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Topiramate modulates pH of hippocampal CA3 neurons by combined effects on carbonic anhydrase and Cl^-/HCO_3^- exchange

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1 Topiramate (TPM) is an anticonvulsant whose impact on firing activity and intracellular pH (pHi) regulation of CA3 neurons was investigated.

2 Using the 4-aminopyridine-treated hippocampal slice model bathed in bicarbonate-buffered solution, TPM (25–50 μM) reduced the frequency of epileptiform bursts and action potentials without affecting membrane potential or input resistance. Inhibitory effects of TPM were reversed by trimethylamine-induced alkalinization.

3 TPM also lowered the steady-state pHi of BCECF-AM-loaded neuronal somata by 0.18 ± 0.07 pH units in CO_2/HCO_3^--buffered solution. Subsequent to an ammonium prepulse, TPM reduced the acidotic peak but clearly slowed pHi recovery. These complex changes were mimicked by the protein phosphatase inhibitor okadaic acid.

4 Alkalosis upon withdrawal of extracellular Cl^- was augmented by TPM. Furthermore, at decreased pHi due to the absence of extracellular Na^+, TPM reversibly increased pHi. These findings demonstrate that TPM modulates Na^+-independent Cl^-/HCO_3^- exchange.

5 In the nominal absence of extracellular CO_2/HCO_3^- buffer, both steady-state pHi and firing of epileptiform bursts remained unchanged upon adding TPM. However, pHi recovery subsequent to an ammonium prepulse was slightly increased, as was the case in the presence of the carbonic anhydrase (CA) inhibitor acetazolamide. Thus, a slight reduction of intracellular buffer capacity by TPM may be due to an inhibitory effect on intracellular CA.

6 Together, these findings show that TPM lowers neuronal pHi most likely due to a combined effect on Na^+-independent Cl^-/HCO_3^- exchange and CA. The apparent decrease of steady-state pHi may contribute to the anticonvulsive property of TPM.
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Abbreviations: 4-AP, 4-aminopyridine; AZM, acetazolamide; OA, okadaic acid; PKA, protein kinase A; TMA, trimethylamine; TPM, topiramate

Introduction

The novel anticonvulsant topiramate (TPM) is effectively used for the treatment of focal and generalized epilepsies. The broad anticonvulsant property of TPM relies on multiple inhibitory mechanisms, which include voltage-activated sodium channels (Zona *et al*., 1997; De Lorenzo *et al*., 2000), L-type high voltage-activated calcium channels (Zhang *et al*., 2000), and kainate-evoked currents (Gibbs *et al*., 2000). The latter mechanism was suggested to be due to the property of TPM to bind to phosphorylation sites of AMPA and kainate receptors (Angehagen *et al*., 2004). Furthermore, a rapid enhancement of $GABA_A$-evoked hyperpolarizations was described (White *et al*., 1997; 2000).

Aside from affecting membrane currents TPM also inhibits the isoenzymes I–VI of carbonic anhydrase (CA) at therapeutically relevant concentration (Dodgson *et al*., 2000). In this respect, TPM-induced changes of $GABA_{ergic}$ depolarizations were supposed to be based on a decreased intracellular bicarbonate concentration, which may be caused by an inhibition of neuronal CA (Herrero *et al*., 2002). Other CA inhibitors such as sulthiame and acetazolamide (AZM) have previously been shown to lower neuronal intracellular pH (pHi), which effectively reduced epileptiform activity in epilepsy model systems *in vitro* (Leniger *et al*., 2002). Similar to AZM, it has been shown that TPM has a CA-inhibitory efficacy (Dodgson *et al*., 2000), which appears to be subtype specific (Casini *et al*., 2003). Other studies suggest that the anticonvulsive effect of TPM is based on additional mechanisms which clearly differ from CA inhibition (Shank *et al*., 1994; Stringer, 2000; Russo & Constanti, 2004). Therefore, it remains unclear to what extent the anticonvulsive property of TPM could rely on its capability to change neuronal HCO_3^- concentration and/or pHi.

We addressed that point by a combined electrophysiological and pHi imaging study. Inhibitory effects of TPM on epileptiform activity were subjected to alkalotic treatment to make sure that pHi effects indeed play a role in the anticonvulsive property of TPM. In the second part of the

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study, we tested the effects of TPM on the steady-state pHi of individual neuronal somata and looked for effects of TPM on pHi-regulating systems. To that aim, we challenged neuronal pHi regulation using the ammonium prepulse technique, thus investigating Na^+/H^+ exchange, Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchange. To circumvent inter-individual differences between pHi transients of neuronal somata in the presence and absence of TPM, we performed pHi measurements in larger regions of the stratum pyramidale of the CA3 region. Furthermore, we compared the effects of TPM to those of AZM and the phosphatase inhibitor okadaic acid (OA), which has recently been demonstrated to alter neuronal acid extrusion (de la Rosa *et al*., 2002). Parts of this study have been presented as an abstract (Thöne *et al*., 2003).

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 pre-incubated 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 pre-incubation bath to the recording chamber (volume 4 ml) mounted on an inverted microscope (Zeiss ID 03), which was used for electrophysiological recordings. pHi measurements (see below) were carried out at an upright microscope. The recording chamber was continuously perfused (perfusion rate of 4.5 ml min^{-1}) 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 and 95% O_2 (Brett *et al*., 2002). A Na^+-free, CO_2/HCO_3^--buffered solution was prepared with choline-chloride (124 mM) and choline-HCO_3 (26 mM) instead of NaCl and $NaHCO_3$, respectively.

The epileptiform activity of CA3 neurons was induced by 4-aminopyridine (4-AP, 50 μM), which was added to the superfusate to reach a stable state of hyperexcitation characterized by epileptiform bursts and spontaneous $GABA_{ergic}$ hyperpolarizations (Rutecki *et al*., 1987; Leniger *et al*., 2000). Topiramate was provided by the R.W. Johnson Pharmaceutical Research Institute (Spring House, Pennsylvania, U.S.A.) and dissolved in all solutions immediately before the experiment. Okadaic acid (OA) was purchased from Merck-Biosciences (Darmstadt, Germany). Acetazolamide (AZM) and all other chemicals were from Sigma.

Intracellular recording and measurement of neuronal activity

Intracellular recordings were obtained from the somata of CA3-neurons with sharp glass microelectrodes filled with 2 M potassium methylsulphate (150–180 MΩ) as described (Bingmann & Speckmann, 1986). Electrodes were connected to an amplifier (BA-1S, npi-advanced electronics, Tamm, Germany) using the bridge mode. The input resistance of neurons was calculated from the voltage deflection upon hyperpolarizing current pulses (0.1 nA, 200 ms). The analog bioelectric signals were converted and recorded digitally using a DABAS system operated with 12 kHz sampling rate and 10 bit resolution (Widman & Bingmann, 1996). The frequency of action potentials and epileptiform bursts was determined off-line. Therefore, a trigger level was used which 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 TPM, the frequency of action potentials and epileptiform bursts was normalized to the mean value obtained during a 10-min period immediately prior to drug application.

