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EFFECT OF γ-AMINOBUTYRIC ACID ON INTRACELLULAR pH IN THE CRAYFISH STRETCH-RECEPTOR NEURONE

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Summary

The effect of γ-aminobutyric acid (GABA) on intracellular pH (pHi) was examined in the crayfish stretch-receptor neurone using H^+-selective microelectrodes and a two-microelectrode voltage clamp. In the presence of 30 mmol l^{-1} HCO_3^- (pH 7.4), application of GABA (0.5 mmol l^{-1}) produced a mean fall in pHi of 0.26 units. The initial rate of fall of pHi was attributable to a net influx of acid equivalents of 6.3 mmol l^{-1} min^{-1}. In the nominal absence of HCO_3^-, GABA had little effect on pHi. The HCO_3^--dependent acidosis caused by GABA was inhibited by picrotoxin (0.1 mmol l^{-1}) but not by depletion of extracellular and intracellular Cl^-. Acetazolamide (0.1 mmol l^{-1}) decreased the rate of fall of pHi caused by a step increase in CO_2 partial pressure as well as by GABA, which indicates that the neurone contains carbonic anhydrase. In the presence of both Cl^- and HCO_3^-, the reversal potential of the GABA-activated current was more positive than under nominally HCO_3^--free conditions. In line with this, GABA induced a marked HCO_3^--dependent depolarization, and this depolarizing action was enhanced in the absence of Cl^- so as to lead to triggering of action potentials. All these observations support the conclusion that the GABA-induced fall in pHi is due to a net efflux of HCO_3^- through the inhibitory anion channels.

Introduction

An extensive amount of work has shown that an increase in Cl^- conductance plays a dominant role in synaptic inhibition mediated by GABA in invertebrate neurones as well as in vertebrate neurones with $GABA_A$-type receptors (Boistel and Fatt, 1958; Takeuchi and Takeuchi, 1967; for reviews, see Atwood, 1982; Siggins and Gruol, 1986). In line with this, a widely accepted assumption is that the reversal potential of the GABA-induced current (E_{GABA}) is identical to the equilibrium potential of Cl^- (E_{Cl}). However, work on both invertebrate (Kaila and Voipio, 1987; Kaila *et al.* 1989*b*, 1990) and vertebrate preparations (Kelly *et al.* 1969; Inomata *et al.* 1986; Bormann *et al.* 1987) has indicated that GABA-gated

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anion channels are significantly permeable to HCO_3^-. We have recently shown that, in crayfish leg opener muscle fibres in the presence of 30 mmol l^{-1} HCO_3^-, E_{GABA} is up to 15 mV more positive than E_{Cl}. The difference between E_{GABA} and E_{Cl} was accounted for by a HCO_3^-/Cl^- permeability ratio of about 0.3 (Kaila *et al.* 1989*b*). In agreement with the significant HCO_3^- permeability of the inhibitory anion channels, GABA was also found to induce a large fall in intracellular pH (pHi) (Kaila and Voipio, 1987; Kaila *et al.* 1990).

In view of the central role of the crayfish stretch-receptor neurone in the experimental work which has led to the clarification of the basic mechanisms underlying GABA-mediated inhibition (Kuffler and Eyzaguirre, 1955; Kuffler and Edwards, 1958; Roberts, 1986), we found it important to examine whether the effect of GABA on the neurone is influenced by HCO_3^- in a manner similar to that observed in the opener muscle fibre. More specifically, such a comparative investigation is motivated by the following facts. First, it has become increasingly evident that, even within a single animal species, the GABA-gated receptor–channel complex exhibits considerable variation in its properties (Dudel and Hatt, 1976; Yasui *et al.* 1985; Levitan *et al.* 1988). Second, the fall in pHi caused by the efflux of HCO_3^- through inhibitory anion channels is governed by factors such as the relative density of the channels on the cell membrane and the steepness of the dependence on pHi of the active acid extrusion mechanisms of the GABA-sensitive cell (Kaila *et al.* 1990). Clearly, not all the above factors are likely to be identical in the opener muscle fibre and the stretch-receptor neurone of the crayfish and, therefore, the possible existence of a significant GABA-induced acid load and its consequences on pHi regulation in the neurone cannot be deduced from results obtained on the muscle alone.

