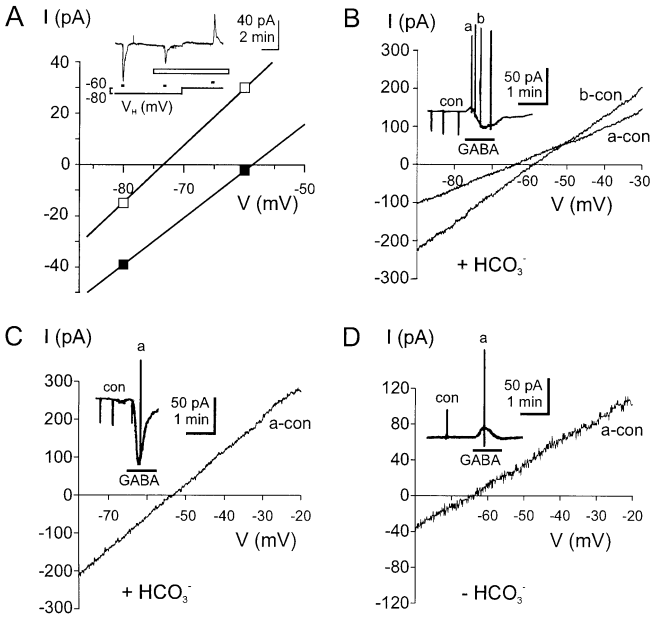

Fig. 6A–D The reversal potential of GABA-induced currents depends on HCO_3^-. **A** Inset: GABA was applied in the GPP mode (V_h=–80 and –60 mV) in the presence and absence of HCO_3^- (indicated by the *open bar*). E_{GABA} was determined by fitting a line through the medians of the current amplitudes obtained in the presence (*filled squares*; n=6 at V_h=–80 mV; n=12 at V_h=–60 mV) and in the absence of HCO_3^- (*open squares*; n=6 at V_h=–80 mV; n=7 at V_h=–60 mV); E_{GABA} was –59 mV in the presence of HCO_3^- and –73 mV in its absence. **B–D** *I-V* relations of GABA-activated currents. Voltage-ramps (from –20 mV to –100 mV, duration 100 ms) were applied before (indicated as *con*) and during the application of 50 µM GABA (indicated by *a* and *b*). Responses to voltage-ramps before GABA applications (*con*) were subtracted from those during the GABA-evoked responses (indicated as *a-con* or *b-con*). **B** Shift of E_{GABA} towards the positive direction during a biphasic current response from –64 mV to –58 mV. **C**, **D** *I-V* relations of monophasic currents in the presence (**C**) and absence of HCO_3^- (**D**). Reversal potentials: –53 mV (**C**) and –65 mV (**D**)

(n=8; Fig. 5A) when the nominally Cl^--free pipette solution (PS3) was used. Only outward currents were also observed in the absence of extracellular HCO_3^- (n=3; Fig. 5B). In the presence of HCO_3^-, but without extracellular *and* intracellular Cl^-, GABA induced inward currents, (n=6; Fig. 5C). In a Cl^-- and HCO_3^--free saline, the GABA-induced currents were suppressed (n=6; Fig. 5D), but reappeared when Cl^- and/or HCO_3^- were added again. The results, summarized in Fig. 5E, indicate that both Cl^- and HCO_3^- anions contribute to GABA-induced currents in cultured cortical neurons.

Reversal potential of the GABA-induced current

The reversal potential of the GABA-induced current (E_{GABA}) in cultured cortical neurons was determined by applying GABA while the membrane potentials was held at different values (V_m). Membrane current responses were recorded in the gramicidin-perforated-patch configuration in the presence and absence of extracellular HCO_3^-. In HCO_3^--buffered saline at a V_h of –80 mV, GABA induced inward currents in five neurons and an outward current in one (median: –40 pA; range –134 to 8 pA; Fig. 6A), while at a V_h of –60 mV inward currents and outward currents (median: –3 pA; range –96 to 102 pA; n=12) were recorded. In the absence of HCO_3^- at a V_h of –80 mV, GABA evoked monophasic inward currents in five out of six neurons (median: –15 pA; range –73 to 6 pA; Fig. 6A). At a V_h of –60 mV currents were inwardly directed in two of seven neurons, but reduced in amplitude compared to those recorded at a V_h of –80 mV or outwardly directed (median: 30 pA; range –124 to 100 pA; n=7; Fig. 6A). Although there was variation in the GABA-induced current polarity in the presence of HCO_3^-, GABA-activated currents were mostly outwardly directed or significantly reduced in amplitude, if they were inwardly directed. Current amplitudes were plotted as a function of V_h. Since voltage-ramp experiments (see below) showed linear *I-V* relations of the GABA-activated current in the potential range between –80 and –60 mV, E_{GABA} was determined by fitting a line through the medians obtained in the absence and presence of HCO_3^-, respectively (Fig. 6A). In the HCO_3^--free saline E_{GABA} was close to –73 mV, while it was shifted to a more positive value in the HCO_3^--buffered saline (to –59 mV, determined by extrapolation; Fig. 6A).

