

Proconvulsive effect of hydrochlorothiazide in an *in vitro* rat seizure model

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ARTICLE INFO	ABSTRACT
<p>Article type: Original article</p> <hr/> <p>Article history: Received: Dec 19, 2013 Accepted: Jul 14, 2014</p> <hr/> <p>Keywords: Blood-brain-barrier Diuretics Epilepsy Hippocampal slices</p>	<p>Objective(s): Protective effects of diuretics, particularly of hydrochlorothiazide (HCT), for the development of epilepsy have been described <i>in vivo</i>. However, its mechanism of action is unknown.</p> <p>Materials and Methods: Extracellular field potentials were recorded from the CA1- and CA3-subfields of the hippocampus of rats. Epileptiform discharges were induced by omission of Mg²⁺ from the artificial cerebrospinal fluid (ACSF). HCT was added to the ACSF at a concentration of 2 mmol/l, 0.2 mmol/l or 0.02 mmol/l. Frequency, amplitude and duration of the epileptiform discharges were evaluated. Long-term potentiation (LTP) was induced with and without the presence of HCT (n=6; 2 mmol/l). In addition, rats were injected with HCT (n=4) or saline (n=2), and the brain tissue was analyzed using HPLC.</p> <p>Results: Application of 0.02, 0.2, and 2 mmol/l HCT accelerated the frequency of discharges by 50%, 91%, and 100%, respectively. The amplitude of burst discharges also increased by 9%, 54%, and 300%, and the duration of epileptiform discharges increased by 10%, 30% and 120%. All parameters returned close to the basal levels after 60min washout of the substance. HCT increased the electrical evoked potentials but did not affect the LTP in hippocampal tissues. There was no evidence of HCT in the rat brain after intraperitoneal injection.</p> <p>Conclusion: Exposure of hippocampal slices to HCT enhanced epileptiform activity in a dose-dependent manner. In addition, HCT does not seem to cross the blood brain barrier in rats. Thus, the anticonvulsive effect of HCT most likely is not through direct neuronal effect.</p>

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Introduction

Approximately 20 to 30% of the patients with epilepsy, ultimately develop refractory seizures that do not respond adequately to pharmacological treatment (1). These patients have only a small chance of obtaining seizure control with one of the established anticonvulsant drugs. Therefore, anticonvulsant drugs with different and new mechanisms of action are needed. One of the groups of drugs that have come into focus recently is the group of diuretics (2). Although the first reports of anticonvulsant effects of the loop diuretic furosemide date back nearly thirty years (3, 4), it was discovered only a decade ago that furosemide in high concentrations shows anticonvulsant properties *in vitro* (5). In the following, anticonvulsant effects of diuretics, particularly of furosemide, were shown using a variety of *in vitro* models of epilepsy (5-8). At the same time, there was epidemiological evidence that diuretic use could protect from the risk of new-onset seizures in a community-based sample of elderly patients (9).

Most investigators have focused on furosemide, most likely because of its high diuretic potency and the availability of solutions for intravenous or intraperitoneal injections. This is in contrast to the fact that in the population-based study as well as in the maximum electroshock model, the protective effect of thiazide diuretics was stronger than of furosemide (9). In addition, it has been shown that HCT as well as other benzothiazide derivatives potentiate the effect of low-dose phenytoin on the seizure threshold in the maximum electroshock model. The same study also showed that there is a tendency to elevate the seizure threshold by HCT alone (10).

The mechanisms of anticonvulsant effect of HCT remain to be elucidated. Whereas, there is increasing evidence that furosemide has direct effect on neurons and glia via the electroneutral Na⁺-K⁺-2Cl⁻-co-transporters (NKCC) that modulate intracellular chloride concentration and cell volume (11, 12). Thiazides do not have influence on the NKCC or the

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electroneutral K⁺-Cl⁻ - cotransporters (KCC), either. However, it inhibits the carbonic anhydrase (13, 14). Carbonic anhydrase (CA) regulates the diffusion of HCO₃⁻ and protons across the cell membrane (2, 15) and its inhibition increases the hyperpolarizing effect of long-lasting GABA-A currents (16). Thus, the most favoured hypothesis is that the epidemiological effect of seizure protection by HCT intake may at least partially be an effect of CA inhibition (2).