In this work, we have examined the influence of HCO_3^- on the actions of GABA on membrane potential and on pHi in the slowly adapting stretch-receptor neurone of the crayfish with the aid of conventional and H^+-selective microelectrodes. The results indicate a significant HCO_3^- permeability of GABA-gated channels which has a marked influence on both the electrical behaviour and pHi regulation of the neurone.

Materials and methods

The experiments were carried out on the slowly adapting stretch-receptor neurone of the crayfish *Astacus astacus* L. The stretch receptor with its muscle was isolated as described previously (Brown *et al.* 1978). The preparation was pinned on the bottom of a flow-through chamber, which was perfused at a rate of about 2.5 ml min^{-1} and earthed with an agar/3 mol l^{-1} KCl bridge. The volume of this chamber was about 300 μl. All experiments were carried out at room temperature (20–21 °C). The input resistance of the neurones used in the present experiments ranged from 1.6 to 3 MΩ and they had action potentials with an overshoot of 10–20 mV.

Measurement of membrane potential and of E_{GABA}

Dry-bevelled microelectrodes (Kaila and Voipio, 1985) filled with 0.62 mol l^{-1} K_2SO_4 and 8.0 mmol l^{-1} KCl were used for measuring membrane potential and passing current. They had a resistance of 15–40 MΩ. The reversal potential of the GABA-activated current (E_{GABA}) was measured using a two-microelectrode voltage clamp and short pulses of GABA (0.5 mmol l^{-1}).

Ion-selective microelectrodes

H^+-selective microelectrodes were constructed and calibrated as described previously (Kaila *et al.* 1989*a*,*b*). Briefly, dry-bevelled micropipettes pulled from tubing without a fibre (GC150, Clark Electromedical) were silanised and back-filled with 100 mmol l^{-1} NaCl plus 20 mmol l^{-1} Hepes (pH 7.6). A short column of the H^+ sensor Fluka 82500 was drawn into the tip. In a few experiments, the H^+-selective microelectrodes were made using non-bevelled micropipettes pulled from thin-walled tubing (GC150T, Clark Electromedical). The resistance of these electrodes was about 70–100 GΩ. A few measurements of the rate of depletion of intracellular Cl^- in a chloride-free solution were made using Cl^--selective microelectrodes fabricated in the above manner (sensor, Corning 477913; resistance, 10 GΩ).

Solutions

The nominally CO_2/HCO_3^--free crayfish saline contained (in mmol l^{-1}): NaCl, 207; KCl, 5.4; $CaCl_2$, 7.0; $MgCl_2$, 2.6; Hepes, 10 (pH 7.4; adjusted with NaOH). In making HCO_3^--containing solutions, 30 mmol l^{-1} NaCl was replaced by an equivalent amount of $NaHCO_3$ to yield a pH of 7.4 when equilibrated with air or oxygen containing 5 % CO_2. Cl^--free solutions were made by isomolar substitution of Cl^- by methanesulphonate, or, when using Cl^--selective microelectrodes, by glucuronate with the Ca^{2+} concentration elevated to 17.5 mmol l^{-1} (see Kaila *et al.* 1989*b*). GABA (Sigma) was added from a stock solution made up in water. Acetazolamide (Sigma) and picrotoxin (Sigma) were directly dissolved into the saline.

Calculation of net fluxes and intracellular concentrations of HCO_3^-

The GABA-induced net HCO_3^- efflux ($J^e_{HCO_3}$; mmol l^{-1} min^{-1}) was obtained from (see Kaila *et al.* 1990):

$$J^e_{HCO_3} = J^i_H = -\beta \mathrm{dpHi}/\mathrm{d}t, \quad (1)$$

where J^i_H is the net influx of acid equivalents, β is the intracellular H^+ buffering power of the neurone and t is time. In the presence of CO_2, β is the sum of the non-CO_2 buffering power (assumed to be 10 mmol l^{-1}; Szatkowski and Thomas, 1989) and of the CO_2/HCO_3^- buffering power which is $2.3[HCO_3^-]_i$ (see Roos and

Boron, 1981). The intracellular HCO_3^- concentration ($[HCO_3^-]_i$) was obtained from:

$$[HCO_3^-]_i = 10^{(pHi-pHe)} [HCO_3^-]_e, \quad (2)$$

where pHe is the extracellular pH.