Measurement of pHi

To analyse 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 pre-incubation saline. Slices were transferred to 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 with an optical fibre. 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, England). For background capturing slices not loaded with BCECF-AM were mounted and processed the same way 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 the experiment the ratio 440/490 was calibrated by a standard curve, which 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 not contribute to the fluorescence signal, nor did they influence the pH of the superfusate. Spontaneous pHi deflections (observed to be in the range of ± 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 for the rest of the recording. TPM was added about 20 min later for 20–45 min usually in one concentration step unless otherwise stated. Trimethylamine (TMA) was washed in as an alkalizing agent after at least 20 min of constantly diminished activity due to TPM. Input resistance was measured in periods lasting several minutes shortly before TPM application and after 10–20 min of treatment (see, e.g. Figure 1c).

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 photo-bleaching of single neuronal somata (estimated by the intensity of 440 nm images) was $<0.5\%\ min^{-1}$, indicating that structures under investigation were in good condition. Excitation light was reduced to a

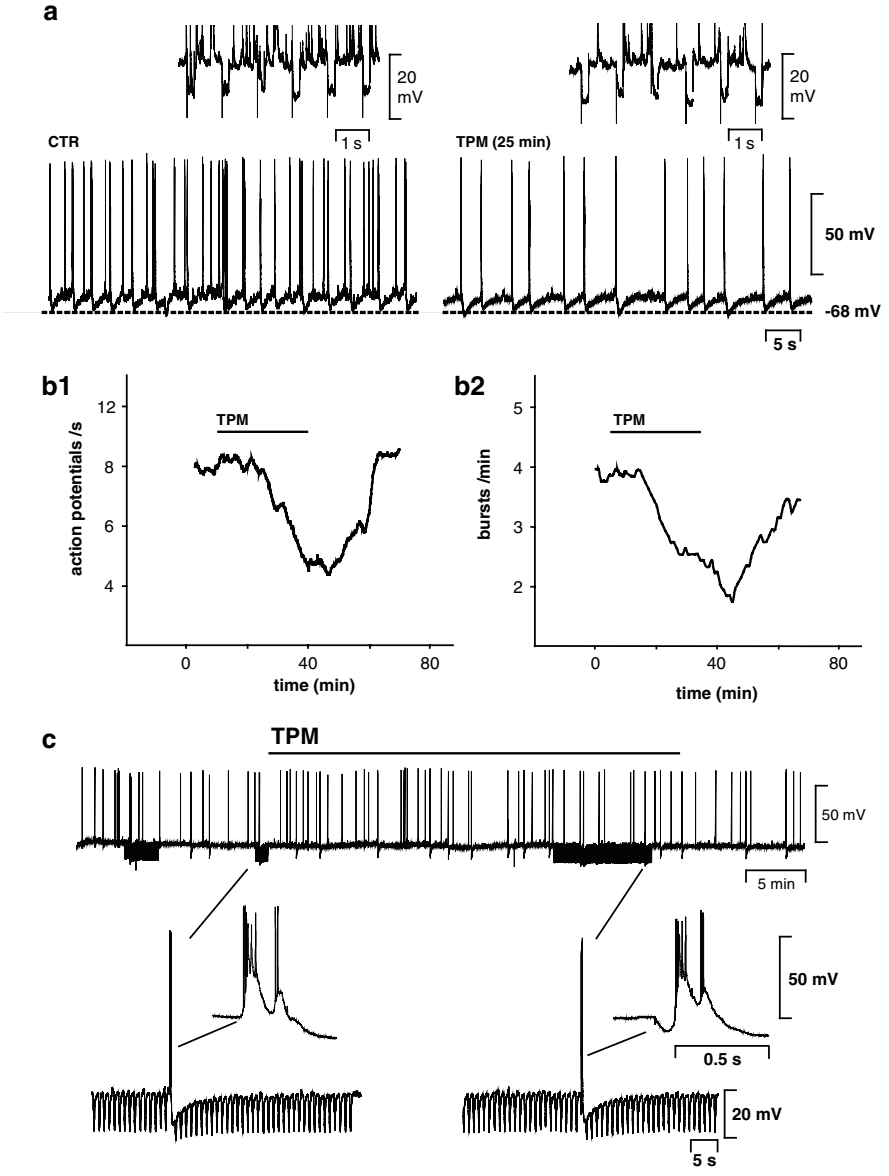

Figure 1 Effect of topiramate (TPM, 50 μM) on membrane potential of 4-AP-treated neurons. (a) Frequency of action potentials and epileptiform bursts was decreased by TPM without apparent alteration of membrane potential (a) and input resistance, as is shown for another neuron in the respective upper tracings (current pulses: -0.1 nA, 200 ms). Parts b1 and b2 show numerical evaluations of the effect of TPM on the frequency of action potentials and epileptiform bursts. (c) TPM treatment was carried out in the nominal absence of extracellular CO_2/HCO_3^- (2 h pre-treatment with Hepes-buffered solution). Under these conditions, application of TPM for 30 min (indicated by bar) had no inhibitory effect on bioelectric activity. Membrane potential, input resistance (current pulses: -0.1 nA, 200 ms, applied during three periods), and shape of epileptiform bursts remained unchanged. Time points of expanded potential tracings are related to the upper recording by lines.

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.

To quantify pHi changes following brief NH_4Cl application, we defined an acidotic shift rate ($\Delta pHi/\Delta t$), a pHi recovery rate ($\Delta pHi/\Delta t$), and an acidotic peak (ΔpHi). Acidotic shift rate and pHi recovery rate rates were linearly extrapolated on the basis of a least-square regression fit to pHi data points sampled every 20 s. To calculate the acidotic shift rate, data points were taken from a time interval which commenced with the most alkalotic value during wash-in of ammonium chloride and ended with the acidotic peak value. Intervals were therefore variable in duration and comprised 8–12 data points. The pHi recovery rate was determined subsequent to intracellular acidification and reflected a relative measure for the net activity of acid extrusion (Raley-Susman *et al*., 1991; Schwiening & Boron, 1994). Data points for the pHi recovery rate were taken from a 10-min interval starting at the acidotic peak. Although complete pHi recovery could be described by an exponential function, we found it more helpful to describe the initial steep pHi increase by a linear function. Height of the acidotic peak was defined as maximal ΔpHi (ΔpHi max.), which was measured as the difference between baseline pHi (averaged from three values immediately before wash-in of ammonium) and the acidotic peak (averaged from three consecutive values including the maximum value).

Repetitive ammonium prepulses were applied with an interval of at least 35 min, to ensure that baseline pHi was reached again. In the absence of any treatment, we found that up to five repetitive ammonium prepulses yielded largely identical pHi deflections, that is, variation of acidotic shift rate, pHi recovery rate, and acidotic peak were less than 3%.

In experiments withdrawing Cl^- from the extracellular solution, the alkalotic shift rate was calculated from a 5-min period beginning with the onset of alkalosis. The pHi recovery rate in these experiments was calculated from a 5-min period beginning at the end of the plateau value. The height of alkalotic plateau was defined as maximal ΔpHi (ΔpHi max.), which was measured as the difference between baseline pHi (averaged from three values immediately before withdrawal of Cl^-) and the alkalotic peak (averaged from three consecutive values including the maximum value).

