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Levetiracetam inhibits Na^+-dependent Cl^-/HCO_3^- exchange of adult hippocampal CA3 neurons from guinea-pigs

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1 The novel anticonvulsant levetiracetam (LEV) was tested for effects on bioelectric activity and intracellular pH (pHi) regulation of hippocampal CA3 neurons from adult guinea-pigs.

2 In 4-aminopyridine-treated slices, LEV (10–100 μM) reduced the frequency of action potentials and epileptiform bursts of CA3 neurons by 30–55%, while the shape of these potentials remained largely unchanged. Suppressive effects were reversed by an increase of pHi with trimethylamine (TMA).

3 Using BCECF-AM-loaded slices, we found that LEV (10–50 μM) reversibly lowered neuronal steady-state pHi by 0.19 ± 0.07 pH units in the presence of extracellular CO_2/HCO_3^- buffer. In the nominal absence of extracellular CO_2/HCO_3^- or in Na^+-free CO_2/HCO_3^--buffered solution, LEV had no effect on steady-state pHi.

4 Recovery of pHi subsequent to ammonium prepulses remained unchanged in the absence of CO_2/HCO_3^- buffer, but was significantly reduced by LEV in the presence of CO_2/HCO_3^- buffer. These findings show that LEV inhibits HCO_3^--dependent acid extrusion, but has no effect on Na^+/H^+ exchange.

5 LEV did not affect Na^+-independent Cl^-/HCO_3^- exchange because intracellular alkalosis upon withdrawal of extracellular Cl^- remained unchanged.

6 These data show that LEV at clinically relevant concentrations inhibits Na^+-dependent Cl^-/HCO_3^- exchange, lowers neuronal pHi, and thereby may contribute to its anticonvulsive activity.
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Keywords: Levetiracetam; Na^+-dependent Cl^-/HCO_3^- exchange; intracellular pH; epileptiform activity

Abbreviations: LEV, levetiracetam; TMA, trimethylamine; 4-AP, 4-aminopyridine

Introduction

The novel anticonvulsant levetiracetam (LEV) shows a good clinical efficacy in the treatment of focal and secondarily generalized epilepsies (Ben-Menachem & Falter, 2000; Cereghino *et al*., 2000; Shorvon *et al*., 2000). However, the antiepileptic mechanisms of LEV are largely unclear, although many attempts have been made to uncover the effects of LEV on inhibitory or excitatory pathways. For instance, $GABA_{ergic}$ currents were not influenced by LEV in a paired-pulse study of field potentials (Margineanu & Klitgaard, 2003). Nevertheless, LEV inhibited the blockade of bicuculline on $GABA_{ergic}$ currents and, by this, decreased bicuculline-induced neuronal hyperexcitability (Margineanu & Wulfert, 1995). In line with this, Rigo *et al*. (2002) found that LEV could oppose the effects of negative modulators of $GABA_A$-mediated currents. However, the anticonvulsive mechanism of LEV underlying the modulation of $GABA_{ergic}$ currents (Poulain & Margineanu, 2002) remains obscure.

LEV also failed to block voltage-gated Na^+ and low-voltage-activated Ca^{2+} currents as well as NMDA receptors (Zona *et al*., 2001; Gorji *et al*., 2002). Nevertheless, there was an incomplete inhibition of voltage-operated K^+ currents (Madeja *et al*., 2003) and N-type Ca^{2+} channels of hippocampal CA1 neurons whose high-voltage-activated calcium currents were reduced by about 20% (Lukyanetz *et al*., 2002). Although the latter channels were overexpressed after kindling (Bernstein *et al*., 1999), their contribution to the genesis of epileptic potentials is still unclear. Recently, it was shown that kindling-induced alterations in gene expression in temporal lobe of rats were modified by LEV (Gu *et al*., 2004). Taking these studies together, LEV seems to affect synchronization of epileptiform events rather than affecting synaptic transmission (Birnstiel *et al*., 1997).

Earlier studies have shown that an acidification of neuronal tissue reduces neuronal excitability, whereas alkalinization increases it (Aram & Lodge, 1987; Chesler, 1990). Especially, changes of the intracellular pH (pHi) were successfully used to reduce epileptiform activity in epileptic model systems (Bonnet *et al*., 1998; 2000a, b, c; Bonnet & Wiemann, 1999). Based on these findings, we looked for alternative modes of action of LEV and focused on the effects of neuronal pHi as a possible factor to reduce bioelectric activity.