Statistics

All values are given as mean±S.E.M.

Results

Effect of GABA on membrane potential and on intracellular pH

In a nominally HCO_3^--free solution, exposure of the stretch-receptor neurone to a near-saturating concentration of GABA (0.5 mmol l^{-1}) produced a change in membrane potential (E_m) of +3.2±0.67 mV (N=14). A hyperpolarizing response was seen in only one of the preparations examined. This observation is in agreement with previous results (e.g. Ozawa and Tsuda, 1973), which report both depolarizing and hyperpolarizing responses upon application of GABA. We did not find any correlation between the type of response (depolarizing/hyperpolarizing) and the various criterion parameters (cell input resistance, action potential overshoot, etc.) which have often been used as indices of cell viability (Deisz and Lux, 1982). The small GABA-induced depolarization observed in the absence of bicarbonate was paralleled by a very slight fall in pHi (0.027±0.006 units; N=7).

As illustrated in Fig. 1, exposure of the preparation to a solution containing 30 mmol l^{-1} HCO_3^- induced a rapid fall in pHi which recovered promptly close to the original baseline level. Application of GABA at this stage produced a depolarization of 9.6±0.78 mV (N=7), which was much larger than that seen in the absence of HCO_3^-. The depolarization was now associated with a fall in pHi of up to 0.3 units (mean 0.26±0.02; N=7). Both effects of GABA were fully reversible.

The initial net influx of acid equivalents caused by exposure to GABA, as well as the steady-state flux which takes place at plateau acidosis, were calculated using equation 1. The steady-state flux was estimated on the basis of the rate of change of pHi measured upon removal of GABA after attainment of plateau acidosis (see Kaila *et al.* 1990). These measurements yielded a value of 6.3±0.31 mmol l^{-1} min^{-1} (N=7) for the initial acid load and 1.6±0.15 mmol l^{-1} min^{-1} (N=6) for the steady-state acid load in the presence of HCO_3^-. The slight GABA-induced fall in pHi seen under nominally HCO_3^--free conditions was attributable to an initial acid load of only about 0.06–0.08 mmol l^{-1} min^{-1}.

Effects of GABA in the absence of Cl^-

Experiments of the kind shown in Fig. 1A do not provide information on the behaviour of Cl^- during the application of GABA. Therefore, one might argue that the effect of GABA on pHi is due to a redistribution of Cl^- which acts on

