Ionic Mechanisms of Spontaneous GABAergic Events in Rat Hippocampal Slices Exposed to 4-Aminopyridine

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Lamsa, Kari and Kai Kaila. Ionic mechanisms of spontaneous GABAergic events in rat hippocampal slices exposed to 4-aminopyridine. J. Neurophysiol. 78: 2582–2591, 1997. Ion-selective (H+ and K+) microelectrode techniques as well as conventional extracellular recordings were used to study the ionic mechanisms of propagating spontaneous GABAergic events (SGEs) in rat hippocampal slices exposed to 4-aminopyridine (4-AP, 50–100 μM). All experiments were made in the presence of antagonists of ionotropic glutamate receptors [10 μM 6-nitro-7-sulphamoyl-benzozquinoline-2,3-dione (NBQX) and 40 μM 6-nitro-2-amino-5-phosphono-3-1-4 benzoxazine (AP5)]. The SGEs were composed of a negative-going change in field potential with a temporally coincident increase (0.7 ± 0.3 mV; mean ± SE) in extracellular K+ ([K+]e) and an alkaline transient (0.01–0.08 units) in extracellular pH (pHext) in stratum radiatum of the area CA1. Simultaneous intracellular recordings showed a triphasic hyperpolarization-depolarization–late hyperpolarization response in pyramidal cells. Application of pentobarbital sodium (PB, 100 μM) decreased the interevent intervals between SGEs from a mean value of 35 ± 20 s and shortened the period of refractoriness of stimulus-evoked propagating events. This was accompanied by a decrease in the amplitude of the field potential response of the [K+]e and the pHext shifts and of the depolarizing phase of the pyramidal-cell response. The SGEs were completely blocked by the γ-aminobutyric acid-A (GABAA) receptor antagonist, picrotoxin (PTX; 100 μM). The amplitudes of the negative-going field potential and of the depolarizing phase of the pyramidal-cell response as well as the ion shifts associated with SGEs were strongly suppressed in the nominal absence of CO2/HCO3−. There was a five-fold increase in the interevent interval, and propagating SGEs could not be evoked by stimuli given at intervals shorter than ~2–3 min. Exposure to inhibitors of carbonic anhydrase, benzolamide (BA; 10 μM) or ethoxyzolamide (EZA; 50 μM) fully blocked the alkaline pHtransients and turned them into acid shifts. The poorly membrane-permeant BA had no discernible effect on the other components of the SGEs, but application of EZA had effects reminiscent to those of CO2/HCO3−-free medium. Addition of the GABAA receptor–permeant weak-acid anion, formate (20 mM) reestablished the SGEs that were first suppressed by exposure to the CO2/HCO3−-free medium. No SGEs were seen in the presence of a similar concentration of the GABAA receptor–impermeant anion propionate. Unlike the alkaline transients associated with HCO3−-driven SGEs, those supported by formate were not blocked by BA. The present data suggest that an inward current carried by bicarbonate is necessary for the generation of SGEs and that the GABAA receptor–mediated excitatory coupling among GABAergic interneurons is essentially dependent on the availability of intracellular bicarbonate.

INTRODUCTION

Work on hippocampal slices has uncovered an increasing number of situations where postsynaptic responses mediated by γ-aminobutyric acid-A (GABAA) receptors are by nature depolarizing rather than hyperpolarizing (Alger and Nicoll 1982; Avoli and Perreault 1987; Ben-Ari et al. 1989; Grover et al. 1993; Kaila et al. 1997; Staley and Mody 1992; Staley et al. 1995; Taira et al. 1997; Xie and Smart 1991). One of the most intriguing recent findings is the mutual excitatory coupling that is mediated by GABAA receptors among interneurons (Michelson and Wong 1991, 1994). This excitatory coupling results in a high degree of synchronization of the activity of individual interneurons, which makes populations of interneurons behave in a networklike manner (Aram et al. 1991; Michelson and Wong 1991, 1994; Perreault and Avoli 1992). Factors that shape the collective behavior of interneuronal networks are of much interest because synchronized interneuronal activity appears to be capable of promoting long-lasting excitatory responses in pyramidal neurons (Kaila et al. 1997; Staley et al. 1995; Taira et al. 1997). In particular, this raises the possibility that GABAA receptor–mediated responses may sometimes enhance rather than inhibit the induction of long-term potentiation at glutamatergic synapses, and perhaps also accentuate the generation of epileptiform activity (Avoli et al. 1996; Higashima et al. 1996; Louvel et al. 1994; Taira et al. 1995a).