We used 4-aminopyridine (4-AP) treatment to investigate the effects of LEV on spontaneous and epileptiform activity of hippocampal CA3 neurons. As with other epileptic model

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systems, hippocampal slices treated with 4-AP respond to a lowering of neuronal pHi by decreased bioelectric activity (Bonnet *et al*., 2000a, c; Leniger *et al*., 2002). The effects of LEV on pHi were studied in parallel using BCECF-AM-loaded slices. Transients of pHi were recorded from individual neuronal somata and also from larger regions of the stratum pyramidale. The effects of LEV on pHi regulation were further tested by ammonium prepulses. This was carried out in the presence and absence of extracellular CO_2/HCO_3^-, and by withdrawal of Cl^- and Na^+ from the bath solution in order to differentiate between potential mechanisms involved in the control of pHi. Our results showed that clinically relevant concentrations of LEV affect Na^+-dependent Cl^-/HCO_3^- exchange, which is of biological significance for pHi regulation of hippocampal neurons (Baxter & Church, 1996; Brett *et al*., 2002).

Methods

Tissue preparation

Transverse hippocampal slices (200–400 μm thick) were prepared from brains of ether or isoflurane anaesthetized adult guinea-pigs (300–400 g). Slices were preincubated in a CO_2/HCO_3^--buffered solution containing (in mM): NaCl (124)), KCl (3), $CaCl_2$ (0.75), $MgSO_4$ (1.3), KH_2PO_4 (1.25), $NaHCO_3$ (26) and glucose (10) at 28°C; pH was adjusted to 7.35–7.40 by gassing with 5% CO_2, 95% O_2. After 1–2 h, slices were transferred from the preincubation bath to the recording chamber (volume 4 ml) mounted onto an inverted microscope (Zeiss ID 03), which was used for electrophysiological experiments. The recording chamber was continuously superfused (perfusion rate of 4.5 ml/min) with the CO_2/HCO_3^--buffered solution in which, however, $CaCl_2$ was elevated to 1.75 mM. Temperature was kept at 32 ± 1°C in all experiments.

Solutions and chemicals

For pHi measurements, a CO_2/HCO_3^--free solution was used in which $NaHCO_3$ was replaced by equimolar amounts of Na^+-HEPES, pH 7.4. This solution was gassed with O_2. To obtain a Cl^--free CO_2/HCO_3^--buffered solution, NaCl, KCl and $CaCl_2$ were isosmotically replaced by the respective gluconate salts and gassed with 5% CO_2, 95% O_2 (Brett *et al*., 2002). A Na^+-free CO_2/HCO_3^--buffered solution was prepared with choline-Cl (124 mM) and choline-HCO_3 (26 mM) instead of NaCl and $NaHCO_3$, respectively.

Epileptiform activity of CA3 neurons was induced by 4-AP (50 μM), which was added to the CO_2/HCO_3^--buffered solution to reach a stable state of hyperexcitation for hours, characterized by epileptiform bursts and spontaneous $GABA_{ergic}$ hyperpolarizations (Rutecki *et al*., 1987).

LEV was kindly provided by UCB S.A. (Braine-l'Alleud, Belgium) and dissolved in experimental solutions immediately before the experiment. 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS) and all other chemicals were from Sigma.

Intracellular recording and measurement of neuronal activity

Intracellular recordings were obtained from somata of CA3 neurons with sharp glass microelectrodes filled with 2 M potassium methylsulfate (150–180 MΩ) as described (Bingmann & Speckmann, 1986). The analogue bioelectric signals were converted and recorded digitally using a DABAS system operated with 12 kHz sampling rate and 10 bit resolution (Widman & Bingmann, 1996). Frequency of action potentials and epileptiform bursts were determined off-line. Therefore, a trigger level was used that was about 40 mV more positive than the resting membrane potential. By this, single action potentials and those riding on epileptiform bursts were counted. The frequency of epileptiform bursts was determined in 1 min intervals from the original tracings. To quantify the effects of LEV, action potential frequency and frequency of epileptiform bursts were normalized to the mean value obtained during a 10 min period immediately prior to drug application.