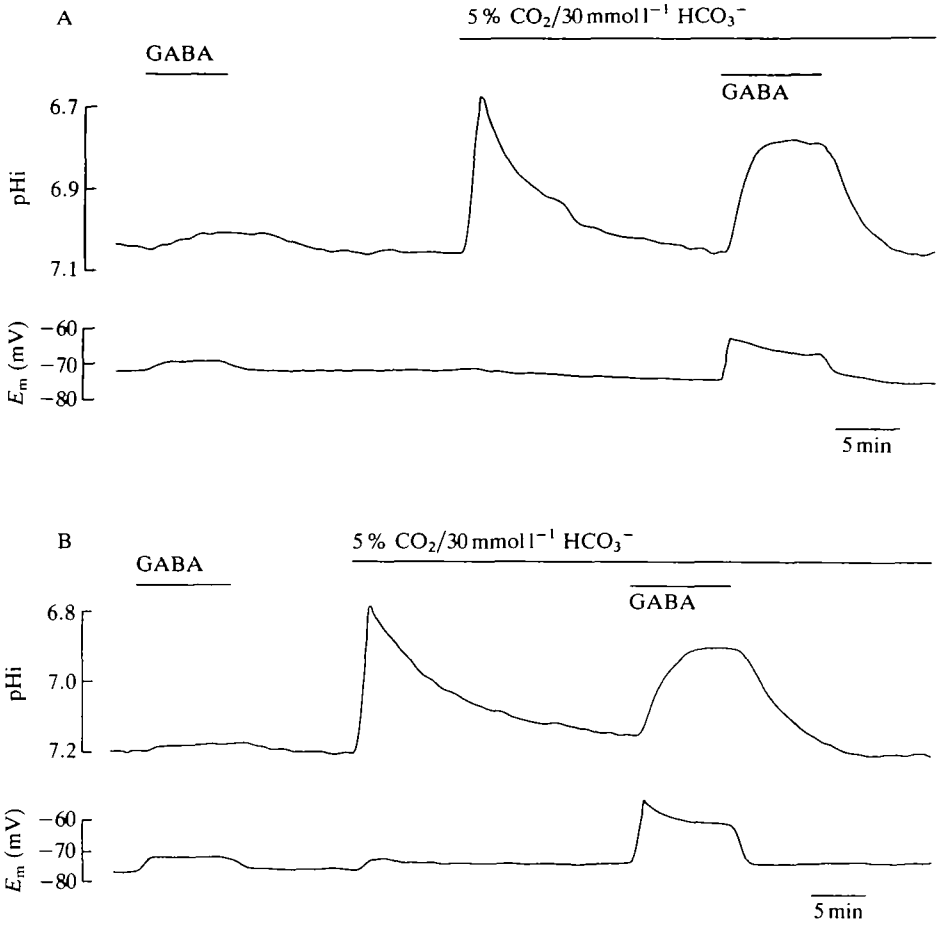

Fig. 1. (A) Dependence on HCO_3^- (30 mmol l^{-1}) of the fall in pHi caused by GABA (0.5 mmol l^{-1}). Note the enhancement of the GABA-induced depolarization in the presence of HCO_3^-. (B) Absence of an influence of Cl^- depletion on the HCO_3^--dependent acidotic effect of GABA (0.5 mmol l^{-1}). The preparation had been kept for 25 min in a Cl^--free solution before the start of the recording. E_m, membrane potential.

Na^+-dependent Cl^-/HCO_3^- exchange (Thomas, 1977; Moody, 1981), thereby affecting pHi. This possibility was examined by studying the effects of GABA in the absence of both extracellular and intracellular Cl^-.

Measurements with Cl^--selective microelectrodes indicated that, upon exposure of the neurone to a Cl^--free solution, the intracellular Cl^- activity fell in a roughly exponential manner with a time constant of about 3.5–4.5 min. This means that a virtually complete depletion of Cl^- could be achieved in about 20 min. Accordingly, the preparation was exposed to a Cl^--free solution for about 20–30 min before the effects of GABA were examined. As shown in the initial part of Fig. 1B, GABA had little effect on pHi in the absence of both Cl^- and HCO_3^-.

However, in a Cl^--free, HCO_3^--containing solution, application of GABA produced a large fall in pHi.

In the absence of both Cl^- and HCO_3^-, GABA produced only a relatively small depolarization. The depolarizing effect of GABA was dramatically augmented in a solution containing HCO_3^- but no Cl^-, and it was usually big enough (20 mV or more) to trigger a train of action potentials (see Fig. 2B).

The above results indicate that the acid load induced by GABA is independent of Cl^-/HCO_3^- exchange. A similar conclusion applies to the opener muscle (Kaila *et al.* 1990).

An interesting observation made in the above experiments was that the recovery of pHi following a CO_2-induced or GABA-induced acidosis was not blocked in the absence of Cl^-. This clearly indicates that acid extrusion in the crayfish stretch-receptor neurone is not solely due to Na^+-dependent Cl^-/HCO_3^- exchange, as has been claimed (Moser, 1985).