In hippocampal slices as well as in various other preparations, application of 4-aminopyridine (4-AP) induces spontaneously propagating waves of neuronal activity, which occur in the presence of antagonists of ionotropic glutamate receptors, but are abolished by GABAA receptor antagonists (Aram et al. 1991; Perreault and Avoli 1992). These spontaneous GABAergic events (SGEs) are attributable to the activity of the interneuronal network, and each of them is associated with an extracellular K+ ([K+]ext) transient and with a long-lasting depolarization of pyramidal neurons within the activated area (Avoli et al. 1996; Louvel et al. 1994; Perreault and Avoli 1992). The 4-AP–induced depolarizations in pyramidal neurons are reminiscent of those evoked by brief trains of high-frequency stimulation of inhibitory afferents (cf. Grover et al. 1993; Kaila et al. 1997; Staley et al. 1995).

At present, little is known about the ionic mechanisms underlying the excitatory coupling among GABAergic interneurons that are required for the generation and propagation of the 4-AP–induced SGEs. While shifts in [K+]ext may be involved in the synchronization of neuronal activity during an SGE (Avoli et al. 1996), the GABAA receptor–mediated interneuronal coupling must be based primarily on currents carried by GABAA receptor–permeant anions, which under physiological conditions are Cl− and HCO3− (Kaila 1994).

In the present work we focused on the role of HCO3− in
the generation of SGEs. The transmembrane distribution of this anion is set by the plasmalemmal pH gradient (Roos and Boron 1981; Thomas 1984) and hence, when activated in a resting neuron, bicarbonate-mediated currents are inwardly directed (i.e., depolarizing) (Kaila and Voipio 1987; Kaila et al. 1990). The present data obtained with the use of conventional electrophysiological techniques as well as ion-selective microelectrodes sensitive to pH and K⁺ show that SGEs induced by 4-AP are tightly linked to a cellular efflux of HCO₃⁻ mediated by GABAₐ receptors and that the generation of the GABAergic wave is critically dependent on the availability of HCO₃⁻. A direct causal role for a HCO₃⁻ as current carrier required for excitatory interneuronal coupling gained further support from the finding that, in the absence of CO₂/HCO₃⁻, SGEs were promoted by formate, another weak-acid anion that is permeant across GABAₐ receptors (Bormann et al. 1987; Kaila 1994; Mason et al. 1990), but not by propionate, an impermeant weak-acid anion. Some preliminary findings were published in abstract form (Lamsa and Kaila 1996).

METHODS

Slice preparation and maintenance

Brain slices (400 μm) were prepared as described elsewhere (Voipio et al. 1995) from hippocampi of 30- to 45-day-old male Wistar rats (100–120 g). The standard physiological solution used contained (in mM) 124 NaCl, 3.0 KCl, 2.0 CaCl₂, 25 NaHCO₃, 1.1 NaH₂PO₄, 2.0 MgSO₄, and 10 D-glucose. The solution was gassed continuously with 95% O₂-5% CO₂ (pH 7.4 at the experimental temperature of 32°C). The slices were allowed to recover for ~1 h at room temperature before placing them into the interface-type recording chamber. In experiments done in the nominal absence of CO₂/HCO₃⁻, NaHCO₃ was replaced by 20 mM N-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 7.4 with NaOH). In some of these experiments, 20 mM Cl⁻ was replaced by an equal amount of formate or propionate. To ensure that the decrease in extracellular Cl⁻ had no influence on the actions of formate or propionate, control experiments were done where 20 mM Cl⁻ was first replaced by methanesulphonate (a GABAₐ receptor–impermeant strong-acid anion) (Kaila 1994) and thereafter the methanesulphonate was replaced by one of the weak-acid anions. No differences could be seen in the results obtained with and without the intermediate methanesulphonate step. All solutions devoid of HCO₃⁻ were equilibrated with 100% O₂ and their pH was adjusted to 7.4.

Recordings

Extracellular field potentials and ion-activities (H⁺, K⁺) were recorded in the stratum radiatum of the CA1 region. Intracellular recordings from CA1 pyramidal neurons were obtained with the use of sharp microelectrodes filled with a solution containing 1.0 M K-acetate, 1.5 M K-methyl sulphate, and 6 mM KCl (pH 7.0). They had a resistance of 60–100 MΩ and were coupled to an amplifier with an active bridge circuit. The cells used for measurements had a stable membrane potential of at least ~60 mV and an input impedance of 20–80 MΩ.