No *in vitro* experiments investigating the anticonvulsant properties and mechanisms of thiazide diuretics in epilepsy models have been published so far. The aim of the present study was to evaluate whether hydrochlorothiazide (HCT), probably the most common used thiazide diuretic, plays anticonvulsant role in brain tissues *in vitro*. Furthermore, we investigated if HCT could pass blood-brain-barrier (BBB) to clear if its effect on CNS is a neuronal modulatory action or a systemic effect.

Materials and Methods

Preparation of the slices and solutions

The experiments were performed on rat hippocampal slices. They have been performed according to national guidelines for animal research. The principles outlined in the Basel declaration have been considered when planning the experiment. After introduction of deep pentobarbital anesthesia, the animal was decapitated and the brain was removed. The hippocampi were dissected and cut into slices of 500 µm thickness. Then the slices were preincubated in artificial cerebrospinal fluid (ACSF) at 28°C for 1 hr under continuous equilibration with 5% CO₂. The preincubation ACSF contained (in mmol/l): NaCl 124, KCl₄, CaCl₂ 1.0, NaH₂PO₄ 1.24, MgSO₄ 1.3, NaHCO₃ 26, and Glucose 10. After preincubation, CaCl₂ was elevated to 2.0 mmol/l and the slices were transferred into an interphase-type recording chamber. In the experimental chamber, the temperature was increased to 32°C. During the experiments, the pH, the bath temperature and the flow rate (1.5 to 2 ml/min) remained constant. For the preparation of the zero-magnesium solution, we omitted MgSO₄.

Hydrochlorothiazide (HCT) solution was prepared by adding dimethylsulfoxide (0.5 ml per mmol/l of the crystalline HCT substance) to achieve water solubility. Crystalline HCT was purchased by Sigma-Aldrich Germany, Munich, Germany.

Electrophysiological recordings

Extracellular field potential recordings were recorded with glass microelectrodes (Resistance 2-5 MΩ) in the stratum radiatum of the CA1 and CA3 hippocampal subregions connected with DC amplifiers. Extracellular recordings were obtained using a custom made differential amplifier (with band-pass filters at 0.5–10 kHz, sampling rate 0.3–100 Hz). The reference electrode and the connection to the

microelectrode were symmetrical Ag-Ag-KCl bridges. Field potentials were recorded by an ink-writer (capable to record between ± 10 V) as well as by a digital oscilloscope. Traces were digitized by Nicolet 460 digitizing oscilloscope (Nicolet Instruments) and analyzed with accompanying software.

Evoked field potentials and long-term potentiation (LTP)

A continuous single pulse of electrical stimulation was applied through a bipolar platinum electrode attached to the Schaffer collaterals of hippocampal slices. Evoked field excitatory post-synaptic potentials (fEPSP) were recorded in hippocampal CA1 region (stratum radiatum). The fEPSP was elicited by adjusting the intensity of stimulation to ~50% of the maximum response (0.1-0.5 mA). The amplitude of fEPSP 1 m sec after the onset was measured for data analysis. In LTP experiments, the slice was sequentially stimulated once every min. Tetanic stimulation (100 Hz, for 1 sec, at baseline stimulation intensity) was operationally defined as the mean change in fEPSP amplitude for five stimuli given 30 min after tetanic stimulation compared with the mean fEPSP to five test pulses given immediately before the stimulation. Thus % potentiation = [(posttetanus amplitude/baseline amplitude) - 1] × 100. Tetanic stimulation was applied 60 min after application of HCT (2 mmol/l).

Experimental protocol

The protocol for the zero-magnesium model experiments consisted of four periods: 1) Superfusion with ACSF for 30 min to test for spontaneously occurring epileptiform field potentials (EFP; control period). Slices with spontaneously occurring EFP were excluded from further analysis. 2) Superfusion with zero-magnesium ACSF until there was a stable EFP frequency and amplitude for at least 20 min (baseline period). 3) Addition of HCT (2 mmol/l, n=5; 0.2 mmol/l, n=5; 0.02 mmol/l, n=5) to the magnesium-depleted ACSF for 60 min (HCT period). 4) Superfusion with zero-magnesium ACSF for 60 min (wash-out period).