Effect of picrotoxin

Picrotoxin (PTX) is a non-competitive inhibitor of crayfish GABA-gated anion channels (Takeuchi and Takeuchi, 1969; Aickin *et al.* 1981). As shown in Fig. 2A, 0.1 mmol l^{-1} PTX has a strong blocking effect on both the depolarization and the acidosis produced by 0.5 mmol l^{-1} GABA in a HCO_3^--containing solution. PTX exerts its inhibitory effect whether Cl^- is present or not (Fig. 2B). These results further support the view that the HCO_3^--dependent fall in pHi caused by GABA is due to a net efflux of HCO_3^- through the inhibitory postsynaptic channels.

Effect of acetazolamide

As has been explained in detail elsewhere (Kaila *et al.* 1990), a channel-mediated net efflux of HCO_3^- leads to a net transmembrane influx of CO_2. The acidifying effect of this influx depends on the hydration of CO_2 (Roos and Boron, 1981), a reaction which in several types of cells is catalyzed by the enzyme carbonic anhydrase (Maren, 1967; Deutsch, 1987).

To determine whether carbonic anhydrase is present in the stretch-receptor neurone, the cell was exposed to the CO_2/HCO_3^--containing solution under control conditions and in the presence of the carbonic anhydrase inhibitor acetazolamide (0.1 mmol l^{-1}). Owing to the rapid transmembrane equilibration of CO_2 (see Fig. 1A,B), experiments of this kind are well-suited for detection of the presence of an intracellular carbonic anhydrase. Acetazolamide caused a marked decrease in the initial rate of fall of pHi seen in these experiments (Fig. 3A).

Acetazolamide also slowed down the initial rate of the GABA-induced acidosis by 28±6.2% (N=4) (Fig. 3B). This indicates that, in the absence of intracellular carbonic anhydrase activity, the acidosis caused by 0.5 mmol l^{-1} GABA is rate-limited by the hydration of CO_2 and not by the HCO_3^- conductance. A similar conclusion was made in previous work on crayfish muscle fibres (Kaila *et al.* 1990).