H⁺- and K⁺-selective microelectrodes were made from double-barreled borosilicate glass pipettes using techniques described before (Voipio et al. 1995). The silanized nonfilamented barrel was backfilled with solution containing (in mM) 100 NaCl, 200 HEPES, 100 NaOH in H⁺ electrodes, and 150 NaCl and 3 KCl in K⁺ electrodes. A short column of the H⁺ (Fluka 95291) or K⁺ sensor (Fluka 60398) was taken by suction into the tip of the silanized nonfilamented barrel. The filamented reference barrel was filled with 150 mM NaCl. The resistance of the reference and ion-selective barrels was 10–20 MΩ and 10–20 GΩ, respectively. The K⁺ electrode responses were calibrated in terms of free concentration, and they had a slope of ~57 mV per decade change. The H⁺-selective electrode had a slope of 55–58 mV/pH unit.

In some experiments, stimuli (2–5 V, 50–100 μs) were delivered with a bipolar electrode placed in s. radiatum at a distance of ~1.5–2 mm from the site of recording.

Drugs

All experiments were made in the presence of ionotropic glutamate receptor antagonists, 6-nitro-7-sulphamoylbenzoquinoxalin-2,3-dione (NBQX; 10 μM), and d-2-amino-5-phosphonopentonic acid (AP5; 40 μM). Pentobarbital sodium (PB) and picROTOXIN (PITX) were applied in some experiments at a concentration of 100 μM. Benzolamide (BA; 10 μM), a poorly permeant inhibitor of carbonic anhydrase, and ethoxyzolamide (EZA; 50 μM), a membrane-permeant inhibitor, were applied in the bath. The synaptic receptor antagonists were from Tocris Cookson. All other chemicals were from Sigma.

RESULTS

In agreement with previous studies (Aram et al. 1991; Avoli et al. 1996; Louvel et al. 1994; Perreault and Avoli 1992), application of 4-AP (50–100 μM) resulted in the appearance of SGEs that were composed of temporally coincident changes in the extracellular field potential, in the pyramidal cell membrane potential, and in [K⁺]o (Fig. 1). The field potential responses recorded in s. radiatum were usually seen as negative deflections, and the changes in the membrane potential of the pyramidal neurons were triphasic, with an initial fast hyperpolarizing component followed by a slow depolarization and, thereafter, by a slow hyperpolarization (Fig. 1A). This late GABAₐ receptor–mediated response (Michelson and Wong 1994) was not further examined in the present work.

The mean interevent interval of SGEs under control conditions, taken from all slices, was 33 ± 5 (SE) s (n = 46). At the peak of the potassium transients in s. radiatum, the increase in [K⁺]o from the baseline (close to 3 mM) was 0.7 ± 0.3 mM (n = 35 slices). In the following, we will put much emphasize the quantification of the K⁺ transients, because they are likely to reflect the gross activity of a large number of cells, thereby serving as a useful index of the level of synchronous activation of the local inhibitory network (cf. Avoli et al. 1996; Louvel et al. 1994). This probably applies also to the long-lasting depolarizations (LLDs) recorded from pyramidal neurons, which are likely to result from the GABAergic [K⁺]o shift that leads to a depolarizing shift in the reversal potential of the GABAₐ receptor–mediated current and may also have a direct depolarizing action of its own. It is therefore worth emphasizing here that although the GABAergic events in the pyramidal neurons provide a useful additional index of the time course and level of activation of the interneuronal network, they are not thought to provide direct information on the specific mechanisms that are responsible for functional coupling within the network.

In addition to the responses that have been described in earlier work (e.g., Avoli et al. 1996; Perreault and Avoli 1992).
observation by Alger and Nicoll (1982) that PB has a potent facilitatory action on depolarizing GABA_A receptor–mediated responses in pyramidal neurons.

The interval between the SGEs in PB (after 40-min wash-in) was shortened from $35 \pm 4$ s to $21 \pm 4$ s ($n = 8$ slices), and the amplitude of the depolarizing phase of the pyramidal-cell response attained values of $>10$ mV with a marked (up to 10-fold) increase in its duration to several seconds. The descending phase of this depolarization eventually masked the late GABA_B-mediated hyperpolarization (Fig. 1A). The peak $[K]_o$ shift increased to $165 \pm 45\%$ ($n = 4$ slices) and the amplitude of the alkaline pH_o shift increased to $170 \pm 30\%$ ($n = 3$ slices).

The time course of the effect of PB on the amplitude and frequency of the $[K]_o$ shift is illustrated in Fig. 2. As shown below in experiments involving exogenous stimulation (see Fig. 7), under constant experimental conditions, there is an inverse relationship between the frequency and the amplitude of the various components of the GABAergic responses; hence the potentiating action of PB on the amplitude of the $[K]_o$ transient is somewhat suppressed by the increase in frequency that takes place simultaneously.