EFP repetition rate was measured by the number of events per min. The EFP amplitude was measured peak-to-peak and EFP duration was determined as the width at half-maximal amplitude. Owing to the small group sizes that make normal distribution and variance homogeneity unlikely, we chose median and 25/75 percentiles for cohort description. The data were processed and analyzed using commercially available software (SPSS Version 12.1, Chicago, Ill., USA). Interval- and ordinal-scaled data were compared using the Wilcoxon test, and nominal-scaled data with the chi-square test or Fisher's exact test (2x2 tables). Significance level was set at *P*<0.05 for two-tailed tests.

Brain tissue of 6 rats was used for HPLC analysis. Four animals were injected intraperitoneally (IP) with HCT (2 mmol/l, 5 ml), two animals only received saline. Two hr later, the animals were sacrificed with high doses of isoflurane followed by decapitation. The brain tissue was stored at -20° C for 12 to 48 hr until HPLC analysis.

HPLC was performed using a method developed by Zendelovska *et al* (17) for determination of HCT in human plasma. The system included a LiChrospher 100 RP8 column (125x5, 5 μ) with the mobile phase consisting of 0.025 M phosphate buffer (adjusted to a pH of 5 with triethylamine) and acetonitrile at a relation of 85/15, a pump, an UV-detector with variable wavelength and interface and dedicated software (LaChrom series, Merck-Hitachi, Darmstadt) running on a personal computer with Intel pentium III processor. The brain homogenate was centrifuged at 13000 rotations/min. The clear supernatant was transferred into an Eppendorf cap, and 40 μl were injected into the HPLC system. The flow rate was 1ml/min, and the detection wave length was 230 nm.

Results

The effect of HCT on 0-Mg²⁺-induced EFP

Only slices without spontaneous epileptiform discharges during control period were investigated. Application of Mg²⁺-free ACSF resulted in EFP after a median latency of 41 min (2 mmol/l HCT), 47 min

(0.2 mmol/l HCT) and 43 min (0.02 mmol/l HCT), respectively. After reaching a stable level over 15 min, median baseline repetition rate was 13/min (2 mmol/l HCT), 11/min (0.2 mmol/l HCT) and 10/min (0.02 mmol/l HCT) respectively. The differences were not significant. Addition of HCT to the bath resulted marked increase of repetition rate, amplitude and duration of the epileptiform field potentials (Figure 1). After 1 hr, the increase of the repetition rate was highest with 2 mmol/l HCT (median 26/min, 25-75 quartiles 21.5/min-38/min),

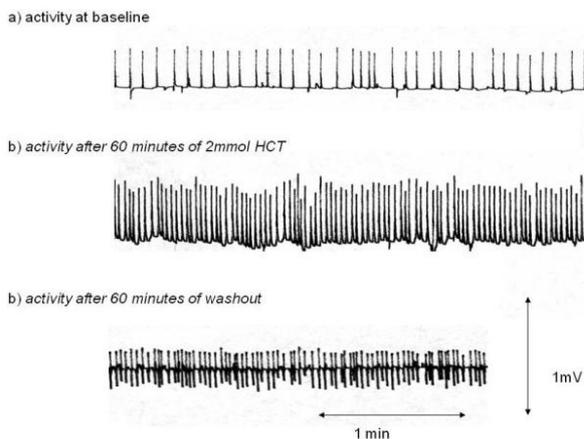


Figure 1. Extracellular field potential recordings from a rat hippocampal slice using the zero magnesium model; at baseline (a), after addition of HCT (b) and during wash-out (c)