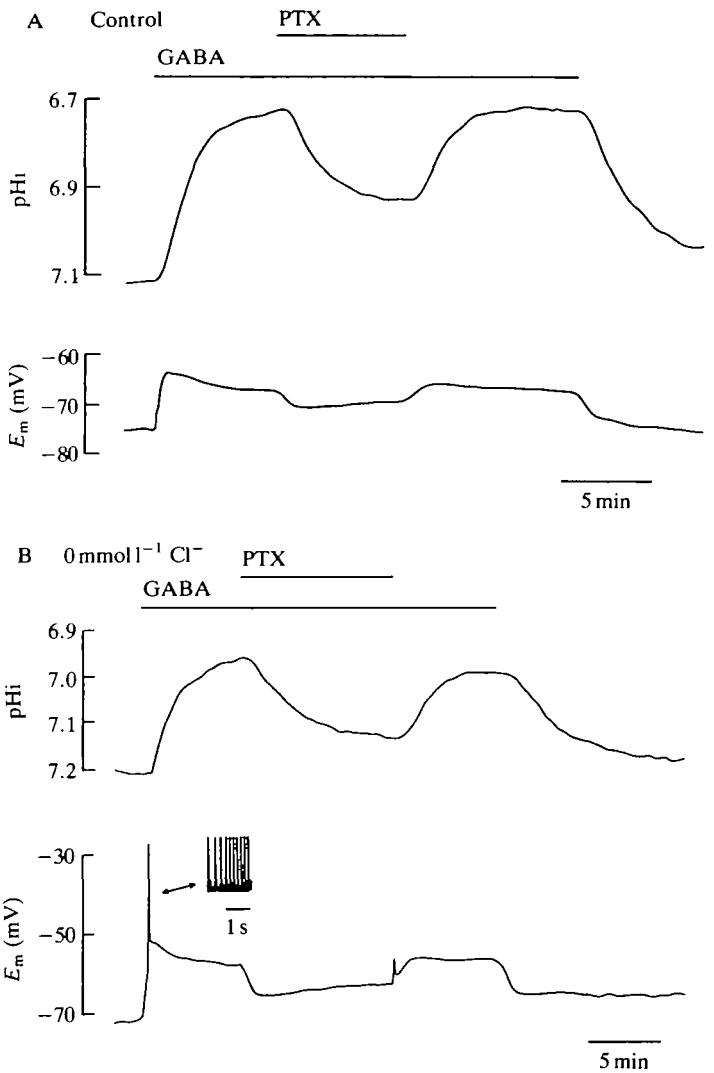

Fig. 2. Inhibition by picrotoxin (PTX, 0.1 mmol l^{-1}) of the HCO_3^--dependent acidosis and depolarization caused by GABA (0.5 mmol l^{-1}) in the presence (A) and absence (B) of Cl^-. In B, the preparation had been kept for 30 min in a Cl^--free solution before exposure to GABA (0.5 mmol l^{-1}). Owing to the limited bandwidth of the membrane potential recording, the train of truncated action potentials (inset), which are triggered by GABA in the presence of HCO_3^-, is seen as a transient depolarizing deflection.

Influence of bicarbonate on E_{GABA}

The above results are in agreement with the idea that the GABA-induced fall in pHi is caused by a net efflux of HCO_3^- mediated by the inhibitory postsynaptic channels. Measurements of the voltage-dependence of the GABA-activated current showed that, in the presence of 30 mmol l^{-1} HCO_3^-, E_{GABA} was about

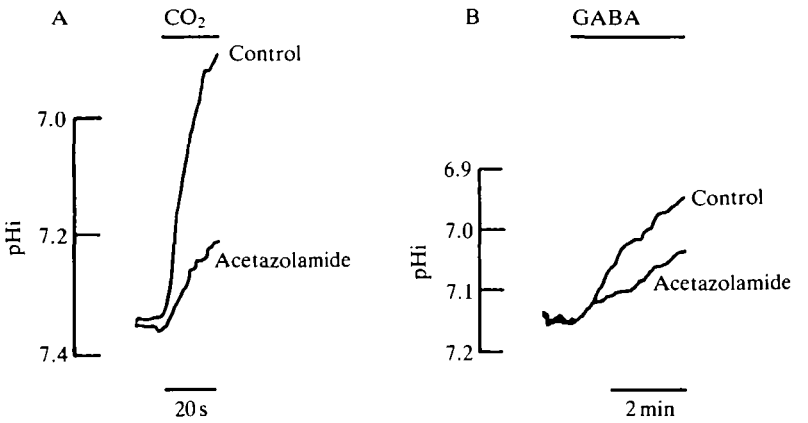

Fig. 3. Effect of 0.1 mmol l^{-1} acetazolamide on the fall in pHi induced by application of (A) CO_2/HCO_3^- and (B) 0.5 mmol l^{-1} GABA in the presence of HCO_3^-.

6–9 mV (N=3) more positive than in the absence of bicarbonate (Fig. 4). This HCO_3^--dependent positive shift in E_{GABA} is consistent with a significant bicarbonate permeability of the GABA-activated channels.

Discussion

The present work shows that the postsynaptic inhibitory channels of the crayfish stretch-receptor neurone are significantly permeable to HCO_3^-. It will be of interest to see whether future work on other transmitter-sensitive neuronal anion channels, such as those gated by acetylcholine (Tauc and Gerschenfeld, 1962) and by glutamate (Marder and Paupardin-Tritsch, 1978) will reveal a significant role for HCO_3^- as a carrier of inhibitory currents.

Active regulation of intracellular pH leads to a non-equilibrium transmembrane distribution of H^+, such that the equilibrium potential of H^+ (E_H) is much more positive than the resting membrane potential (Roos and Boron, 1981; Thomas, 1984). In the presence of CO_2/HCO_3^-, the equilibrium potential of HCO_3^- is equal to that of protons, i.e. $E_{HCO_3}=E_H$ (Kaila and Voipio, 1990). Therefore, a significant HCO_3^- permeability of the GABA-gated channels is expected to lead to a shift in E_{GABA} towards more positive potentials, as was observed in the present work. This shift cannot be due to a redistribution of Cl^- caused by an action of CO_2/HCO_3^- on Na^+-dependent Cl^-/HCO_3^- exchange (Thomas, 1977), because, if anything, such an effect would cause a decrease in intracellular Cl^- concentration and thereby a negative shift in E_{GABA}.