Measurement of pHi

To analyze pHi changes, hippocampal slices were loaded with 0.5–1.0 μM 2′,7-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM, Molecular Probes, Leiden, Netherlands) for 3 min in the preincubation saline. Slices were transferred onto the optical recording chamber (volume 3 ml), which was mounted on the stage of an upright microscope (Olympus Bx50Wi). Measurements of individual CA3 somata (identified by their apical dendrites) were carried out with a $\times$60 water-immersion objective (Olympus), which dipped into the fluid of the recording chamber. For optical recordings from larger regions of the stratum pyramidale of the CA3 region, a $\times$20 objective was used (Olympus). Slices were illuminated with alternating light (440 and 490 nm) provided by a 100 W halogen lamp and a computer-operated filter wheel (Sutter Instruments), which was connected to the microscope by an optical fiber. Light from both wavelengths was dimmed by appropriate neutral density filters to obtain a BCECF excitation ratio 440/490 of about 1.0 at pH 7.0. Fluorescence image pairs were captured every 20 s by an intensified CCD camera (PTI, Surbiton, Surrey, U.K.). For background capturing, slices not loaded with BCECF-AM were mounted and processed like the stained slices using the same camera and illumination settings. Background correction and image processing was performed with a CARAT system (Dr. O. Ahrens, Bargteheide, Germany). At the end of an experiment, the ratio 440/490 was calibrated by a standard curve that was obtained by the *in vitro* calibration method (Boyarsky *et al*., 1996) adapted to water-immersion optics (Bonnet *et al*., 1998; Bonnet & Wiemann, 1999). All drugs used in this study did neither contribute to the fluorescence signal nor did they influence the pH of the superfusate. Spontaneous pHi deflections (observed to be in the range of $\pm$0.05 pH units) and noise were eliminated from the curves by calculating sliding averages of three consecutive values. Changes of the pHi (averaged from a 10 min lasting period before drug application) were regarded to be drug mediated if they exceeded 0.05 pHi units, occurred upon drug application and were at least partly reversible after washout.

Experimental procedures

Electrophysiology After obtaining a stable intracellular recording, 4-AP (50 μM) was applied to increase neuronal excitability. About 20 min later, LEV was washed in for

20–45 min usually in one concentration step unless otherwise stated. TMA was washed in as an alkalizing agent after at least 20 min of constantly diminished activity due to LEV treatment.

pHi measurements After transfer to the optical recording chamber slices were superfused with CO_2/HCO_3^--buffered solution for at least 20 min to remove free dye. Loss of dye from the tissue and photobleaching of single neuronal somata (estimated by the intensity of 440 nm images) was $<0.5\%$/min, indicating that structures under investigation were in good condition. Excitation light was reduced to a minimum to enable optical recordings of up to 4 h. The optical plane was controlled throughout the experiment and focus corrections were made when shifts in the z-axis of more than 3 μm had occurred. Care was taken to apply exactly the same dose of NH_4Cl (variation less than 1%) to make pHi regulation curves comparable. The pHi recovery rate ($\Delta pH/\Delta t$) from intracellular acidification was taken as a relative measure for transmembrane acid extrusion assuming that the total intracellular buffer concentration remained largely stable.

Data analysis

All data were expressed as mean $\pm$ standard deviation (s.d.). In experiments where ammonium prepulses were used to study pHi regulation, a pHi recovery rate was defined that was calculated on the basis of a least-squares regression fit to data points after converting ratio 440/490 values to pHi values. The data points for the pHi recovery rate were collected 10 min after the acidotic peak.

The t-test for paired samples was used to compare differences in pHi recovery rates. Differences were considered significant when $P \leqslant 0.05$ (Statistical Package for the Social Sciences (SPSS) 11.0).

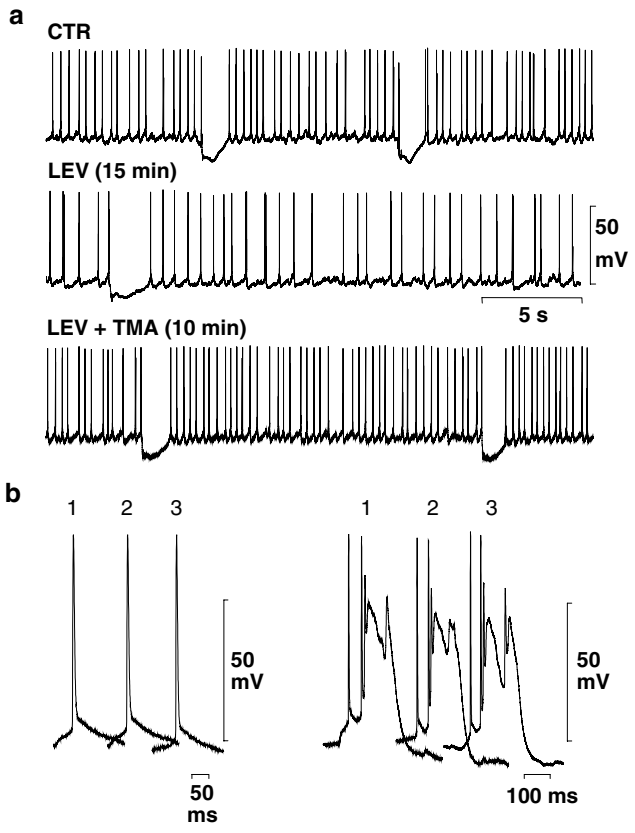

Figure 1 Effect of LEV and TMA on the frequency of action potentials, bursts and $GABA_{ergic}$ hyperpolarizations of CA3 neurons. (a) Firing pattern under control conditions (CTR) evoked by 4-AP (50 μM). LEV (50 μM) reduced bioelectric activity after 15 min. LEV together with 5 mM TMA (LEV + TMA) restored the bioelectric activity seen under control conditions after 10 min. Membrane potential of this neuron was ca. -57 mV. (b) Shapes and amplitudes of action potentials and epileptiform bursts are largely identical under control conditions (1), after 15 min LEV-treatment (2) and after 10 min treatment with LEV and TMA (3).