**Effects of a CO₂/HCO₃⁻-free medium**

GABA_A receptor–mediated depolarizing postsynaptic potentials evoked in pyramidal neurons by high-frequency stimulation are inhibited in solutions nominally devoid of CO₂/HCO₃⁻ (Grover et al. 1993; Kaila et al. 1997; Staley et al. 1995). Hence, it was of interest to examine whether the spontaneous 4-AP–induced GABAergic responses show a similar dependence on bicarbonate. As is evident from Fig. 3A, in the HCO₃⁻-free HEPES-buffered solution the depolarizing transient accumulation of extracellular potassium ($[K]_o$) and an alkaline shift in extracellular pH ($pH_o$). Both ionic transients are potentiated in PB. Traces in A and B are from continuous recordings (breaks in traces 35 and 40 min, respectively).

1992), the spontaneous activity was also associated with transient alkaline shifts in pH_o (Fig. 1B), which had a range of 0.01–0.08 and a mean amplitude of 0.04 pH units ($n = 16$ slices). Both types of extracellular ionic shifts had a time-to-peak of $\sim 1.0–1.5$ s and a duration to 50% decline of $\sim 7.5$ s for the $[K]_o$ shift and 4.0 s for the alkaline shift.

**Effects of pentobarbital**

That activation of GABA_A receptors is necessary for the generation of the SGEs induced by 4-AP was evident from the complete block of the extracellular and intracellular voltage responses and of the ionic shifts following application of 100 µM PiTX (cf. Aram et al. 1991; Avoli et al. 1996; Michelson and Wong 1991, 1994). To further explore the role of GABA_A receptors we exposed the preparation to 100 µM PB, a positive allosteric modulator of GABA_A receptors (Ransom and Barker 1975; Macdonald et al. 1989). As shown in Fig. 1, application of PB resulted in an enhancement of the extracellular and intracellular voltage responses as well as of the $[K]_o$ and pH_o shifts. In the pyramidal cell response, the depolarizing component appeared to be selectively enhanced, which fits nicely with the well-known
As described before (Church 1992; Kaila et al. 1997), the input resistance of pyramidal neurons decreases in a bicarbonate-free medium, but it is obvious that this decrease, which amounted to 30–50% (n = 4 slices), cannot account for the selective decrease in the amplitude and slowing of the time course of the depolarizing phase. There was also a marked suppression of the negative-going field potential responses (Fig. 3A) and of both the [K⁺]₀ and pHᵢ shifts (Fig. 3B). All the effects of the HCO₃⁻-free solution were fully reversed following reintroduction of the CO₂/HCO₃⁻ medium.

That spontaneous activity was not completely blocked in the HEPES solution may be due to the fact that even in the absence of ambient CO₂/HCO₃⁻, metabolic production of CO₂ leads to a CO₂ tension within the slice that is roughly equal to that of a solution equilibrated 1% CO₂ (J. Voipio, P. Paalasmaa, and K. Kaila, unpublished data; cf. Voipio and Kaila 1993). It is worth noting here that the GABAₐ receptor–mediated depolarization of pyramidal neurons evoked by high-frequency stimulation is also not fully suppressed in a HEPES-buffered solution (Grover et al. 1993; Kaila et al. 1997).

**Effects of inhibitors of carbonic anhydrase**

The results in the previous section suggest that bicarbonate movements are closely linked to the generation of the SGEs. To gain further information on the role of HCO₃⁻, we examined the effects of two kinds of inhibitors of carbonic anhydrase. As described above (Church 1992; Kaila et al. 1997), the input resistance of pyramidal neurons decreases in a bicarbonate-free medium, but it is obvious that this decrease, which amounted to 30–50% (n = 4 slices), cannot account for the selective decrease in the amplitude and slowing of the time course of the depolarizing phase. There was also a marked suppression of the negative-going field potential responses (Fig. 3A) and of both the [K⁺]₀ and pHᵢ shifts (Fig. 3B). All the effects of the HCO₃⁻-free solution were fully reversed following reintroduction of the CO₂/HCO₃⁻ medium.