The HCO_3^- permeability also explains the marked fall in pHi caused by GABA in a solution equilibrated with 5 % CO_2. A fall in pHi due to the efflux of HCO_3^- across transmitter-sensitive anion channels has previously been observed in the crayfish opener muscle (Kaila and Voipio, 1987; Kaila *et al.* 1990) and in the acinar cells of mammalian salivary glands (Melvin *et al.* 1988; Brown *et al.* 1989). In the

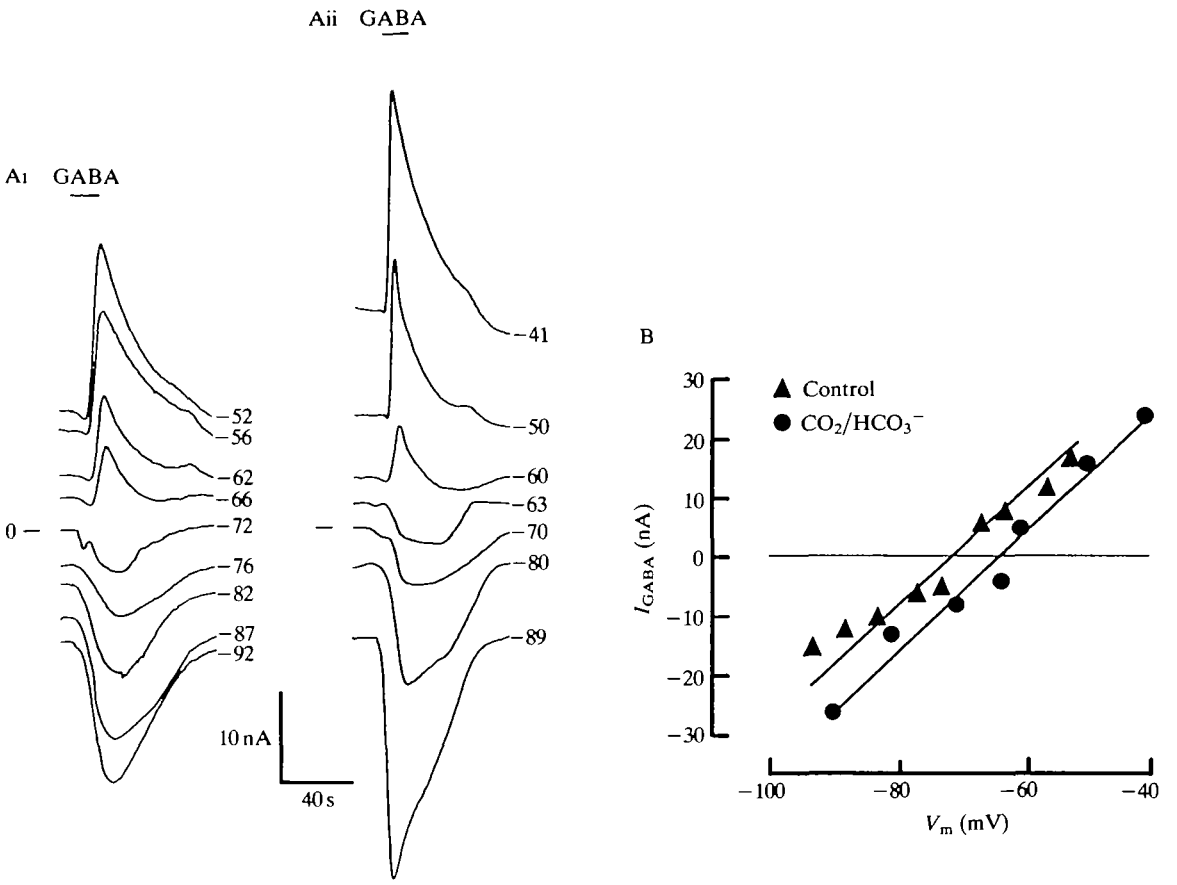

Fig. 4. Influence of HCO_3^- on E_{GABA}. (A) Specimen recordings of GABA-activated currents at various holding potentials in the absence (i) and presence (ii) of HCO_3^-. The holding potential (in mV) is indicated on the original recordings. (B) Dependence on holding potential (V_m) of peak currents (I_{GABA}) activated by GABA in control solution (▲) and in the presence of HCO_3^- (●).