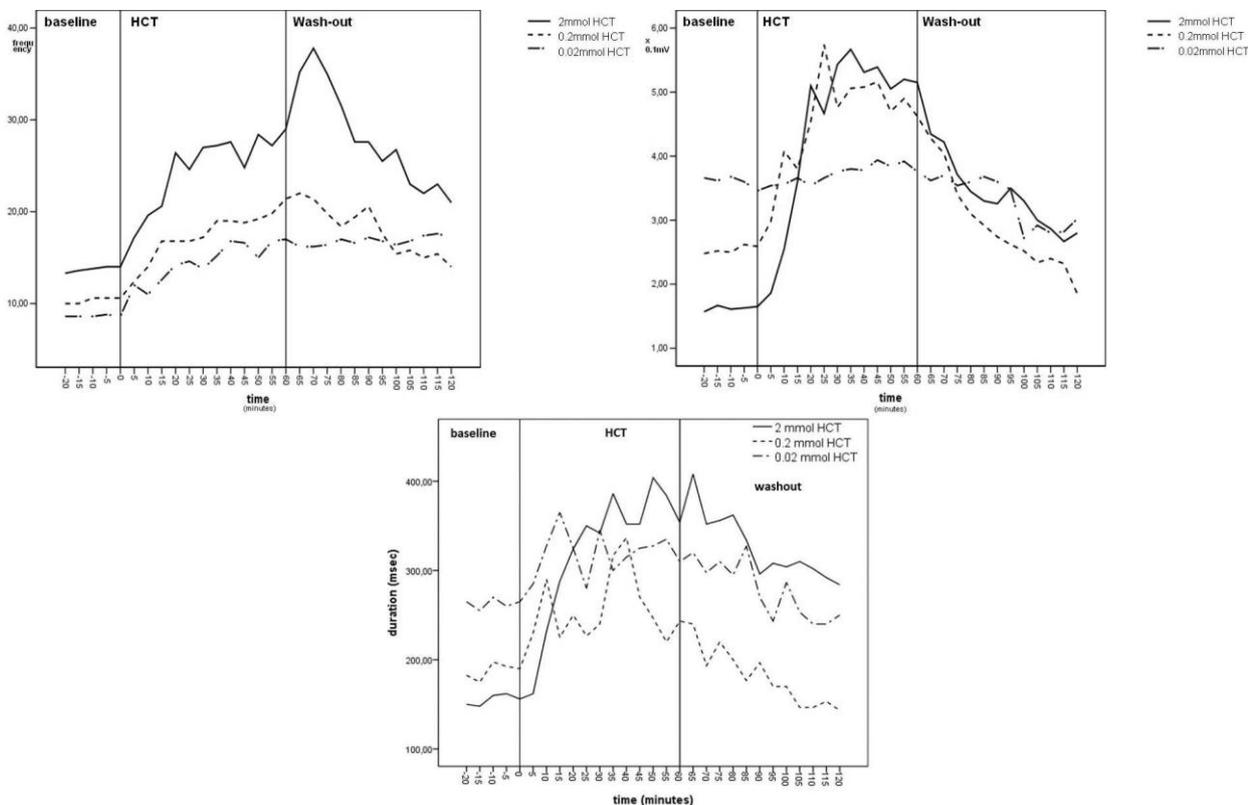


Figure 2. Mean discharge repetition rate (a) and mean discharge amplitude of the field potential recordings of three groups of rats (n=5) using 0.02 mmol, 0.2 mmol and 2 mmol HCT solution