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**Fig. 3.** Dependence of SGEs on presence of CO₂/HCO₃⁻. A: strong attenuation of the field potential signal and LLD in the CO₂/HCO₃⁻-free medium, accompanied by drastic reduction in frequency of SGEs. * Typical pyramidal cell responses are shown on expanded timescale on right; note selective attenuation of the depolarizing phase in the absence of CO₂/HCO₃⁻. In this experiment, resting Vm was kept constant (-68 mV) by using a small depolarizing current (0.1–0.2 nA) in the N-hydroxyethylpiperezine-N'2-ethanesulfonic acid (HEPES) medium. B: SGE-linked [K⁺]₀ and pHᵢ shifts are also strongly and reversibly suppressed in HEPES medium. Traces in A and B are from continuous recordings (breaks in traces 25–30 min).

The two phases of the pyramidal-cell response in the presence and absence of CO₂/HCO₃⁻, the resting membrane potential of the neuron was held at a constant level under the two experimental conditions (Fig. 3A). It should be noted that in the absence of current injection, the resting potential of the neuron was more negative in the HEPES solution and the peak of the LLD was slightly depolarizing. This was also the case in the experiments with EZA (cf. Fig. 5B).
drase. Benzolamide is a poorly permeant inhibitor that can be used for selective inhibition of the extracellular (interstitial) carbonic anhydrase activity, whereas ethoxyzolamide is a permeant blocker that will inhibit both interstitial and intracellular isoforms (Kaila et al. 1992; Maren 1977; Pasternack et al. 1993).

Exposure of the slice to 10 μM benzolamide had no clear effect on the amplitude of the spontaneous field or intracellular potentials (not illustrated). Neither were the [K+]o shifts affected, as shown in Fig. 5A. However, in sharp contrast to this lack of effect, the alkaline pHo transients were fully inhibited by benzolamide, and in fact turned into acid shifts. This is a significant observation because a sensitivity of the above kind to inhibition by blockers of extracellular carbonic anhydrase activity is a hallmark of GABA<sub>A</sub> receptor–mediated transmission (Kaila et al. 1992; Taira et al. 1995b; Voipio et al. 1995).

Application of 50 μM ethoxyzolamide had effects reminiscent to those of the HEPES medium; there was a decline in the amplitude of both the depolarizing phase of the pyramidal cell response (Fig. 5B) and of the [K+]o transient (Fig. 5C). These two effects developed in parallel, and within ~50 min the [K+]o shift was reduced to 50 ± 10% from its control value (n = 4 slices; Fig. 6). The negative-going field potential response also declined with a similar time course and, as could be expected, the pHo transient was affected in a manner similar to that seen with benzolamide (not illustrated). The frequency declined to ~80% from control in EZA.

Figure 6 demonstrates the time course of the action of EZA on the amplitude and frequency of the [K+]o shifts. In comparison with the effects of HEPES, those of EZA are smaller and develop much more slowly, which can be explained by the fact that a near-complete inhibition of carbonic anhydrase is usually needed to have significant effects on acid-base turnover within various tissue (Maren 1967). Another difference between the actions of EZA and HEPES medium is that EZA seems to have a preferential inhibitory action on the amplitude of the [K+]o shifts whereas the withdrawal of bicarbonate has its major effect on the frequency of the SGEs. At the present, we can offer no explanation for this difference.

### GABAergic events paced by electrical stimuli

A problem inherent in a strict quantification of the SGEs in terms of the amplitudes of its various components is that under conditions where, for instance, a suppression of both amplitude and frequency takes place, the lower frequency will facilitate restoration of the relevant ionic gradients necessary for the generation of the events. This situation was clearly evident in the experiments with the HCO<sub>3</sub>-free solution, where a marked fall in the frequency to 20% often took place, but the GABAergic events were associated with [K+]o transients that were still ~70% of the control level. Direct evidence for the role of the interevent interval in the reestablishment of the ionic gradients was provided by experiments where propagating GABAergic events were triggered by using weak electrical stimuli applied at a distance of 1.5–2 mm from the recording site and at a rate that exceeded the inherent pace-making activity under any given condition (cf. Aram et al. 1991; Perreault and Avoli 1992).

Experiments of this type demonstrated that increasing the rate of pacing under various conditions led to a prompt decrease in the depolarizing phase of the pyramidal cell response. As illustrated in Fig. 7, under control conditions a decrease of the interevent interval from 30 to 25 and 20 s produced a decline in the amplitude of the depolarization of about 30 and 60%, respectively. Complete refractoriness of the propagating GABAergic events to exogenous stimulation was typically seen at intervals of ~15 s.

In the HEPES medium, propagating GABAergic events could be evoked at interstimulus intervals of ~2–3 min. Qualitatively similar effects to those seen in the absence of bicarbonate were observed following the application of EZA. In view of the wide time window (up to several minutes) of the partial and complete refractoriness seen in the HEPES medium and in the presence of EZA, it is unlikely that intrinsic mechanisms that control the plasticity of GABAergic transmission (see Discussion) played a significant role here.