present context, it is of interest that glutamate agonists (*N*-methyl-D-aspartate, quisqualate and kainate) have been shown to induce a fall in pHi in frog motoneurones (Endres *et al.* 1986). However, the mechanism underlying the acidotic action of glutamate agonists has not yet been clarified.

Because an open CO_2/HCO_3^- H^+-buffer system operates within the neurone, the GABA-induced efflux of HCO_3^- gives rise to an equivalent influx of acid (see equation 1). Despite the obvious structural and functional differences between the stretch-receptor neurone and the opener muscle fibre, which has been studied previously (Kaila *et al.* 1990), it is evident that, in the presence of HCO_3^-, GABA produces an instantaneous HCO_3^- efflux which is surprisingly similar in these two kinds of cells. It is slightly smaller in the neurone (6.3 mmol l^{-1} min^{-1}) than in the muscle (8.0 mmol l^{-1} min^{-1}). Likewise, the fall in pHi caused by GABA in the

neurone was somewhat smaller (0.26 units) than in the muscle (0.43 units). However, it is worth pointing out here that this kind of between-cells correlation of the two parameters is not obligatory, i.e. a smaller acidosis is not necessarily linked to a smaller acid load. This is because, at steady state, the fall in pHi produced by GABA is determined by the net efflux of HCO_3^- through the inhibitory channels as well as by the net efflux of acid equivalents mediated by plasmalemmal acid-extrusion mechanisms. A steady state (plateau acidosis) is attained when both fluxes are of equal magnitude.

An unexpected observation made in the present work was that, in sharp contrast to the conclusions made by Moser (1985), acid extrusion in the crayfish stretch-receptor neurone is not strictly dependent on the presence of Cl^-. This suggests that there is no difference between the receptor neurone and central neurones, which have been shown to employ two separate mechanisms for acid extrusion: a Na^+/H^+ exchange and a Na^+-dependent Cl^-/HCO_3^- exchange (Moody, 1981).

The observation that acetazolamide decreased the initial rate of the fall in pHi caused both by CO_2/HCO_3^- and by GABA (Fig. 3) clearly indicates that the stretch-receptor neurone contains an intracellular carbonic anhydrase. Immunohistochemical work has recently shown that certain vertebrate neurones contain carbonic anhydrase (Droz and Kazimierczak, 1987; Wong *et al.* 1987; Aldskogius *et al.* 1988) and, on purely structural grounds, it has been postulated that carbonic anhydrase may play a role in synaptic transmission (Aldskogius *et al.* 1988). A catalyzed hydration of CO_2 will tend to accentuate postsynaptic pHi transients produced by HCO_3^- movements across inhibitory channels, and it is possible that such pHi changes may have a modulatory role in synaptic transmission (see Kaila and Voipio, 1990).

Since most of the work on the stretch-receptor neurone has been done under nominally HCO_3^--free conditions (Kuffler and Eyzaguirre, 1955; Ozawa and Tsuda, 1973; Deisz and Lux, 1982), the demonstration of a significant HCO_3^- permeability of the inhibitory channels does not necessitate major revisions of the conclusions made in previous work on the effects of GABA on membrane potential. However, under conditions *in vivo*, a HCO_3^- permeability is bound to lead to a marked difference between E_{Cl} and the reversal potential of the inhibitory postsynaptic potential IPSP (E_{IPSP}), especially when the HCO_3^- concentration of the haemolymph is high (see Gaillard and Malan, 1983).

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