Results

Effects of LEV on neuronal activity

Intracellular recordings were obtained from 12 CA3 neurons (12 different slices, eight guinea-pigs). Experiments were carried out in the continuous presence of 4-AP to analyze the effects of LEV on a constant bioelectric activity consisting of epileptiform bursts, intermittent action potentials and spontaneous $GABA_{ergic}$ hyperpolarizations. Neurons selected for drug application had resting membrane potentials of at least -50 mV and action potential amplitudes exceeding 50 mV. The depth of the neurons within the slice ranged between 10 and 150 μm.

Bioelectric activity was markedly reduced by LEV in 10 of 12 neurons. In six experiments, a concentration of 50 μM LEV suppressed action potential firing and epileptiform bursting within 10–20 min (Figure 1a). With 10 or 100 μM (each $n=2$) the same type of suppression was achieved after at least 30 min of application, such that we decided to pool the data of the whole concentration range used (10–100 μM) for statistical evaluation. Thus, after 30 min action potential frequency was reduced by $39 \pm 9\%$ vs control. The frequency of epileptiform bursts decreased concomitantly by $48 \pm 7\%$. As shown in Figure 1b, the shape and amplitude of action potentials and epileptiform bursts remained largely unchanged upon LEV. Membrane resistance and resting membrane potential were unaltered such that the inhibitory effect of LEV could not be attributed to a change of these parameters. LEV-mediated inhibition was reversible upon washout within 7–29 min. Two neurons did not change their firing pattern even after more than 45 min of continuous treatment with up to 100 μM LEV.

The putative acidifying effect of LEV (50 μM) could be reversed with the membrane permeant base TMA (5 mM), such that the action potential firing and epileptiform bursting reincreased ($n=3$, Figure 1a). This suggested that alkalosis due to TMA compensated for the effect of LEV.

Effects of LEV on pHi

Steady-state pHi To examine whether LEV induced a neuronal acidification, we used BCECF-AM-loaded slices and observed the pH-dependent fluorescent signal from single neurons or expanded regions of the stratum pyramidale (Figure 2a and b). In CO_2/HCO_3^--buffered solution, steady-state pHi measured in neuronal somata was 7.05 ± 0.16 ($n=11$). Application of LEV (50 μM) lowered pHi in eight of 11 neurons (Figure 2a). After a stable steady state had been

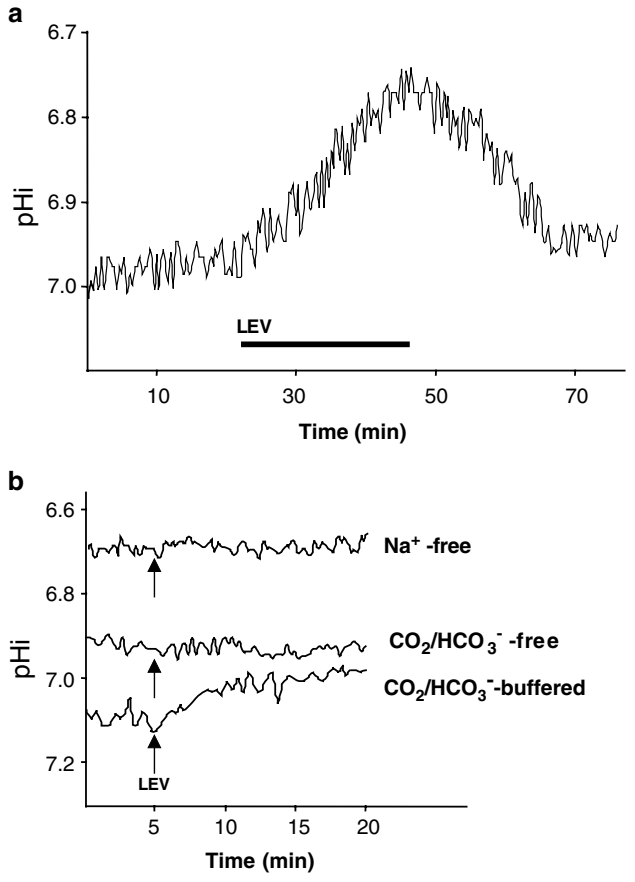

Figure 2 Effect of LEV on the neuronal steady-state pHi. (a) pHi of a BCECF-AM-loaded CA3 neuron was reversibly lowered by 50 μM LEV in CO_2/HCO_3^--buffered solution. (b) Effect of LEV (50 μM) on pHi measured in the stratum pyramidale (size of each region was approximately $200 \times 40\ \mu m^2$). In the absence of extracellular Na^+ (Na^+-free) and in the absence of extracellular CO_2/HCO_3^- (CO_2/HCO_3^--free), pHi remained unchanged while LEV decreased pHi in CO_2/HCO_3^--buffered solution. Arrows indicate the onset of LEV treatment. pHi transients were from three different slices, each representative of five similar experiments.