In another set of experiments, the K^+ conductance of the neurons was inhibited by using a Cs^+-containing pipette solution in order to improve space-clamp conditions under gramicidin-perforated-patch conditions. E_{GABA} was determined with the voltage-ramp method (see Materials and methods). Therefore, the current responses evoked by voltage-ramp commands recorded in the absence of GABA were subtracted from those in its presence. In the HCO_3^--buffered saline GABA induced biphasic currents in two cells (Fig. 6B), and inward currents in four cells (Fig. 6C). In the HCO_3^--free saline GABA evoked inward currents in three neurons and in three other cells outward currents (Fig. 6D). E_{GABA} was determined for outwardly directed and inwardly directed current responses which were recorded either in the presence of HCO_3^- or in its absence. E_{GABA} was significantly more positive in the presence of HCO_3^- (–46±4 mV) than in its absence (–59±5 mV, paired *t*-test; $P<0.02$; $n=6$). It should be noted, however, that GABA-evoked inward currents also occurred in the absence of HCO_3^-.

Discussion

When using the gramicidin-perforated-patch configuration to measure membrane currents in cultured cortical neurons, GABA primarily produced inward currents or depolarizations, and biphasic current or biphasic membrane potential changes, which are mediated by Cl^- and HCO_3^-. GABA-activated inward currents and, in particular, the inward component of biphasic current responses were dependent on the presence of HCO_3^-. In addition, the activation of $GABA_A$ receptors frequently led to a significant increase in $[Cl^-]_i$.

In our study we have used the gramicidin-perforated-patch configuration, because this allows recording without interference with the physiological changes of $[Cl^-]_i$ [18] which may occur after the activation of ligand-gated receptor channels permeable to Cl^-. Since the pipette solution contained a high Cl^-concentration it was also possible to reject experiments when an unintended conventional whole-cell-clamp configuration occurred.

Mechanism underlying monophasic GABA-activated currents

The application of GABA evoked three types of membrane responses in cultured cortical neurons. The majority of neurons responded with inward currents or biphasic current responses. GABA-induced inward currents and depolarizations could be explained by assuming that E_{GABA} is more positive than the resting membrane potential. The activation of $GABA_A$ receptors would then lead to a Cl^- efflux resulting in an inward current that would produce a depolarization. Indeed, GABA evokes depolarizing responses during early developing stages, e.g. in hippocampal neurons [4, 7], in neocortical neurons [19], in hypothalamic neurons [6], in embryonic and early postnatal neocortical cells [22] and in spinal cord neurons [23, 24, 27, 29], which was attributed to an active intracellular Cl^- accumulation in these neurons.

An elevated resting $[Cl^-]_i$ would require the expression of transport mechanisms such as the Na^+-K^+-Cl^- co-transporter, which has been identified in rat sympathetic neurons [2], or the Cl^-/HCO_3^- exchange system as described in rat cerebellar Purkinje cells [12], or the Na^+-dependent Cl^-/HCO_3^- co-transporter found in Rohon-Beard spinal neurons of *Xenopus* larvae [24].

The mechanism underlying biphasic GABA-activated currents

GABA-activated inward currents and the inward component of biphasic current responses disappeared in the absence of HCO_3^- indicating that HCO_3^- contributes to these responses. Since $GABA_A$ receptors are permeable to Cl^- and, to a lesser extent, also to HCO_3^- [5, 15], E_{GABA} does not exactly match E_{Cl}, but is given by the following equation:

$$E_{GABA}=(RT/F)\ln\{([Cl^-]_e+b[HCO_3^-]_e)/([Cl^-]_i+b[HCO_3^-]_i)\} \quad (1)$$

where R is the gas constant, T is the absolute temperature, F is the Faraday constant, $[Cl^-]_e$, $[Cl^-]_i$ are the extra- and intracellular Cl^- concentrations, respectively, $[HCO_3^-]_e$, $[HCO_3^-]_i$ are the extra- and intracellular HCO_3^- concentrations, and b represents P_{HCO_3}/P_{Cl} [16], which was calculated to be 0.18 in cultured spinal cord neurons [5]. Assuming an intracellular pH of 7.2 [9] and according to $E_{HCO_3}=E_H=(RT/F)(pH_i-pH_o)$ (pH_o is extracellular pH) [16], E_{HCO_3} and $[HCO_3^-]_i$ can be estimated to be –12 mV and 16 mM, respectively. Because E_{HCO_3} is significantly more positive than the resting membrane potential, the activation of $GABA_A$ receptors evokes a HCO_3^- efflux leading to an inward current, because of the stronger driving force for HCO_3^- (E_{Cl} is close to the resting membrane potential). This mechanism is supported by several of our findings. First, GABA-activated currents are still present in the nominal absence of intra and extracellular Cl^- but, second, in the absence of HCO_3^- and Cl^- hardly any current response remains. Third, in the presence of HCO_3^- E_{GABA} is shifted towards more positive values. We therefore conclude that a subpopulation of cortical neurons expresses $GABA_A$ receptors which are characterized by a significant HCO_3^- permeability. It should be mentioned that a P_{HCO_3}/P_{Cl} in the range 0.18 to 0.44 [5, 11] is sufficient to produce a depolarization in a cell even if E_{Cl} is identical or slightly more negative than the resting membrane potential [16].