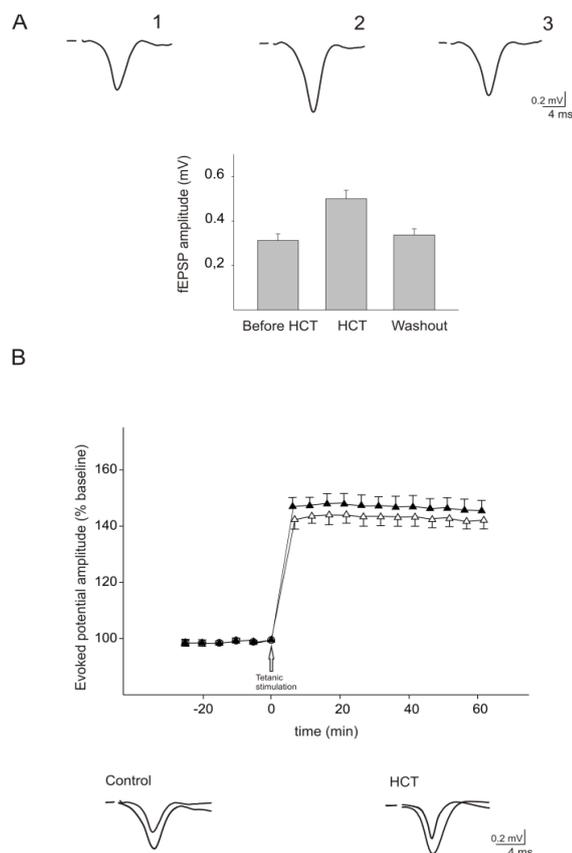


Figure 3. Long-term potentiation (LTP) of the evoked field excitatory post-synaptic potentials (fEPSP) in neocortical preparations. (a) Tetanic stimulation ((100 Hz, for 1 sec, at baseline stimulation intensity) produces a rapid and stable potentiation in the amplitude of the evoked field potentials, calculated as a percentage of baseline mean response amplitude. Solid and open triangles show the evoked fEPSP after application of hydrochlorothiazide (HCT, 2 mmol/l) and control, respectively. Arrow shows the time of tetanic stimulation, 60 min after application of HCT and artificial cerebrospinal fluid (control). The time points given refer to LTP induction. (b) Representative examples of the fEPSP before and after tetanic stimulation in control and HCT affected slices

slightly lower with 0.2 mmol/l HCT (median 21/min, 25-75 quartiles 18.5-24.5/min) and with 0.02 mmol HCT (median 15/min, 25-75 quartiles 14.5-20.5/min). The inter-group differences were statistically significant, as were the differences between baseline and 1 hr exposition within each group ($P < 0.05$). The amplitude increased from a median of 0.18 mV to a median of 0.55 mV in the 2 mmol group, from a median of 0.24 mV to a median of 0.37 mV in the 0.2 mmol/l group, and from a median of 0.21 mV to a median of 0.23 mV in the 0.02 mmol/l group. Only the within-group differences of the 2 mmol/l and the 0.2 mmol/l groups were significant ($P < 0.05$). Discharge duration also increased from 120 msec to 340 msec (median) in the 2 mM group, from 190 msec to 230 msec (median) in the 0.2 mmol/l group, and from 270 msec to 320 msec (median) in the 0.02 mmol/l group. Differences within groups, but not inter-group differences were statistically significant. After 60 min

of wash-out, repetition rate, discharge amplitude and discharge duration gradually returned to baseline levels (Figure 2).

The effect of HCT on LTP

A conditioning tetanic stimulation was delivered to the Schaffer collaterals pathway followed by pulses with stimulation parameters identical to control values. The evoked fEPSP was stable for at least 30 min (with mean amplitude of 0.3 ± 0.03 mV) before application of tetanic stimulation (less than 10% variation; Figure 3). Administration of tetanic stimulation produced a rapid, stable, and lasting enhancement of the amplitude of the fEPSP in all tested preparations ($n=6$, $147 \pm 1.6\%$ control; Figure 3 B). The potentiation rose within 2 to 3 min and stabilized within 5–10 min after the train of stimulations. Application of HCT (2 mmol/l; $n = 8$) 60 min before tetanic stimulation did not change LTP induction in all tested slices ($146 \pm 1.4\%$ control, Figure 3B). Enhancement of LTP after application of HCT did not significantly differ from control slices ($P < 0.05$).