However, experiments with PB clearly showed that up-modulation of factors that control the intrinsic efficacy of GABA<sub>A</sub> receptor–mediated transmission will lead to an enhancement
anion of a weak acid. Because the conjugate acid (carbonic acid) and in particular, the anhydride (CO₂), are highly permeant across cell membranes, the distribution of HCO₃⁻ is governed by the plasmalemmal pH gradient (Kaila 1994; Kaila and Voipio 1990). Because pHᵢ in all nucleated animal cells, including neurons, is maintained at a level more alkaline than a thermodynamically passive distribution for HCO₃⁻ (or H⁺) would predict (Roos and Boron 1981; Thomas 1984), activation of HCO₃⁻-permeable channels leads to a net efflux of bicarbonate and, consequently, HCO₃⁻-mediated currents evoked in a resting cell are inwardly directed (Kaila 1994). If these two basic features of HCO₃⁻ (being a conjugate anion of a weak acid and permeant across GABA_A receptors) are critical for its action to promote SGEs, then any other anion with these two properties would be expected to mimic the effects of bicarbonate.

There is another anion, formate, that shares the two key properties of HCO₃⁻. Formate has a relative permeability versus Cl⁻ that is slightly higher than that of bicarbonate (Bormann et al. 1987; Kaila 1994; Mason et al. 1990). Hence, it was exciting to find that exposure to the formate-containing solution reestablished SGEs that were strongly suppressed or abolished by withdrawal of CO₂/HCO₃⁻ (Fig. 8). The amplitude and frequency of the [K⁺]₀ transients of both the frequency and amplitude of the various components of the SGEs (cf. Figs. 1 and 2). Figure 7 shows representative examples of the relationship between the amplitude of the pyramidal-cell depolarization and the interstimulus interval under various experimental conditions (exposure to PB, HCO₃⁻-free medium, and ethoxyzolamide).

**Formate, a GABA_A receptor-permeant weak-acid anion, is capable of promoting SGEs**

The peculiar properties of HCO₃⁻ as a current carrier across GABA_A receptor channels is based on its being an

![Graph of BA/EZA](attachment:image1.png)

**Fig. 6.** Time course of effects of carbonic anhydrase (CA) inhibitors on amplitude and frequency of spontaneous [K⁺]₀ transients. Although BA has no clear effects, EZA inhibits mainly the amplitude of SGE-linked [K⁺]₀ shift with only a small effect on the rate of their occurrence. Data quantified as described in Fig. 2 (n = 4 ± 4 slices).

![Graph of LLD amplitude at steady state](attachment:image2.png)

**Fig. 7.** Amplitude of LLD associated with propagating GABAergic events is dependent on the interevent interval. LLDs with steady-state amplitude were induced by applying weak single-pulse stimuli at a constant frequency at a distance of 1.5–2 mm from recording site. Under all experimental conditions, LLD amplitude was strongly reduced upon a decrease in the interevent interval. As expected, LLDs evoked at any given frequency were suppressed in the presence of EZA and in the absence of CO₂/HCO₃⁻, whereas the opposite is true for PB. In PB, LLDs could occasionally be evoked even at intervals as brief as 10 s. In the CO₂/HCO₃⁻-free medium no LLDs were seen until after a 2–3 min refractory time. In the graph, LLDs are normalized with reference to the maximum steady-state LLD seen under control conditions (7–10 mV). Each symbol (○, ●, ▽, ▼, △, and ■) refers to a continuous experiment on a single neuron/slice.

![Graph of LLD amplitude](attachment:image3.png)

**Fig. 8.** GABA_A receptor-permeant weak-acid anion formate is capable of promoting SGEs. A: addition of formate (20 mM) reestablishes the spontaneous [K⁺]₀ transients that were strongly suppressed by exposure to the CO₂/HCO₃⁻-free HEPES medium. B: no SGEs were seen in GABA_A receptor-impermeant propionate (20 mM) containing HEPES-buffered solution. C: unlike the CO₂/HCO₃⁻-dependent alkaline shifts that are changed into acid transients by BA (cf. Fig. 5A), the formate-dependent alkaline shifts take place in the presence of BA. Traces in A, B, and C are from continuous recordings (breaks in traces 20–40 min).
Disc U sion