Data analysis

All data are given as means $\pm$ standard deviation of at least three independent experiments on different slices. The *t*-test for paired samples was used to compare differences in acidotic and alkalotic shift rates, pHi recovery rates, and ΔpHi max. values. Significance was considered for a *P*-value $\leqslant 0.05$.

Results

Effects on neuronal activity

To analyse effects of TPM on neuronal activity, intracellular recordings were obtained from 23 different neurons (23 slices from 18 animals), which were superfused by CO_2/HCO_3^--buffered solution ($n = 20$). In a limited number of experiments ($n = 3$), we switched to Hepes-buffered solution at least 60 min before application of TPM. Depth of the neurons within the tissue ranged from 9 to 150 μM. A stable pattern of augmented bioelectric activity consists of an increased action potential firing rate and regular epileptiform bursts, which developed within 10–20 min upon application of 4-AP (50 μM). Resting membrane potential was at least -50 mV, input resistance ranged from 50 to 110 MΩ, and amplitudes of action potentials exceeded 50 mV. Switching to Hepes buffer in the presence of 4-AP largely abolished the firing of action potentials and bursts (see Bonnet *et al*., 1998). However, rarely occurring epileptiform bursts remained in three neurons, enabling us to look at effects of TPM also under these conditions.

We found that TPM (25 and 50 μM) reduced the frequency of epileptiform bursts and action potentials in 16 of 20 neurons in CO_2/HCO_3^--buffered solution. As demonstrated in Figure 1a, we were unable to detect any hyperpolarization or change in input resistance measured under current clamp conditions. These neurons which reduced their bioelectric activity (BA) upon TPM will be termed BA-responding neurons. Four neurons, however, were not affected by TPM treatment even after a prolonged wash-in (>45 min) and/or when the concentration of TPM was raised to 500 μM, in order to facilitate better tissue penetration. In BA-responding neurons, the suppressive effect on action potentials (Figure 1b1) and epileptiform bursts (Figure 1b2) typically developed within 10–15 min. Since the change of bioelectric activity induced by TPM appeared identical regardless of whether a low (25 μM) or high concentration (50 μM) was used, data from both groups were pooled for quantification: The frequencies of action potentials and epileptiform bursts, when normalized to a value taken immediately before drug application (mean of a 10-min period), were equally reduced by 46.1 ± 13.4 and 48.9 ± 15.3%, respectively ($n = 13$). All effects of TPM were at least partly reversible upon washout within 5–15 min. No differences were found concerning amplitude or shape of either action potentials or epileptiform bursts, as long as these were selected on the basis of identical resting membrane potential (Figure 1a and 2b).

Inhibitory effects of TPM were not found in the absence of CO_2/HCO_3^- buffer, that is, when Hepes-buffered solution was used. The sparsely occurring epileptiform bursts remained unchanged in frequency and shape including after-hyperpolarization (Figure 1c). Also, under these conditions, there were no alterations of membrane potential and input resistance.

Since the attenuation of 4-AP-induced burst firing in CO_2/HCO_3^--buffered solution resembled the effect of a mild neuronal acidification, for example, due to inhibitors of carbonic anhydrase (Bonnet *et al*., 2000a, b; Leniger *et al*., 2002), we speculated that one of the anticonvulsive properties of TPM might involve changes in free intracellular proton concentration. To test this hypothesis, we counteracted the putative acidification with a low concentration of trimethylamine (TMA, 5 mM). As is shown in Figure 2c this treatment reversibly increased tissue pH of the stratum pyramidale by 0.1 ± 0.03 pH units within 3–5 min ($n = 3$). TMA reversed the suppressive effect of TPM (50 μM) and re-established the firing pattern observed before TPM treatment (Figure 2a, $n = 3$), without altering shapes of action potentials and epileptiform bursts (Figure 2b). The same finding was made even in the

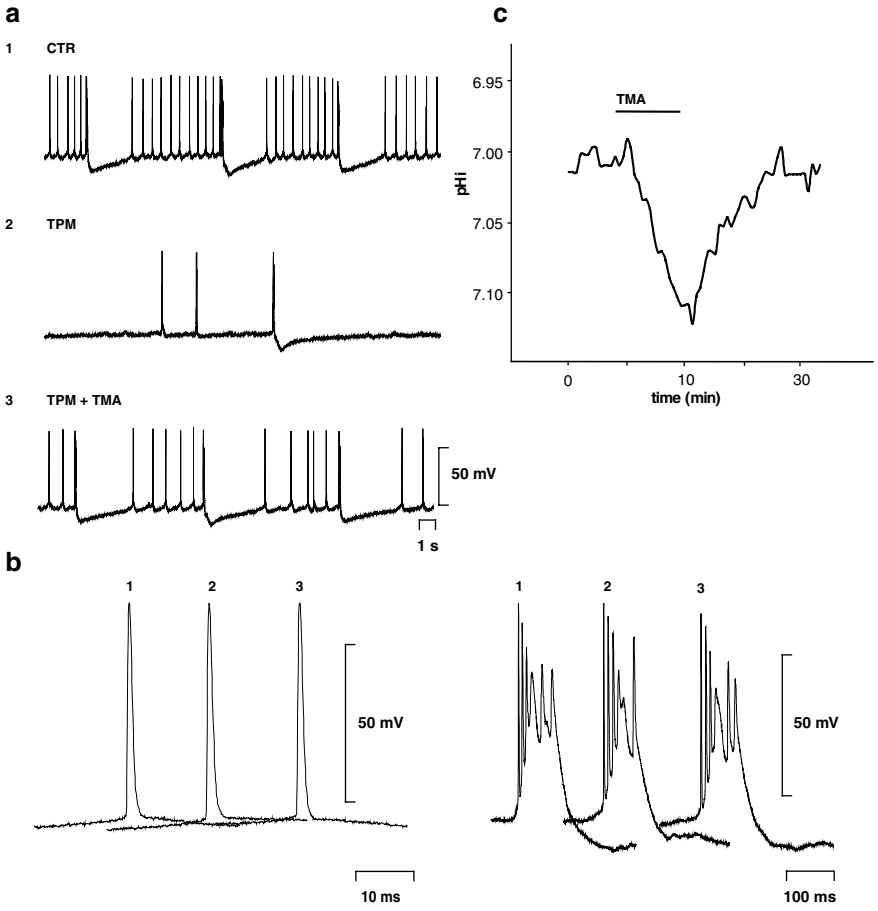

Figure 2 The suppressive effect of topiramate (TPM, 50 μM) on the frequency of action potentials and epileptiform bursts of a 4-AP-treated CA3 neuron is antagonized by mild alkalinization with trimethylamine (TMA, 5 mM). (a) Membrane potential recording showing firing patterns under control condition (1), after 20 min TPM (2), and after 10 min TPM + TMA (3). (b) Action potentials and typical epileptiform bursts (1, control condition) remained unchanged during TPM-mediated inhibition (2) or in combination with TMA (3). (c) TMA (5 mM) reversibly alkalized a neuronal CA3 soma bathed in CO_2/HCO_3^--buffered solution ($n=3$).

presence of excessive TPM concentration (500 μM, data not shown), making it unlikely that TMA only competes for TPM-binding sites.