reached (after 5–18 min), the mean acidification during LEV amounted to 0.14 ± 0.09 pH units ($n = 11$). This value increased to 0.19 ± 0.07 pH units ($n = 8$) if the three nonresponding neurons were excluded. A further increase of LEV concentration to 100 μM was without additional effect ($n = 4$). Small shifts of the steady-state pHi were also observed upon 10 μM LEV ($n = 3$); 1 μM LEV was without any effect on pHi ($n = 4$). Thus, the concentration of LEV relevant for changes of pHi in slice experiments ranged between 10 and 50 μM. Therefore, further experiments were carried out with 50 μM LEV. Washout of LEV increased pHi within 20–32 min to values being slightly decreased compared to the control phase before LEV (Figure 2a).

In the further course of the study, we looked at pHi in larger regions of the stratum pyramidale using a low magnification objective ($\times 20$). This allowed us to maintain the focal plane if the optical signal had to be followed during profound changes of the extracellular ion composition. In CO_2/HCO_3^--buffered solution application of LEV (50 μM) decreased pHi of larger regions by 0.11 ± 0.04 pH units ($n = 5$, Figure 2b). In the nominal absence of extracellular CO_2/HCO_3^- and in the presence of HEPES (pH 7.4), the decrease of pHi upon LEV was abolished ($n = 5$, Figure 2b). We next tested whether LEV could affect steady-state pHi in Na^+-free CO_2/HCO_3^--buffered solution. Withdrawal of Na^+ from the bath solution decreased pHi in the stratum pyramidale by 0.5 ± 0.14 pH units to a new steady state. This value was, however, not further influenced by LEV (50 μM, $n = 5$, Figure 2b). These experiments show that LEV-mediated lowering of the steady-state pHi depends on inwardly directed gradients of both HCO_3^- and Na^+.

pHi regulation in CO_2/HCO_3^--free, HEPES-buffered solution In the next set of experiments, we took advantage of the ammonium prepulse technique to study the effects of LEV on pHi regulation. Slices loaded with BCECF-AM in CO_2/HCO_3^--buffered solution were equilibrated with HEPES-buffered solution for at least 20 min before the first ammonium prepulse (20 mM, 3 min) was applied. Under control conditions, an ammonium prepulse typically resulted in an alkalinization phase, followed by an acidification upon NH_4Cl washout, and a final phase of pHi recovery due to proton extrusion (Figure 3b2). To examine the effects of LEV (50 μM), the drug was washed in 15 min before the second ammonium prepulse. Similar to its lacking influence on steady-state pHi (Figure 2b), LEV did not change the ammonium prepulse-induced pHi regulation in CO_2/HCO_3^--free solution (Figure 3a). pHi recovery rate was $1.61 \pm 0.59 \times 10^{-2}$ pH units/min and $1.78 \pm 0.36 \times 10^{-2}$ pH units/min in the absence and presence of LEV, respectively ($n = 5$, $P = 0.43$). These findings rule out that LEV at this concentration inhibits HCO_3^--independent acid extrusion, such as Na^+/H^+ exchange.

pHi regulation in CO_2/HCO_3^--buffered solution In the presence of extracellular CO_2/HCO_3^- regulation of pHi was clearly affected by LEV (50 μM, pretreatment 15 min). Within the stratum pyramidale LEV significantly decreased pHi recovery from $0.85 \pm 0.10 \times 10^{-2}$ pH units/min to $0.26 \pm 0.16 \times 10^{-2}$ pH units/min ($P = 0.005$, $n = 5$, Figure 3b1). Also, in individual neuronal somata the same concentration of LEV lowered pHi recovery from $1.27 \pm 0.44 \times 10^{-2}$ pH units/min to $0.68 \pm 0.25 \times 10^{-2}$ pHi units/min ($P = 0.003$, $n = 4$). Figure 3b2 shows a typical experiment. After pHi recovery subsequent to the first ammonium prepulse (control), preincubation with LEV lowered the steady-state pHi to a value of 6.82. The second ammonium prepulse increased pHi by ca. 0.2 pH units, thus reaching the value it had adopted upon NH_4Cl application in the absence of LEV. After the acidotic peak, the pHi recovery rate was clearly slowed. Wash out of LEV then initiated the increase of pHi. It should be pointed out that LEV did not inhibit pHi recovery of two neurons, in which steady-state pHi did not fall during preincubation with LEV.