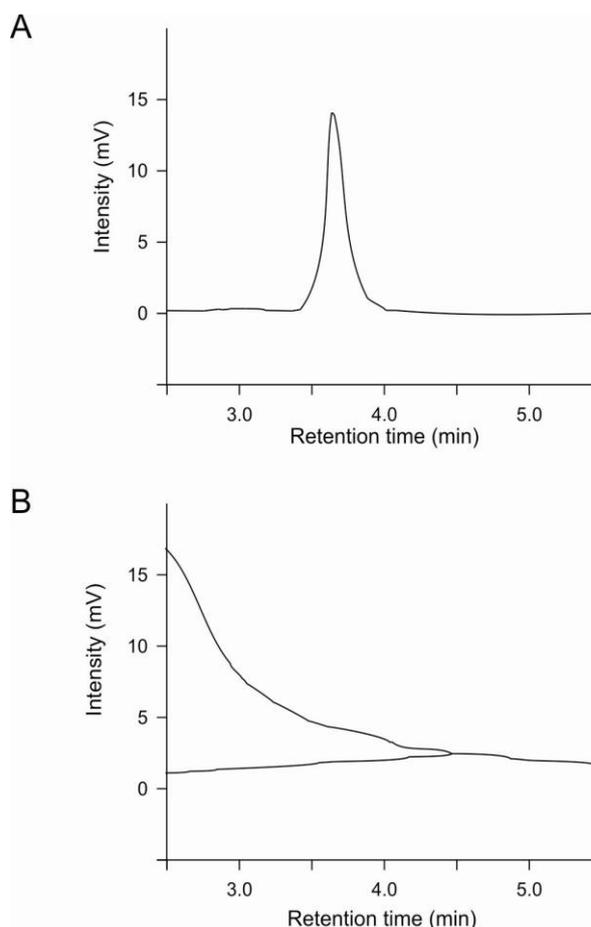


Figure 4. A) HPLC detection curve after injection of 1 µg HCT solved in 100 µl water. HCT retention peak occurred after 3.5 min. B) HPLC detection curve after injection of brain homogenate of a rat who had received injection of HCT 2 mM and later Isoflurane for anesthesia

Measurement of HCT concentration in the brain after IP injection

In HPLC, the peak of HCT appeared at a retention time of approximately 3.5 min. There was a linear dose-concentration curve 0.5 µg and 100 µg HCT. When brain homogenate of HCT-treated rats was injected, HPLC showed no significant peak at the HCT retention time (Figure 4). There was also no peak when the brain of rats injected with saline (control) was analyzed.

Discussion

In contrast to our expectations, HCT increased repetition rate, amplitude and duration of the epileptiform discharges elicited by omission of Mg^{2+} from the bath solution in a dose-dependent manner. HCT also increased the amplitude of electrically evoked field potentials, but did not have any influence on the LTP induced by tetanic stimulations applied to Schaffer collaterals. When intraperitoneally injected, HPLC analysis did not show evidence of HCT in the brain even at high doses.

Our recordings used the zero- Mg^{2+} model of epileptiform activity (18-20). This model initiates robust epileptiform activity in both normal and lesioned cortex by augmenting the NMDA-component of EPSP, without pharmacological blockade of synaptic receptors (i.e., $GABA_A$ receptors in the bicuculline model), and represents a model for pharmacoresistant status epilepticus. This model represents a well-controlled analysis of drug effects independent of systemic influences (18-20). This model quite closely imitates *in vivo* conditions because recurrent and lateral inhibition remains intact. In addition, low-magnesium activity induces post-synaptic calcium influx, resulting in activation of the same calcium dependent second messenger mechanisms that underlie activity-dependent neuronal plasticity (20).

It has been found that diazoxide, another benzothiazide diuretic, is a potent and reversible gating modifier of AMPA-type glutamate receptor channels (22) that inhibits the rapid desensitization of the response to a constant agonist concentration. The most potent agent of the group of thiazide desensitizers is cyclothiazide (CTZ) (23, 24) that recently has been proposed as an *in vitro* epilepsy model (25). Although there is chemical similarity between different benzothiazide diuretics, the effect on quisqualat-induced AMPA-desensitization has been shown to be quite variable. In concentrations of 500 µmol, HCT also potentiated the steady-state component of quisqualat-activated currents (23), but to a much smaller amount than cyclothiazide. It remains undetermined whether HCT also - like CTZ - increases pre-synaptic glutamate release (26) or directly inhibits $GABA_A$ receptors (27).