Together, results presented so far suggest that a decrease of pHi could contribute to the anticonvulsive activity of TPM in a bicarbonate-dependent fashion.

Effects of TPM on intracellular pH

Steady-state pHi of individual neuronal somata In the next part of the study we, therefore, investigated the effect of TPM on steady-state pHi of BCECF-AM-loaded CA3-somata ($n=14$, from 10 slices, eight animals) in CO_2/HCO_3^--buffered solution. The mean steady-state pHi amounted to 7.03 ± 0.18. Upon application of TPM (50 μM) there was a clear intracellular acidification, which developed within 5–10 min in 10 out of 14 neurons, which will be termed pHi-responding neurons. The mean acidification of pHi-responding neurons amounted to 0.18 ± 0.07 pH units ($P<0.001$). This mean value was reduced to 0.13 ± 0.09 pH units ($P<0.001$), when non-responding neurons were included. Washout of TPM increased pHi to the steady-state pHi, and this was mostly completed within 20–25 min (Figure 3). In line with the effects presented in the electrophysiological part of this investigation, we found that the acidifying effect could be equally elicited with 25 μM TPM ($n=4$, Figure 3).

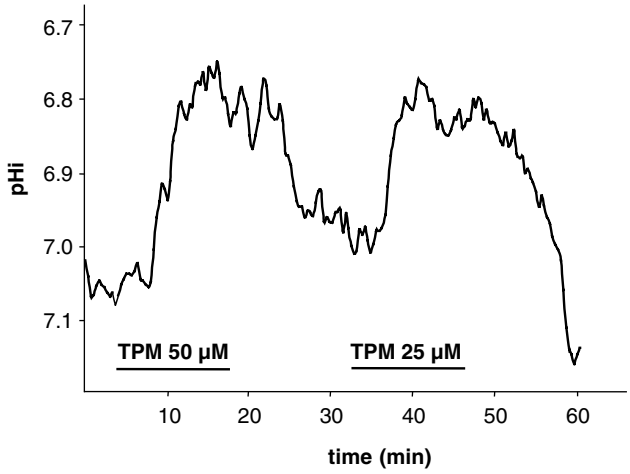

Figure 3 Effect of topiramate (TPM) on the steady-state pHi measured in a BCECF-AM-loaded neuronal CA3 soma superfused with CO_2/HCO_3^--buffered solution. TPM reversibly lowered the pHi and this effect occurred equally with 50 and 25 μM TPM.

pHi regulation

To unravel the underlying mechanism of this TPM-mediated neuronal acidification, we focused on neuronal pHi regulation using the ammonium prepulse method to challenge neuronal acid extrusion in CO_2/HCO_3^--free and CO_2/HCO_3^--buffered solution. pHi-transients measured in individual neuronal somata yielded only weak TPM-induced differences (data not shown), which were hard to interpret due to superimposed pHi deflections resulting from noise and/or bioelectric activity. Therefore, we decided to use a low-magnification objective ($\times 20$) and to define larger regions of interest covering areas of ca. $200 \times 40\,\mu m^2$ within the stratum pyramidale to collect a BCECF-fluorescence signal from the whole pyramidal cell layer. This type of pHi measurement turned out to be more stable and allowed to reveal small differences between pHi curves obtained after repeated ammonium prepulses. For these experiments, 40 slices from 15 animals were used.

Experiments in CO_2/HCO_3^--free, Hepes-buffered solution

Slices were equilibrated with Hepes-buffered solution at least 20 min before the first ammonium prepulse (20 mM, 3 min) was applied. In 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. To examine the effects of TPM (50 μM), the drug was applied 20 min before the second ammonium prepulse. TPM did not change steady-state pHi in CO_2/HCO_3^--free solution and had only minor, although significant, effects on ammonium prepulse-induced pHi regulation, which are shown in Figure 4a ($n = 5$). While the alkalinization was unchanged, there was an accelerated acidotic shift (Figure 4a,b) and a more pronounced acidotic peak (Figure 4a,c). Recovery of the pHi was slightly steeper in its initial phase, as is outlined in Figure 4a,b. These findings provided no evidence for a TPM-mediated inhibition of sodium–proton exchange. However, the increase in peak acidification and pHi recovery rate could be due to a lowered intracellular buffer capacity resulting from a reduced amount of intracellular bicarbonate concentration. Since TPM is also a CA inhibitor (Dodgson *et al*., 2000; Masereel *et al*., 2002; Casini *et al*., 2003), we repeated the experiments with the CA inhibitor AZM (0.5 mM) instead of TPM and obtained similar results. Peak acidification was slightly enlarged and the pH recovery rate was equally augmented by 38.1% (vs 38.5% by TPM, $n = 5$, see inset Figure 4a). These findings suggest that TPM, similar to AZM, slightly lowered the total intracellular buffer capacity in the nominal absence of extracellular bicarbonate.

Experiments in CO_2/HCO_3^--buffered solution

pHi regulation in the presence of extracellular CO_2/HCO_3^- was significantly affected by TPM (50 μM, pre-treatment 20 min, Figure 5a1). In contrast to Hepes-buffered solution, the initial alkalotic shift during wash-in of ammonium was augmented by TPM. Although the acidotic shift rate was increased (Figure 5a2), the acidotic peak was diminished (Figure 5a3) and the pHi recovery rate was decreased by TPM vs control