However, the HCO_3^- permeability of $GABA_A$ receptors alone does not explain why GABA-activated currents or membrane potential changes reverse their polarity *during* the response. An appropriate mechanism cannot be derived from Eq. 1 alone, but is conceivable under the assumption that the Cl^- and/or HCO_3^- gradient chang-

es during the GABA-mediated response. In this study we have shown by measuring SPQ-fluorescence that the activation of $GABA_A$ receptors in cultured cortical neurons in fact leads to an accumulation of Cl^-. This shifts E_{GABA} into the positive direction (see Eq. 1). If E_{GABA} becomes more positive than the resting membrane potential, the GABA-activated response will change its direction from an outward to an inward current or from a hyperpolarization to a depolarization, respectively. In addition, $[HCO_3^-]_i$ could be maintained by the free diffusion of CO_2 across the cell membrane which is converted into HCO_3^- by the activity of the enzyme carbonic anhydrase. This is supported by our observation that in 92% of cultured cortical neurons (n=105) loaded with the membrane-permeable pH-sensitive dye bicarboxy-ethylene-carboxyfluoresin-acetoxy-methylester (BCECF-AM), the application of 100 µM GABA did not induce a change of pH_i (unpublished data), indicating the fast intracellular replacement of lost HCO_3^- and the strong buffering power of intact cells. Thus, the HCO_3^- gradient will be less affected [26]. An efflux of HCO_3^-, however, would further depolarize the cell membrane, thereby enhancing Cl^- influx and shifting E_{Cl} and consequently E_{GABA} even more into the positive direction. Without the contribution of HCO_3^-, E_{GABA} could never shift to values more positive than the resting membrane potential. GABA-induced currents or membrane potential changes would then never change their polarity, if no other depolarizing processes are coincidentally active.

Interestingly, E_{GABA} was significantly more positive when determined with the voltage-ramp method. This may be due to the voltage-ramps which lead to transient depolarizations of the neurons, and, in the presence of GABA, would drive Cl^- into the cells through open $GABA_A$ receptor ion channels, thus inducing a positive shift of E_{GABA}. A similar mechanism has recently been proposed for biphasic glycine-activated responses in developing auditory neurons of rat brainstem slices [1].

Since the GABA-induced changes in SPQ-fluorescence only indicated a GABA-activated rise in $[Cl^-]_i$ that could not be quantified, the question remains: how much Cl^- has to be accumulated to shift E_{GABA} to values more positive than the resting membrane potential? In the absence of HCO_3^-, E_{GABA} equals E_{Cl} at a potential of –73 mV in our experiments, indicating a $[Cl^-]_i$ of 8 mM (with $[Cl^-]_e$ =133.5 mM). In the presence of HCO_3^-, E_{GABA} was found to be –59 mV. According to Eq. 1 and assuming that the HCO_3^- gradient does not significantly change during the response [26], the $[Cl^-]_i$ should have increased to 11 mM during GABA's action. This shows that a small rise of the $[Cl^-]_i$ above its resting level is sufficient to shift E_{GABA} positive to the resting membrane potential, which will result in the change of the response's polarity. Much larger Cl^- fluxes of up to 16 mM have already been observed in rat sympathetic neurons after the activation of $GABA_A$ receptors [2, 3].

A subpopulation of neurons, however, responded with an outward current, or with a hyperpolarization, even though HCO_3^- was present, suggesting an E_{GABA} that was, and remained, more negative than the membrane resting potential. It might be possible that in these neurons the activation of $GABA_A$ receptors did not induce a large enough Cl^- accumulation. Another possibility is the existence of different $GABA_A$ receptor subtypes [28] which differ in P_{HCO3}/P_{Cl}. However, at present, there is no evidence that this molecular diversity includes different permeability properties.

GABA-activated inward currents were dependent on the presence of HCO_3^- but appeared also in its absence in a subpopulation of cortical neurons, where the E_{Cl} might have been more positive than the resting membrane potential. Thus, depolarizations induced by GABA could be due to two different mechanisms: one characterized by the activity of transporter molecules that maintain an active Cl^- accumulation; another characterized by an acute uptake of Cl^-, that is reinforced by the depolarizing effect of HCO_3^- efflux.

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