Even though the precise mechanism whereby the K⁺-channel blocker 4-AP induces rhythmic GABAergic activity remains yet to be uncovered, this in vitro model provided a valuable experimental tool in the study of various cellular and ionic mechanisms that are likely to be responsible for functional coupling within the GABAergic interneuronal network (Michelson and Wong 1991, 1994; Perreault and Avoli 1992). Spontaneous GABAergic waves of the kind examined in the present work can be induced by application of 4-AP in a variety of preparations (e.g., Aram et al. 1991; Avoli and Perreault 1987; Avoli et al. 1988; Kita et al. 1985; Lücke et al. 1995), which indicates that the underlying mechanisms are widely distributed in the brain. It is also of interest that SGEs are observed in the absence of 4-AP in neonatal preparations, where they may serve the important task of controlling axonal guidance and synapse development (Cherubini et al. 1991). There is also reason to believe that pathological manifestations of interneuronal synchronization can result in the generation of epileptiform activity (see Introduction), particularly in the juvenile brain (Avoli et al. 1996; Louvel et al. 1994; Taira et al. 1997).

To gain an insight into the mechanisms of operation of the interneuronal network, it is evident that an understanding of the cellular and biophysical basis of its workings has to be obtained. There are several studies examining the possible role of the 4-AP-induced [K⁺]ᵣ transients in the synchronization of the activity of the interneuronal network and of principal neurons (Avoli et al. 1996; Louvel et al. 1994; Lücke et al. 1995), yet little is known about the ionic mechanisms involved.

The present study was conducted to examine the role of bicarbonate in the generation of SGEs. The two questions we addressed were 1) whether the excitatory coupling of GABAergic interneurons as reflected by the generation and spread of SGEs depends on the availability of HCO₃⁻ and 2) if so, is there evidence to suggest that HCO₃⁻ is needed as a carrier of depolarizing GABA A receptor–gated current within the network. The data obtained suggest a ‘‘yes’’ in response to both questions.

Factors affecting the frequency and amplitude of spontaneous GABAergic responses

It is likely that both the rate of spontaneous pacing as well as the amplitudes of the voltage and ionic responses are affected by the following two distinct kinds of factors: 1) by changes in the intrinsic efficacy (i.e., plasticity) of the GABAergic coupling within the interneuronal network (as evidenced by the actions of PB) and 2) by factors that control the dissipation and recovery of the transmembrane...
MECHANISMS OF PROPAGATING GABAERGIC EVENTS

Role of bicarbonate in the generation of the GABAergic events

That bicarbonate is essential for the generation and spread of the SGEs is evident from the results discussed in the previous section. However, the mere inhibitory effect of the HCO₃⁻-free solution does not, in itself, provide further knowledge about the exact mode of action of this anion. In this respect, the block of the alkaline pHₐ shift by benzolamide is an important observation because it lends support to the idea that bicarbonate does, indeed, act as a carrier of absence of HCO₃⁻. Of course, the present data do not exclude the possibility that the experimental manipulations of the availability of HCO₃⁻ have other kinds of actions mediated by changes in synaptic plasticity or in GABA_A receptor function. However, the finding of extremely long periods of refractoriness in the HEPES-medium and in the presence of EZA strongly supports the view that, under these conditions, the availability of bicarbonate was the main rate-limiting factor for the generation of the SGEs.

ionic gradients responsible for driving the SGEs (as evidenced by the actions of the HEPES medium and of EZA). In addition to this, mechanisms affecting interneuronal excitability (see Whittington et al. 1995) are probably also involved in shaping SGEs but, as will be shown in the following paragraphs, most of our results can be qualitatively explained by focusing on mechanisms of type I or 2.

All the experimental manipulations described presently (PB, HCO₃⁻-free medium, and ethoxyzolamide) that had an effect on the frequency of the SGEs also consistently affected the amplitude of the [K⁺]₀ transients, the depolarizing phase of the pyramidal-cell response, and the negative-going field-potential response. As an example of type I factor defined in the previous paragraph, PB had a facilitatory effect on the frequency of SGEs and also significantly decreased the period of refractoriness of the network activity in response to exogenous stimulation.

A different kind of situation was encountered in experiments with the HCO₃⁻-free solution and with intracellular inhibition of carbonic anhydrase, the “excitatory” aspects of the SGEs (the [K⁺]₀ transient and the depolarization of pyramidal neurons) were strongly suppressed. Of course, the present data do not exclude that the experimental manipulations of the availability of HCO₃⁻ have other kinds of actions mediated by changes in synaptic plasticity or in GABA_A receptor function. However, the finding of extremely long periods of refractoriness in the HEPES-medium and in the presence of EZA strongly supports the view that, under these conditions, the availability of bicarbonate was the main rate-limiting factor for the generation of the SGEs.