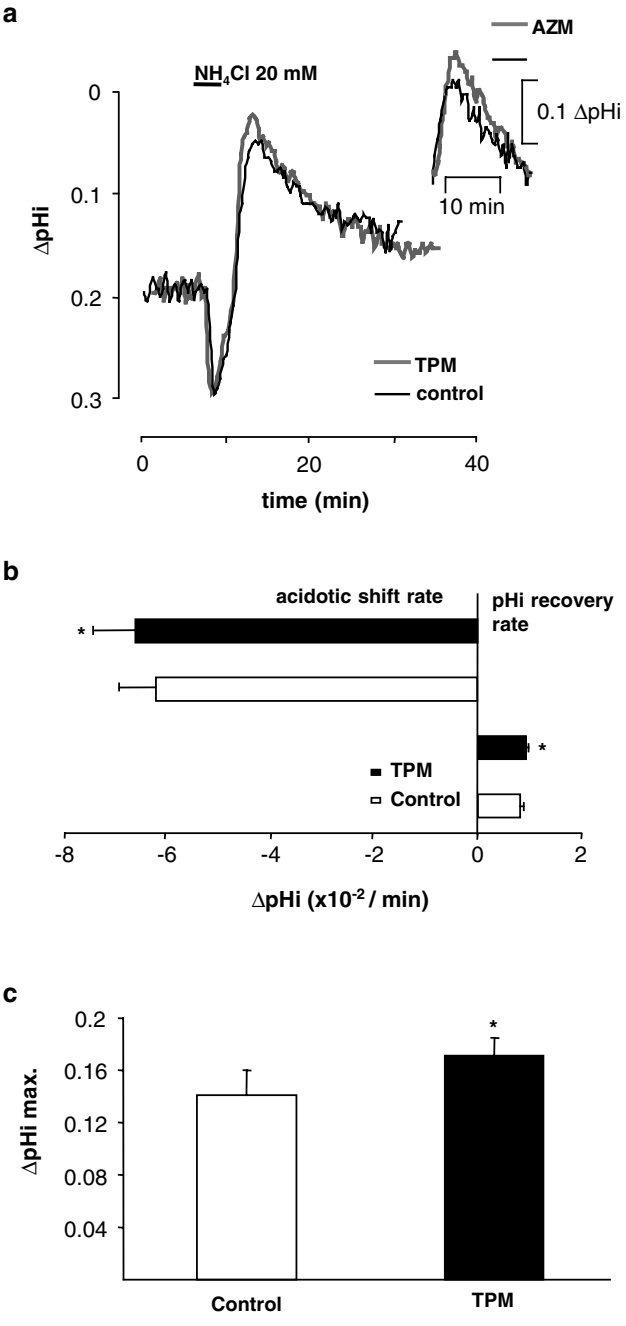

Figure 4 Effect of topiramate (TPM, 50 μM) on pHi regulation challenged by ammonium prepulse (NH_4Cl 20 mM, 3 min) in hippocampal stratum pyramidale bathed in HCO_3^-/CO_2-free, Hepes-buffered solution. (a) Curves from control and TPM (pre-treatment 20 min) were superimposed for clarity reasons. Time interval between the first and second prepulse was 50 min. Starting pHi in both cases was 7.1. TPM slightly accelerated the acidotic shift and the following pHi recovery rate. The effects of the carbonic anhydrase inhibitor acetazolamide (AZM, 0.5 mM) for the same portion of tissue are shown for comparison (see inset, curves superimposed from consecutive applications of NH_4Cl, starting pHi of each curve was 7.1). A similar acidotic over shoot and pHi recovery rate were obtained. (b) Mean rates of acidotic shift, pHi recovery, and (c) ΔpHi max. of the type of experiment illustrated in (a) ($n = 5$). Asterisks in (b) and (c) indicate significant differences vs control ($P < 0.05$).

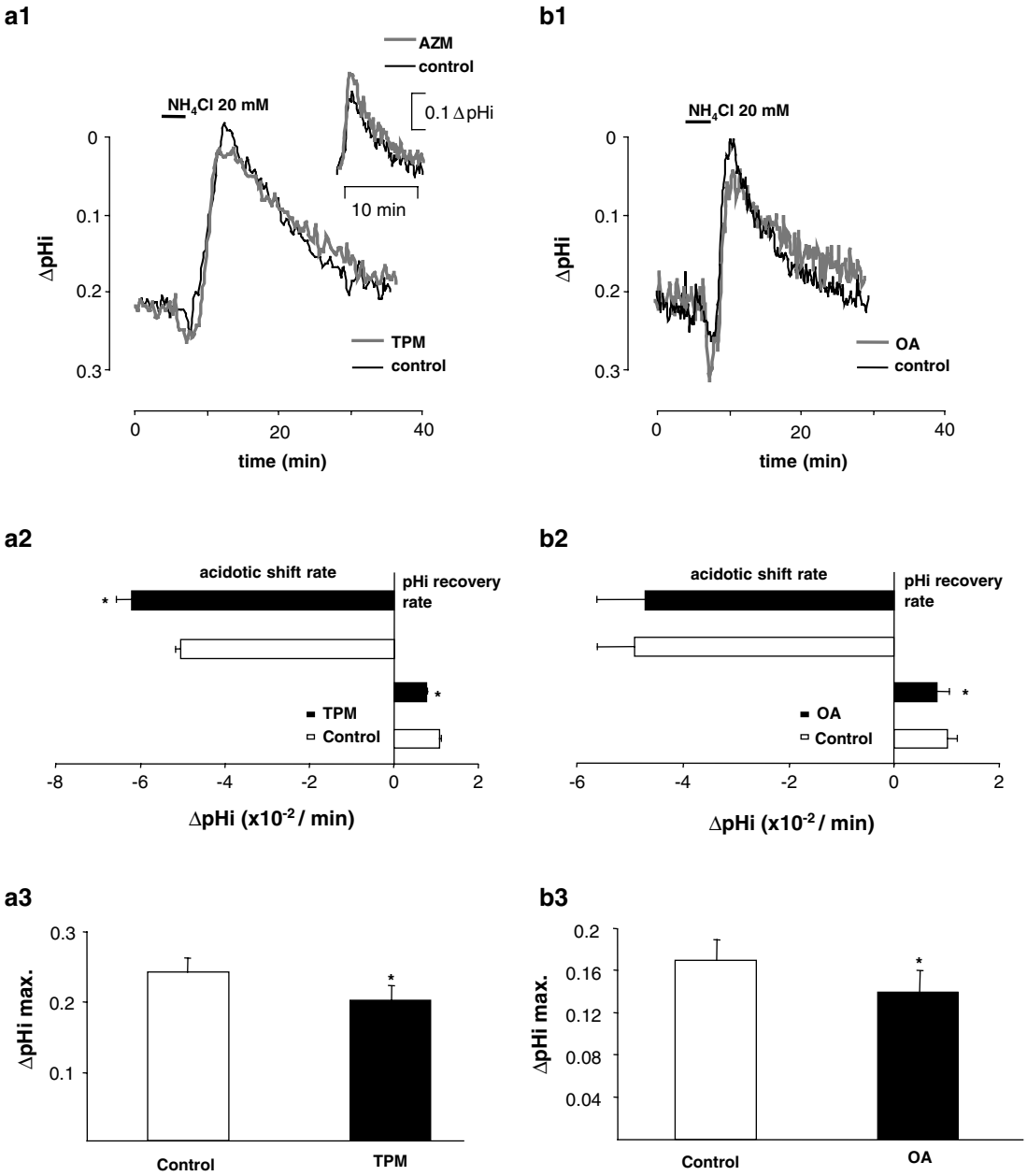

Figure 5 Effect of topiramate (TPM, 50 μM) and okadaic acid (OA, 0.5 μM) on pHi regulation challenged by ammonium prepulse (NH_4Cl 20 mM, 3 min) in hippocampal stratum pyramidale bathed in CO_2/HCO_3^--buffered solution. (a1) TPM augmented the slope of both the alkalotic and the acidotic shift. The acidotic peak was reduced and the following pHi recovery was slowed by TPM. Curves from control and TPM (pre-treatment 20 min) were superimposed for clarity reasons. Time interval between the first and second prepulse was 40 min. Starting pHi was 7.15 (control) and 7.09 (TPM), respectively. The carbonic anhydrase inhibitor acetazolamide (AZM, 0.5 mM) augmented the acidotic peak and accelerated the following pHi recovery (see inset, curves superimposed from consecutive applications of NH_4Cl, starting pHi was 7.13 (control) and 7.05 (AZM), respectively). (b1) Comparable to the effect of TPM, OA reduced the acidotic peak of the acidotic shift and slowed the following pHi recovery. Curves from control and OA (pre-treatment 20 min) were superimposed. Time interval between the first and second prepulse was 40 min. Starting pHi was 7.09 (control) and 6.96 (OA), respectively. (a2,b2) Mean rates of acidotic shift, pHi recovery, and (a3,b3) ΔpHi max. of the type of experiment illustrated in (a1) ($n=5$) and (b1) ($n=5$), respectively. Asterisks in (a2,3) and (b2,3) indicate significant differences vs control ($P<0.05$).