LTP is a phenomenon in which a constant pre-synaptic high stimulation of excitatory amino acids

neuronal pathways results in prolonged enhanced post-synaptic responses. It is well established that NMDA receptors are a molecular detector of the coincidence of both the pre-synaptic release of glutamate and a post-synaptic depolarization at the origin of LTP induction (28). HCT did not affect LTP. In keeping with our data, it has been shown that the HCT (500 µM) potently inhibits non-NMDA receptors desensitization in cultured hippocampal neurons (23). Cyclothiazide (300 µM), a thiazide diuretic and an allosteric inhibitor of non-NMDA receptor desensitization, induced potentiated low Mg^{2+} -induced seizure-like events recorded from the CA3 pyramidal layer of juvenile rat hippocampal slices (29). In line with our results, cyclothiazide administered intracerebral ventricle induced epileptiform activities from an initial of multiple evoked population spikes, progressed to spontaneous spikes and finally to highly synchronized burst activities in hippocampal CA1 neurons (30, 31). In contrast to *in vivo* studies, systemic administration of thiazides, including hydrochlorothiazide (100 mg), enhanced the anticonvulsant action of carbamazepine (32) and gabapentin (33) in the maximal electroshock seizure model in mice.

In our experiments, we could not find evidence that HCT does permeate BBB in the rat. This is in agreement with studies of dogs where dosages as high as 500 mg/kg administered twice daily did not result in measurable quantities (<3 mg/l) of the drug in CSF or brain (34). Unfortunately, there is no data available in the literature regarding the BBB permeability for HCT in humans. In addition, there is no evidence for central nervous effects of HCT even in the setting of overdose intoxication (35). One could argue that HCT may influence ion transport at the BBB without actually permeating it. However, HCT at a relatively high concentration of 1.5 mmol/l does not have any effect on the blood to brain sodium transport *in vitro* (36).

The anticonvulsant effect of HCT *in vivo* most likely is mediated via mechanisms based on effects outside the brain. It seems unlikely that the inhibition of the carbonic anhydrase plays a relevant role. Dashputra and colleagues found that there is no correlation between the effect on the carbonic anhydrase and the potentiation of the anticonvulsant effect of phenytoin in the maximal electroshock model in rats (10). Systemic acidosis seems equally unlikely to be related to the protective effect of HCT and other benzothiazides because acetazolamide raises the seizure threshold before there has been time for substantial bicarbonate loss (37). It is very unlikely that the protective effect of HCT in humans is due to enhancing the action of standard anticonvulsants because the population under study did not suffer from epilepsy (9, 38).

There are limitations to our study. First, we did

not investigate the effect of HCT in other *in vitro* models of epilepsy. Therefore, generalizability is limited. Moreover, we arbitrarily chose HCT concentrations ranging from 0.02 mmol/l to 2 mmol/l. We intended to look across a large window of possible concentrations. However, HCT concentration in plasma (or brain) of humans when exposed to HCT in clinical practice may be much lower (39). Thus, we may have missed relevant effects of concentrations below our lowest concentration, or between concentrations. In addition, the group sizes were rather small. However, the effects we have observed seem rather robust, which makes it unlikely that with larger groups we would have seen different results.

The protective effect of HCT and the other diuretics is rather mild in the epidemiological studies. Moreover, in the animal studies presented by Hesdorffer *et al* (9), HCT (as well as furosemide) was applied in doses that are 100 to 200 times higher than the doses used in clinical practice of hypertension treatment (HCT 165 mg/kg in rats vs. HCT 0.3-1 mg/kg in humans). Therefore, the use of diuretics as alternative convulsant agents cannot be recommended yet. More research is needed to further elucidate possible mechanisms of anticonvulsant activity.

Conclusion

Although HCT seems to have anticonvulsive properties on *in vivo* experimental models, exposure of hippocampal slices to HCT resulted in an enhancement of the epileptiform burst discharges. In addition, HCT at the same concentration did not cross the BBB in rats. Thus, the anticonvulsive effect of HCT most likely is not through direct neuronal effects.

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