These findings point to an LEV-mediated inhibition of transmembrane HCO_3^- influx, which is used by hippocampal neurons to regulate pHi after an acid load.

Cl^-/HCO_3^- exchange In a final set of experiments, we tested whether LEV had any effect on Na^+-independent Cl^-/HCO_3^- exchange, as this electroneutral antiport is also involved in the adjustment of steady-state pHi and pHi regulation of hippocampal neurons (Brett *et al.*, 2002). A DIDS-sensitive alkalinization upon removal of extracellular

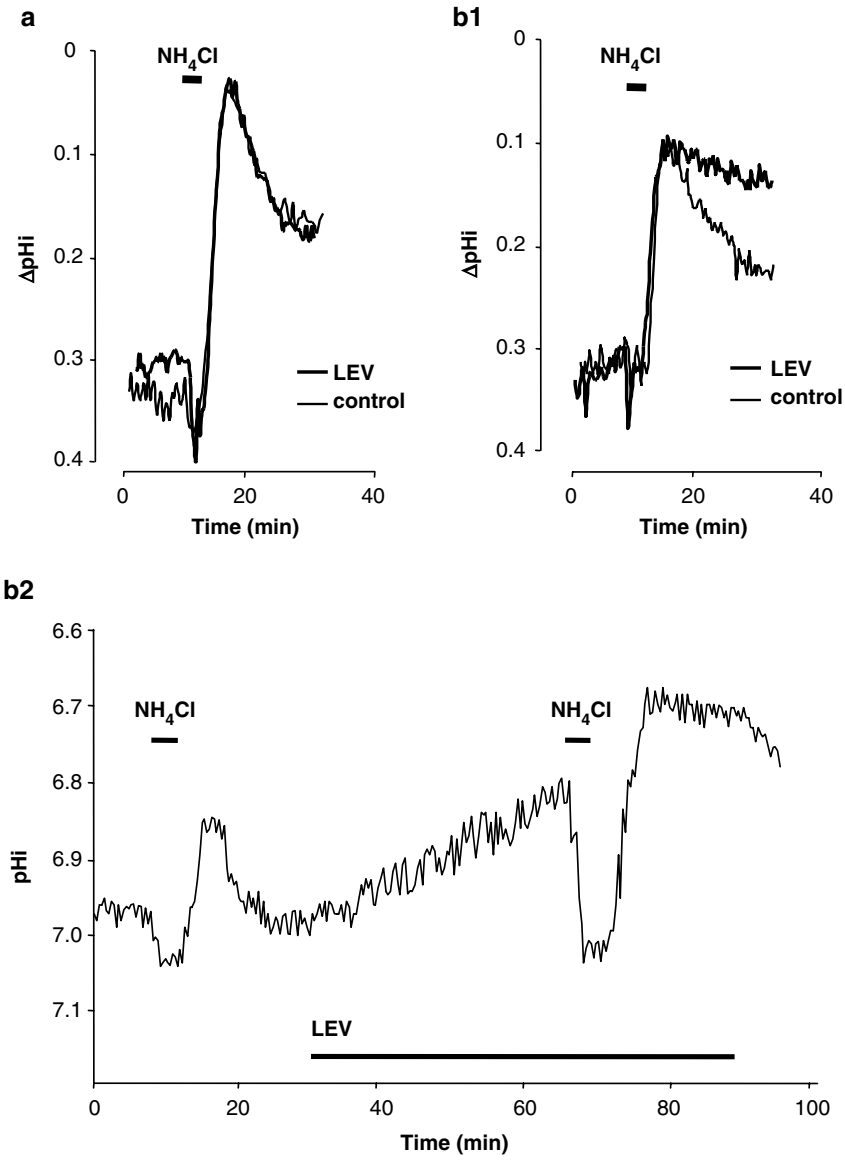

Figure 3 Effect of LEV on hippocampal pHi regulation challenged by ammonium prepulse (NH_4Cl 20 mM, 3 min) in CO_2/HCO_3^--buffered and CO_2/HCO_3^--free solution. (a, b1) pHi measurement in the stratum pyramidale (size of each region was approximately $200 \times 40\ \mu m^2$). (a) In CO_2/HCO_3^--free, HEPES-buffered solution, pHi regulation after pretreatment with LEV for 15 min (bold line) was not different from control ($n = 5$). Starting pHi of the curves shown in (a) were 6.93 (control) and 6.90 (LEV), respectively. (b1) In CO_2/HCO_3^--buffered solution, LEV (bold line) significantly reduced the pHi recovery rate from the acid shift ($P = 0.005$, $n = 5$). Curves starting at pHi of 7.02 (control) and 6.94 (LEV) were superimposed for clarity reasons. (b2) Effect of LEV (50 μM) on pHi regulation of a CA3 neuron bathed in CO_2/HCO_3^--buffered solution. LEV significantly lowered the pHi recovery rate. Trace is representative of four similar experiments.