Formate mimics HCO₃⁻ in the generation of SGEs

When considering the present experiments with formate and propionate, it is relevant to note that the basic physical and chemical mechanisms that govern the transmembrane distribution of HCO₃⁻ are also at work with any other weak-acid anion (A⁻) which has a conjugate acid (the neutral or protonated species, HA) that is readily permeable across cell membranes. Hence, as schematically illustrated in Fig. 9, one might expect that the actions of HCO₃⁻ should be mimicked by other weak-acid anions that show a significant permeability across GABA_A receptors.

The weak-acid nature of the protonated form HA ensures that it is present at sufficiently high concentrations, and the high permeability allows for its rapid transmembrane equilibration. Together, these two factors lead to a situation where, at equilibrium, the intracellular and extracellular concentrations of HA are identical. Because the intracellular pH is actively regulated to a level higher than what is predicted on the basis of a passive thermodynamic distribution of H⁺, an accumulation of internal A⁻ takes place (when compared with the corresponding Nernstian anionic distribution). Hence, as illustrated in Fig. 9A, the equilibrium potential of A⁻ (E_A) will be more positive than the resting membrane potential (E_m) resulting in an inwardly directed current mediated by the efflux of A⁻ on the opening of channels with a significant permeability to A⁻ (for further discussion on factors controlling anion distributions, see Kaila 1994; Kaila and Voipio 1990).

The preceding predictions were confirmed in a very rewarding manner by experiments showing that formate, a weak-acid anion with a somewhat higher relative permeability versus Cl⁻ (0.5) than that of HCO₃⁻ (0.2–0.3) (Bormann et al. 1987; Kaila 1994), was able to support SGEs in the absence of HCO₃⁻ (cf. Fig. 9C). Because no SGEs were observed in the presence of an identical concentration of propionate (Fig. 9D), which has a negligible relative permeability across GABA_A receptors (<0.02) (see Bormann et al. 1987), the bicarbonate-like effects of formate were most likely due to its ability to carry a GABA-gated inward current.

The preceding conclusion gained strong support from the observation that the SGEs evoked in the presence of either HCO₃⁻ or formate were associated with extracellular alkaline transients. In both cases, the alkaline transients were evidently triggered by a transmembrane HA/A⁻ shuttle driven by the channel-mediated net efflux of A⁻. Previous work has shown that HCO₃⁻-dependent GABAergic alkaline transients are blocked upon inhibition of extracellular carbonic anhydrase (e.g., Kaila et al. 1990, 1992; Voipio et al. 1995) and this was also the case with the alkaline shifts associated with bicarbonate-dependent SGEs in the present work (Fig. 9B). In contrast to this, and as expected on the basis of the cellular structures involved with the activity of the inhibitory network. The results of the present work suggest that this assumption is correct. However, it is too early to conclude that the critical carbonic anhydrase activity is located within the inhibitory interneurons. The possibility remains that it is to be found, for instance, within glial cells that are structurally associated with the interneuronal network.
protonation equilibrium between formate and formic acid, the alkaline pH shifts linked with formate-dependent SGEs were not inhibited following inhibition of extracellular carbonic anhydride.

Conclusions

In the light of the present data, it appears that excitatory GABAergic coupling within the interneuronal network (e.g., Michelson and Wong 1994; Perreault and Avoli 1992) is dependent on the availability of bicarbonate. This conclusion has a number of interesting consequences that provide for yet another link between the regulation of brain cell pH and neuronal functions (see Chesler and Kaila 1992; Kaila 1994). In particular, the present results with EZA suggest that the anticonvulsant actions of inhibitors of carbonic anhydrase (Anderson et al. 1989; Oles et al. 1989) may be at least partly due to their actions on GABAergic interneuronal networks.

The present results do not only shed light on the role of bicarbonate in the functions of the interneuronal network but they also suggest a novel basis for the mechanisms underlying long-lasting GABAergic depolarizations in pyramidal neurons. Taken together with results presented elsewhere (Kaila et al. 1997), the observations made presently suggest that activation of the local interneuronal network plays a crucial role in situations where tonic activation of a strictly monosynaptic input into the principal neuron was taken as the synaptic basis of GABAergic excitation (Grover et al. 1993; Staley et al. 1995). We have recently shown that network-driven [K+]o shifts act as an important nonsynaptic mechanism in the generation of long-lasting GABAergic depolarizations in pyramidal neurons (Kaila et al. 1997).

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