($n=5$, Figure 5a2). We repeated the ammonium prepulse experiment with AZM in CO_2/HCO_3^--buffered solution. However, for this drug, the effects on pHi regulation in CO_2/HCO_3^--buffered solution were comparable to those in Hepes-buffered solution, comprising both an increased acidotic peak and pHi recovery rate ($n=5$, see inset in Figure 5a1). The observed differences between the effects of TPM and AZM in CO_2/HCO_3^--buffered solution point to a complex impairment of transmembrane HCO_3^- flux due to TPM treatment.

Cl^-/HCO_3^- exchangers of CA1 neurons are influenced by protein kinase A-dependent phosphorylation (Brett *et al.*,

2002). Further on, effects on Na^+-independent Cl^-/HCO_3^- exchange by the phosphatase inhibitor okadaic acid (OA) have been described (de la Rosa *et al*., 2002). We, therefore, compared the effects of TPM and OA on pHi regulation induced by ammonium prepulse. Treatment of hippocampal slices with OA (0.5 μM) lowered the steady-state pHi by 0.07 ± 0.02 pH units ($n = 5$) within 3–5 min. After 20 min pre-incubation, it also significantly changed the pHi deflections upon ammonium prepulse very similar to TPM (Figure 5b1): (1) It augmented the alkalotic shift (Figure 5b1), (2) it decreased the acid peak (Figure 5b3), and (3) it slightly slowed down the pHi recovery rate vs control (Figure 5b2). Comparable to TPM, the latter effect became most obvious at a pHi value larger than 7.0 ($n = 5$, Figure 5b1). Unlike TPM, however, there was no significant change in acidotic shift rate (Figure 5b2).

Removal of Cl^- and Na^+ from the CO_2/HCO_3^--buffered solution

Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchange take part in the adjustment of steady-state pHi and pHi regulation of hippocampal neurons. While Na^+-independent Cl^-/HCO_3^- antiport is usually activated at more alkalotic conditions (Kurtz & Golchini, 1987; Brett *et al*., 2002), Na^+-dependent Cl^-/HCO_3^- exchange acts as an acid extruder driven by the sodium gradient (Baxter & Church, 1996). Both transporters are influenced by the initial steady-state pHi of the cell, such that non-selective inhibition of Cl^-/HCO_3^- exchange could decrease or increase pHi depending on initial pHi (Brett *et al*., 2002).

To demonstrate the effects of TPM on both types of Cl^-/HCO_3^- exchange, we removed Cl^- from the CO_2/HCO_3^--buffered superfusate. This led to an intracellular alkalinization, indicative of a reversed Cl^-/HCO_3^- exchange and an increase in intracellular HCO_3^- concentration (Raley-Susman *et al*., 1993; de la Rosa *et al*., 2002). TPM (50 μM) significantly changed this transport (Figure 6a): The alkalotic shift rate was accelerated (Figure 6b), the maximal value of the alkalosis was doubled from 0.14 to 0.28 pH units (Figure 6c), and the pHi recovery rate upon Cl^- re-addition was increased (Figure 6b). These findings pointed to an augmentation of Cl^-/HCO_3^- exchange by TPM ($n = 5$).

To test the effect of TPM on Na^+-independent Cl^-/HCO_3^- exchange in relative isolation, we superfused hippocampal slices with Na^+-free, CO_2/HCO_3^--buffered solution. This resulted in a new steady-state pHi, which was reduced by 0.5 ± 0.14 pH units ($n = 5$). Under these conditions, application of TPM reversibly increased pHi by 0.1 ± 0.06 pH units ($n = 5$, $P = 0.029$, Figure 7), suggesting an influx of HCO_3^- in Na^+-free solution.