Cl^- is usually believed to be indicative of the influx of HCO_3^- in exchange for Cl^- (Raley-Susman *et al*., 1993). As shown in Figure 4, LEV (50 μM, preincubation 15 min, $n = 5$) had no effect on this type of alkalinization, whereas DIDS (500 μM, pretreatment 15 min, $n = 3$) completely abolished it. We therefore conclude that LEV at a concentration of 50 μM does not inhibit the Na^+-independent Cl^-/HCO_3^- exchange.

Discussion

The main finding of this study was that LEV, at clinically relevant concentrations, induced the acidification of hippocampal neurons of adult guinea-pigs, an action most likely to be due to an inhibition of the Na^+-dependent Cl^-/HCO_3^- exchange. Electrophysiological experiments carried out in parallel showed that LEV decreased the frequency of action potentials and epileptiform bursting of CA3 neurons. This effect was switched off in the presence of LEV by a compensating intracellular alkalosis due to TMA treatment. Based on our former pHi studies on epileptic model systems (Bonnet *et al*., 1998; 2000a), the anticonvulsive effect of LEV is at least in part attributable to an inhibition of acid extrusion.

To our knowledge, this is the first report suggesting that an anticonvulsive drug inhibits Na^+-dependent HCO_3^- transport of neurons. Evidence is based on the fact that both pHi regulation and steady-state pHi were lowered by LEV in the presence of an inwardly directed gradient of HCO_3^- and Na^+,

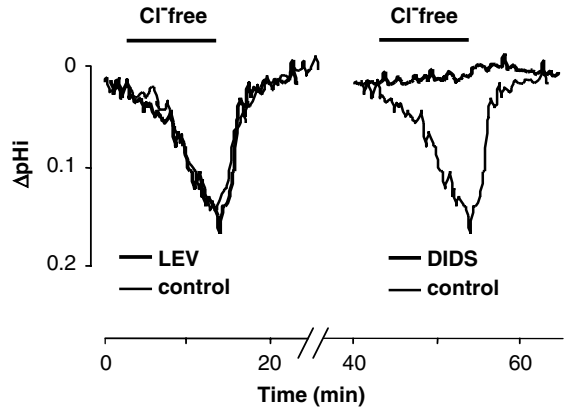

Figure 4 Effect of LEV (50 μM) on hippocampal Cl^-/HCO_3^- exchange challenged by extracellular removal of Cl^-. Successive pHi measurements in the stratum pyramidale in the presence of CO_2/HCO_3^--buffered solution. Increase in ΔpHi reflects an influx of HCO_3^-. Curves were recorded from the same part of tissue (size of the region was approximately $200 \times 40\,\mu m^2$) with a delay of 30–60 min and superimposed for clarity reasons. Left diagram: Pretreatment with LEV (15 min, bold line) did not change the alkalotic shift induced by the removal of extracellular Cl^-. Starting pHi values were 7.05 (control) and 6.91 (LEV), respectively. Right diagram: Pretreatment with DIDS (500 μM, 15 min, bold line) completely blocked intracellular alkalinization. Starting pHi values were 7.01 (control) and 6.87 (DIDS), respectively. Each trace is representative of five similar experiments.

whereas pHi remained unchanged when these ions were absent from the extracellular fluid. However, LEV left the DIDS-sensitive Na^+-independent Cl^-/HCO_3^- transport unchanged. Taking into account that classical inhibitors of Cl^-/HCO_3^- exchange such as DIDS are unable to distinguish between different types of Cl^-/HCO_3^- exchangers, LEV should be examined more closely as to its capabilities to serve as a selective inhibitor of Na^+-dependent Cl^-/HCO_3^- exchange. We cannot exclude that LEV may also influence Na^+/HCO_3^- cotransport that was originally thought to be important, especially for glial cells (Chesler, 1990). However, the abundance of this exchanger within the somata of CA3 neurons is very low (Schmitt *et al*., 2000) and its contribution to the maintenance of steady-state pHi and pHi recovery following acid load remains to be established (Pedersen *et al*., 1998).

The anticonvulsive effect on the 4-AP model consisted in a reduction of the frequency of action potentials and epileptiform bursting by 30–55%. However, LEV did not consistently modulate the shape of these bursts as has been repeatedly observed when pHi was lowered (Bonnet *et al*., 1998; 2000a; Leniger *et al*., 2002). Epileptic bursts remained also unchanged in a high K^+/low Ca^{2+} epileptic model system where LEV similarly reduced the frequency of epileptiform events (Niespodziany *et al*., 2003).