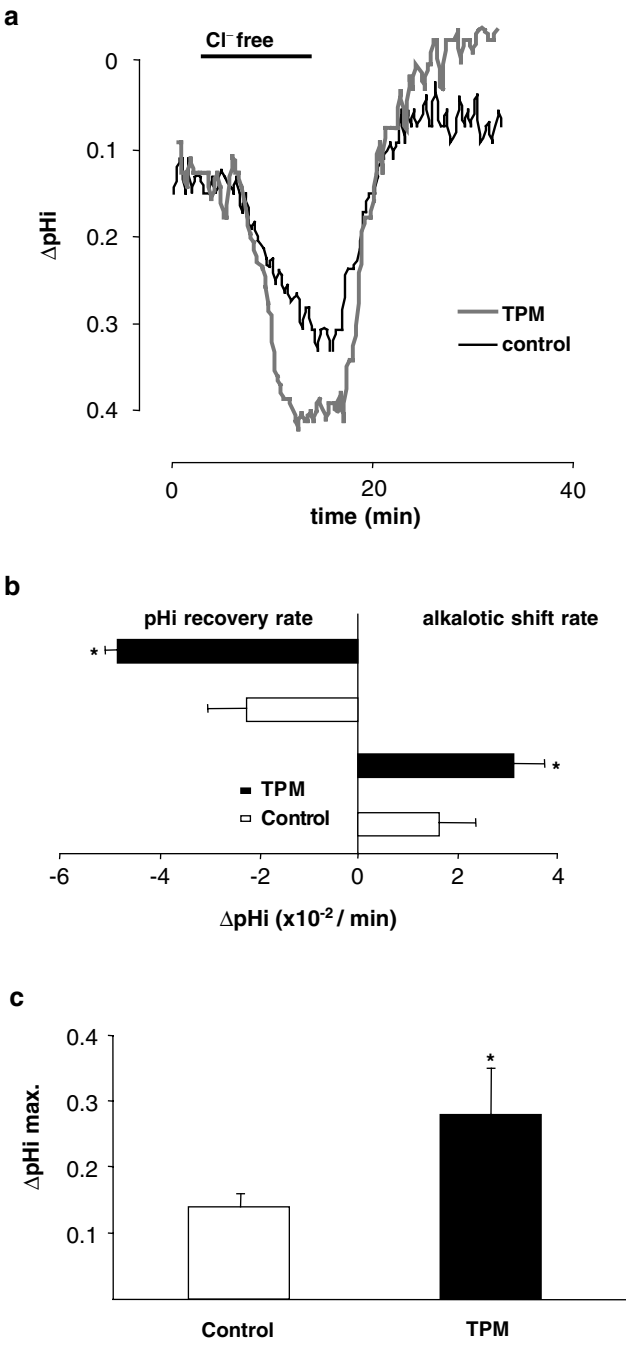

Figure 6 Effect of topiramate (TPM, 50 μM) on Cl^-/HCO_3^- exchange challenged by Cl^- removal for 10 min in hippocampal stratum pyramidale bathed in CO_2/HCO_3^--buffered solution. The pHi increase likely reflects the influx of HCO_3^-. (a) After pre-treatment with TPM for 10 min alkalotic shift rate, alkalotic plateau, and pHi recovery rate were augmented. Curves from control and TPM were superimposed. Time interval between the first and second withdrawal of Cl^- was 30 min. Starting pHi was 7.09 (control) and 7.04 (TPM), respectively. (b) Means of alkalotic shift rate, pHi recovery rate, and (c) ΔpHi max. of the type of experiment illustrated in (a) ($n = 5$). Asterisks in (b) and (c) indicate significant differences vs control ($P < 0.05$).

Discussion

This study showed that TPM at therapeutically relevant concentrations of 25–50 μM (Shank *et al*., 2000) lowers the steady-state pHi of hippocampal neurons. The underlying mechanism is based on a combined effect of TPM on carbonic anhydrase and, as demonstrated for the first time, on Na^+-independent Cl^-/HCO_3^- exchange. In line with this, the suppressive effect on epileptiform activity in the 4-AP epileptic model system could be reversed by an intracellular alkalinization with TMA. Based on our former pHi studies on epileptic model systems (Bonnet *et al*., 1998; 2000b), the anticonvulsive

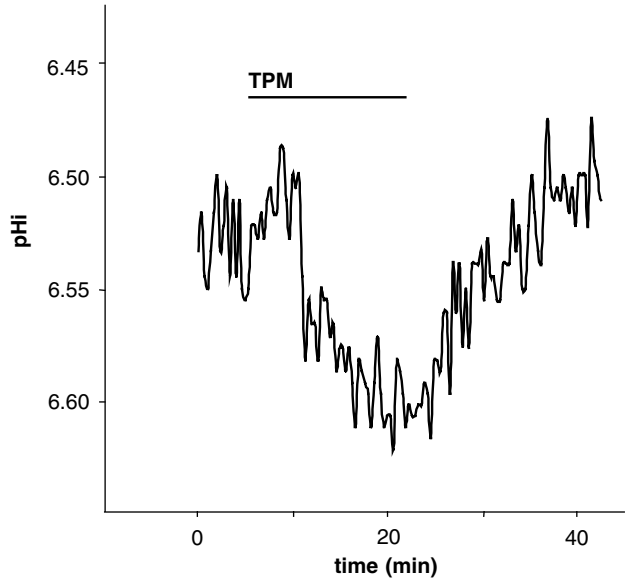

Figure 7 Effect of topiramate (TPM 50 μM) on the steady-state pHi in hippocampal stratum pyramidale bathed in Na^+-free solution. Removal of Na^+ for 30 min led to a decrease in pHi of about 0.6 pH units. Under this condition, TPM reversibly increased pHi. Trace is representative for five similar experiments.

effect of TPM is at least in part attributable to the pHi regulatory effects of TPM.

Effects of TPM on neuronal activity

With respect to the firing pattern established upon TPM-treatment (Figures 1 and 2), we found that concentrations ranging from 25 to 50 μM were effective and that even excessive concentrations (500 μM) added no further effects. This suggests that target structures of TPM responsible for the suppressive effect in the 4-AP epileptic model system were widely saturated at concentrations $\leqslant 50\,\mu$M, which is in the range of concentrations effective for the inhibition of various ion channels permeable to Na^+, Ca^{2+}, or Cl^- (White *et al*., 1997; Zona *et al*., 1997; De Lorenzo *et al*., 2000; McLean *et al*., 2000; Zhang *et al*., 2000). However, despite these multiple effects on ion channels, the shapes of action potentials and epileptiform bursts were not changed in the 4-AP epileptic model system used here. In a study on synaptic transmission (Qian & Noebels, 2003), TPM was similarly found to leave excitatory field potentials unchanged. Nevertheless, the frequency of action potentials and bursts was reduced (Figures 1 and 2), as it was found to occur also in cultured neurons (McLean *et al*., 2000). Similar to our results, this effect took several minutes to develop in the latter study. More importantly, a minor fraction of neurons did not respond. This points to basic properties, which may predefine neurons as TPM responders.

In contrast to the study of Herrero *et al*. (2002), who found a clear hyperpolarization of CA1 neurons and a decrease in input resistance upon 20 and 100 μM TPM, we and others (Hanaya *et al*., 1998) obtained no such changes. Although species- or cell-related differences may account for these differences, we believe them to be based on electrode solution. Importantly, the effect on membrane potential seen by Herrero *et al*. (2002) was mostly absent when electrodes were filled with potassium methylsulphate (as in our study), but were pronounced using potassium acetate. Considering that acetic acid leaves the cell faster than acetate anions, it is tempting to speculate that electrodes filled with buffered potassium acetate may slightly alkalize the cytoplasm. If this was true, the hyperpolarizing effect and the decrease in input resistance by TPM could be based on a pHi-mediated augmentation, for example, of potassium conductance. In the light of our results, namely an effect of TPM on pHi-regulating Cl^-/HCO_3^- exchange, the effect of acetate-filled electrodes on neuronal pHi and TPM sensitivity needs further examination. At least, it underlines the importance of the intracellular milieu for the TPM effect and, by this, makes it unlikely that the TPM effects are restricted to ion channel binding. In line with this, an inhibition of repetitive firing upon bath application of glutamate by TPM (10–100 μM) was clearly not attributable to Na^+ or Ca^{2+} channels (Hanaya *et al*., 1998).

First evidence for the view that pHi changes play a role for the anticonvulsive effect of TPM came from our experiments with TMA. This agent led to an intracellular alkalosis at constant pH of the superfusate (Figure 2c), and did not change action potentials or epileptiform bursts *per se*. TMA clearly antagonized the TPM-induced depression of bioelectric activity, thus making the firing frequency under the influence of TPM plus TMA largely comparable to control (Figure 2a and b). This speaks in favour of the view that it was indeed the intracellular alkalinization which compensated for the putative acidosis produced by TPM.

The 4-AP epileptic model system has been repeatedly used to demonstrate the inhibitory effects of intracellular acidification on epileptiform activity (Bonnet *et al*., 1998; 2000b; Leniger *et al*., 2002). In a recent study, we found that also CA inhibitors such as sulthiame and AZM lowered the pHi of hippocampal neurons, decreased the frequency of 4-AP-induced epileptiform activity, but left the shapes of epileptiform bursts largely unchanged (Leniger *et al*., 2002). These alterations were also characteristic for TPM in our study and, therefore, may be explained at least in part by the well-known property of TPM to inhibit carbonic anhydrase (Stringer, 2000; Casini *et al*., 2003).

Recently, effects of TPM on AMPA/kainate receptor channels were reported (Skradski & White, 2000; Qian & Noebels, 2003) and it was shown that TPM interfered with cAMP-dependent protein kinase pathways involved in channel activation (Shank *et al*., 2000; Angehagen *et al*., 2004). While it is unlikely that AMPA/kainate is itself inhibited by low pHi (c.f. Traynelis & Cull-Candy, 1991), the effect of TPM on cAMP-dependent protein kinases may be considered in this context because pHi-regulatory systems are also regulated by phosphorylation (see below).

Effects on intracellular pH

The change of neuronal steady-state pHi by TPM was the most striking finding in this investigation. Many different possibilities can in principal account for a neuronal acidification. Firstly, there may be an increase in cellular acid production (e.g. lactic acid) upon TPM. This appears unlikely because there was no effect upon adding TPM in Hepes-buffered solution. Secondly, neuronal acidification could

result from increased firing frequency (Trapp *et al*., 1996). However, TPM clearly reduced the firing frequency and this should result in an increase rather than a decrease of pHi. Thirdly, TPM might influence neuronal acid extrusion. In this case, one has to keep in mind that H^+ extrusion against an electrochemical gradient is necessary to maintain steady-state pHi. This transport becomes especially evident during a pHi regulation of a cellular acid load. We, therefore, tested pHi recovery by the ammonium prepulse method. Whereas in the absence of CO_2/HCO_3^- there was no impairment of pHi recovery, we found a reduction of this parameter in CO_2/HCO_3^--buffered conditions. This finding clearly showed that an inhibition of the sodium–proton exchange is not involved in TPM-mediated acidification. Further on, an impairment of Na^+-dependent Cl^-/HCO_3^- exchange (Baxter & Church, 1996) was unlikely because the effect of TPM on the pHi recovery rate in CO_2/HCO_3^--buffered solution was nearly unchanged immediately after the acidotic peak. This contrasts with the effects of classical inhibitors of Na^+-dependent Cl^-/HCO_3^- exchange such as DIDS (Yao *et al*., 1999). However, we cannot fully exclude a limited inhibitory influence of TPM on Na^+-dependent Cl^-/HCO_3^- exchange, as there was a significant effect of TPM on HCO_3^--mediated pHi regulation after ammonium washout at a pHi level more alkaline than 7.0. It is furthermore possible that TPM activates a Na^+-independent Cl^-/HCO_3^- exchange, which acts as an acid loader, since it mediates an efflux of HCO_3^- in more alkaline conditions. This would not only slow pHi recovery, but would also decrease intracellular total buffer capacity (Figure 5a1). Several observations are in line with this suggestion. In CO_2/HCO_3^--buffered solution, the alkalotic shift and the acidification rate were increased by TPM, and this may be explained by a reduced buffer capacity. When Na^+-independent Cl^-/HCO_3^- exchange was forced to operate in reversed mode by removal of extracellular Cl^- (Raley-Susman *et al*., 1993), we found that TPM reversibly augmented this transport, leading to a pronounced alkalosis (Figure 6). Further on, in the absence of extracellular Na^+ – which resulted in a decreased pHi – TPM imposed an alkalosis even in the absence of any further driving force (Figure 7). This increase of pHi is most likely due to an influx of HCO_3^-, as in acidotic conditions the Na^+-independent Cl^-/HCO_3^- exchanger can reverse its polarity (Schwartz *et al*., 2002). This may also explain the decreased acidotic peak after washout of ammonium chloride (Figure 5a).

It is not yet clear how TPM exerts this effect on Na^+-independent Cl^-/HCO_3^-. However, using the phosphatase inhibitor OA, we obtained alterations of the pHi deflection upon NH_4^+ application, which were largely similar to those evoked by TPM, namely a lowered acidotic peak and a lowered pHi recovery rate at more alkaline pH (Figure 5b1). These changes in pHi regulation cannot be attributed to an inhibition of carbonic anhydrase, as this would increase both values due to a lowered buffer capacity.

Aside from these similar pHi changes induced by TPM and OA, there is further evidence speaking in favour of the assumption that TPM effects are mediated at least in part by altered phosphorylation: (i) TPM has been shown to interfere with phosphorylation sites of ion channels (Shank *et al*., 2000; Angehagen *et al*., 2004), (ii) OA has been described to activate Na^+-independent Cl^-/HCO_3^- exchange (de la Rosa *et al*., 2002), and (iii) Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchange is modulated by protein kinase A (PKA) in a pHi-dependent fashion (Brett *et al*., 2002). The latter study is of particular importance as it showed that in the presence of extracellular Na^+ and bicarbonate either activation or inhibition of PKA alkalized neurons. Therefore, TPM cannot be regarded as a PKA activator or inhibitor. However, in the absence of extracellular Na^+, which presumably isolates the Na^+-independent Cl^-/HCO_3^- exchanger, PKA activation/inhibition clearly decreased/increased steady-state pHi in acidic neurons (Brett *et al*., 2002). In our study, TPM increased pHi in Na^+-free solution (at pHi lowered to 6.55, see Figure 7), suggesting that the flux of HCO_3^- *via* Na^+-independent Cl^-/HCO_3^- exchange was stimulated by TPM under these conditions. This appears equivalent to the effect of the PKA inhibitor Rp-cAMPS on acidotic neurons under Na^+-free condition (Brett *et al*., 2002). However, as Rp-cAMPS alkalizes neurons, we suggest that TPM does not directly inhibit PKA, but may exert inhibitory effects on the phosphorylation of Cl^-/HCO_3^- exchanger, as has been shown for certain ion channels (Angehagen *et al*., 2004).

Cells not responding to TPM

As has also been found by others (Skradski & White, 2000), a minor fraction of neurons did not respond to TPM during electrophysiological experiments. We propose that the pHi of these non-responders was not changed by TPM, as it was indeed observed with a similar frequency during the pHi measurements. Indirect evidence underlining this proposal comes from previous experiments showing that all neurons were inhibited when pHi was decreased by physicochemical means (Bonnet *et al*., 1998; 2000c; Bonnet & Wiemann, 1999; Leniger *et al*., 2002). Furthermore, neurons having a pHi of about pH 7.15–7.2 were largely insensitive to a modulation of Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchange (Brett *et al*., 2002). This pHi value, although determined in cultured neurons from newborn rat, may have some relevance for TPM effects on human brain, where pHi values in the range of 7.0–7.1 have been measured by ^{31}P-NMR (Garcia *et al*., 1994; Chu *et al*., 1996).

In conclusion, we found that TPM reduced the steady-state pHi of CA3 neurons in slices. This effect was most likely due to lowered HCO_3^- concentration inside cells and was caused not only by a decreased carbonic anhydrase activity but also by an augmentation of Na^+-independent Cl^-/HCO_3^- exchange. The resulting decrease of pHi was sufficient to explain the suppressive effects of TPM in the 4-AP epileptic model system. In line with this hypothesis, TPM-induced changes of epileptiform activity could be reversed by an alkalosis.

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