It is generally accepted that neuronal pHi decreases with enhanced bioelectric activity and that this pHi shift is due to a Ca^{2+}/H^+ ATPase activity (Trapp *et al*., 1996). Thus, the LEV-induced decline in pHi upon LEV cannot be attributed to inhibited neural activity. *Vice versa*, convincing evidence has been presented that intracellular acidification in the range of 0.1–0.2 pH units diminished spontaneous and epileptiform activity (Bonnet *et al*., 1998; 2000b; Bonnet & Wiemann, 1999; Leniger *et al*., 2002). The way acidification was achieved was of minor importance, for example, inhibition of neuronal carbonic anhydrase, withdrawal of bicarbonate from the superfusate, ammonium prepulse technique or application of propionate were all similarly effective. Also, an inhibition of neuronal acid extrusion by amiloride and DIDS led to an intracellular acidification and suppression of epileptic activity in the 4-AP epileptic model system (Bonnet *et al*., 2000a). It should also be noted that the time courses of acidification and antiepileptic action of LEV were very similar, pointing to a causal relationship of decreased pHi and firing pattern.

Na^+-dependent Cl^-/HCO_3^- exchange has been shown to be of special relevance for the adjustment of pHi in hippocampal neurons (Schwiening & Boron, 1994). In cultured neurons, initial steady-state pHi values ranged from pH 6.4 to 7.6 (Brett *et al*., 2002). Interestingly, the effect of DIDS on steady-state pHi depended on initial pHi. In acidotic neurons, pHi fell on treatment with DIDS, whereas in alkalotic neurons, such treatment increased pHi. This finding is most likely to be explained by the effects on both Na^+-dependent and Na^+-independent, Cl^-/HCO_3^- exchangers, their individual pHi responses and, most importantly, their phosphorylation states (Brett *et al*., 2002). However, if neuronal pHi is in the range of 7.0–7.1, as measured in the human brain (Garcia *et al*., 1994; Chu *et al*., 1996), inhibition of active Na^+-dependent Cl^-/HCO_3^- exchange is most likely to lower the influx of HCO_3^- and decrease neuronal pHi. Changes in pHi upon LEV may also occur in dendritic regions whose pHi regulation involves Na^+-dependent Cl^-/HCO_3^- exchange. In this respect, pHi shifts induced by depolarization are three-fold larger in dendrites than in the soma in CO_2/HCO_3^--buffered solution (Willoughby & Schwiening, 2002). This underlines the relevance of pHi in the processes of synaptic function.

Using physicochemical methods to lower pHi (Bonnet *et al*., 1998; Bonnet & Wiemann, 1999), we previously found that virtually all CA3 neurons tested lowered their firing frequency. However, in this investigation, three of 11 neurons did not respond to LEV with a change of bioelectric activity, which may be attributed to compensatory acid extrusion such as Na^+/H^+ exchange (Raley-Susman *et al*., 1991; Baxter & Church, 1996). In addition, an increased initial pHi may prohibit the activated state of Na^+-dependent Cl^-/HCO_3^- exchange (Brett *et al*., 2002). Therefore, we suggest that those neurons, which did not change firing behavior upon LEV, lacked the appropriate change of pHi.

The effective concentration of LEV found in this study (10–50 μM) was comparatively low. Previously published effects of LEV on K^+ channels were achieved with higher concentrations (100–500 μM). In spite of such high concentration, inhibition of K^+ channels was limited to less than 20% (Madeja *et al*., 2003). Also, high-voltage-activated Ca^{2+} channels were moderately but irreversibly inhibited (−18%) by 100 μM LEV. Among these, N-type Ca^{2+} channel currents were the most sensitive and reduced by ca. 15% (calculated for 50 μM LEV, according to Lukyanetz *et al*., 2002). These minor changes may correspond to the obvious lack of changes observed here for 4-AP-induced epileptiform potentials. On the other hand, some channels are inhibited by intracellular acidosis. Among these are hippocampal KCNQ2/3 potassium channels (Prole *et al*., 2003) and N-type calcium channels (Kiss & Korn, 1999). Thus, effects on distinct currents may be secondary to low pHi. This could also explain why alkalosis

following TMA (Xu & Spitzer, 1994) restored the original bioelectric pattern and counteracted effects of LEV (Figure 1).

In conclusion, LEV acidifies hippocampal neurons in a slice preparation due to an impaired Na^+-dependent Cl^-/HCO_3^- exchange. As this intracellular acidification is sufficient to inhibit spontaneous and epileptiform activity, we suggest that the decrease of pHi by LEV contributes to its anticonvulsive property.

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