Cyclothiazide Prolongs Low [Mg^{2+}]–Induced Seizure-Like Events

Bálint Lasztozi and Julianna Kardos
Department of Neurochemistry, Institute of Biomolecular Chemistry, Chemical Research Center, Hungarian Academy of Sciences, Budapest, Hungary

Submitted 16 March 2006; accepted in final form 15 August 2006

Lasztozi, Bálint and Julianna Kardos. Cyclothiazide prolongs low [Mg^{2+}]–induced seizure-like events. J Neurophysiol 96: 3538–3544, 2006. First published August 16, 2006; doi:10.1152/jn.00287.2006. Here we address the effects of cyclothiazide (CTZ), an allosteric inhibitor of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor desensitization, on low [Mg^{2+}]–induced seizure-like events (SLEs) recorded from the CA3 pyramidal layer of juvenile rat hippocampal slices. CTZ (100 μM) made the period of tonic-like discharges (161 ± 18% of control) and the whole SLE (151 ± 15% of control) longer (in 7 of 9 slices) or induced endless SLE by stabilizing clonic-like bursting (in 2 of 9 slices). CTZ (30 μM) had no significant effects on SLE dynamics (n = 4), whereas 300 μM CTZ induced endless SLEs in four of eight slices. Coapplication of CTZ (100 μM) with 100 μM GYKI-52466, the allosteric inhibitor of AMPA receptor function, restrained the effects of CTZ and shortened SLEs and their tonic phases to 37 ± 4.2 and 47 ± 4.2% of the control, respectively. Effects of GYKI-52466 and GYKI-52466 with CTZ on SLE dynamics were indistinguishable. 4-aminopyridine (4-AP; 50 μM) alone (n = 5) or in combination with CTZ (n = 6) transformed recurrent SLE pattern into incessant epileptiform activity with patterns distinguishable from those under 100 μM CTZ application. The effect of 4-AP may suggest a role for facilitated presynaptic glutamate release in disrupting recurrent dynamics. In contrast, the self-similar slow-down of low [Mg^{2+}]–induced SLE dynamics by CTZ indicate AMPA receptor desensitization as a parameter shaping SLEs.

INTRODUCTION

Recurrent epileptic seizures in vivo and seizure-like events (SLEs) in vitro are characterized by nonstationary trains of synchronized neuronal discharges (Frasnaczuk et al. 1998; McCormick and Contreras 2001; Nyikos et al. 2003; Schiff et al. 2000). It is now widely accepted that a condition characterized by increased neuronal excitability (McCormick and Contreras 2001), decreased effectiveness of inhibitory inputs (Dzhala and Staley 2003; McCormick and Contreras 2001; Perez Velazquez 2003), and emergent synaptic and nonsynaptic coupling of neuronal cell assemblies (Bikson et al. 2003; Khoshmarani et al. 2005; Lasztoczi et al. 2004; Timofeev and Steriade 2004; Traub et al. 2001) may possibly serve the appearance of a seizure. However, equally important, less studies have been undertaken to identify factors that act to sustain or dissipate the state of the seizure. Before and during seizures, an increase in the extracellular concentration of glutamic (iGlut) is documented in vivo (During and Spencer 1993) and can also be conjectured in brain slices (Demarque et al. 2004; Lee et al. 2002). Besides contributing to the SLE waveform by activating various Glu receptors (GluR) (Lee et al. 2002; Lücke et al. 1996; Swann et al. 1993; Traub et al. 1996), such a [Glut] transient could also act to desensitize GluRs, which may in turn have profound effects on the SLE dynamics. Ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors desensitize quickly in response to relatively low (a few micromolar) ambient [Glut] (Hausser and Roth 1997; Robert and Howe 2003; Sun et al. 2002; Zorumski et al. 1996), a process inhibited by cyclothiazide (CTZ) (Brauner-Osborne et al. 2000; Diamond and Jahn 1995; Ishikawa and Takahashi 2001; Partin et al. 1993; Sun et al. 2002; Yamada and Tang 1993). In this study, we tested the hypothesis that AMPA receptor desensitization shapes SLE dynamics, thereby regulating SLE duration. This was addressed by measuring the effects of CTZ (30, 100, and 300 μM) on low [Mg^{2+}]–induced SLEs recorded from the CA3 pyramidal layer of juvenile (P9–P13) rat hippocampal slices. Besides the inhibition of AMPA receptor desensitization, CTZ also increases Glu release (Diamond and Jahn 1995; Ishikawa and Takahashi 2001) and inhibits GABA_A receptors (Deng and Chen 2003). To distinguish between these effects, CTZ was applied in combination with the allosteric inhibitor of AMPA receptor function (GYKI-52466), and CTZ effects were compared with the effects of 4-aminopyridine (4-AP) a drug that enhances presynaptic Glu release by blocking K^+ ion channels.

METHODS

All animals were kept and used in accordance with the European Council Directive of 24 November 1986 (86/609/EEC) and with the Hungarian Animal Act 1998 and associated guidelines. All efforts were made to minimize animal suffering and the number of animals used. Hippocampal slices were prepared, and the recordings were performed as described earlier (Lasztoczi et al. 2004, 2006; Walther et al. 1986). Briefly, 400-μm-thick slices containing the hippocampus proper, the subiculum, the entorhinal cortex, and attached parts of the cortex were prepared from male Wistar rats (Toxicoop, Budapest, Hungary) on P9–P13 in ice-cold preparing solution (in mM: 87 NaCl, 75 sucrose, 25 glucose, 2.5 KCl, 1.25 NaH2PO4, 7 MgSO4, 0.5 CaCl2, and 25 NaHCO3). Slices were incubated for 1 h at 36 °C and thereafter stored at 25 °C under humidified carbon gas atmosphere in an interface-type holding chamber containing artificial cerebrospinal fluid (ACSF; in mM: 129 NaCl, 10 glucose, 3 KCl, 1.23 NaH2PO4, 1.8 MgSO4, 1.6 CaCl2, and 21 NaHCO3; pH 7.4 when equilibrated with 5% CO2–95% O2 gas mixture). Field potential changes were recorded (Multiclamp 700A amplifier, Axon Instruments, Foster City, CA) in a submerge-type recording chamber with glass microelectrodes (4 to 8 MΩ resistance, filled with ACSF) inserted into the CA3 stratum pyramidalis. Data were low-pass filtered at 1 kHz and digitized at 10 kHz (Digitida1320A, Axon Instruments). Epileptiform activity was induced at 36°C by changing the
superfusing solution to low-[Mg$^{2+}$] ACSF (ACSF without added Mg$^{2+}$ ions and [K$^+$] raised to 5 mM). After three SLEs (control), the drugs were applied through the superfusing solution. For testing the pharmacological effects of substances, appropriate amounts of stock solutions of CTZ (100 mM in DMSO), 4-AP (100 mM in water), and GYKI-52466 (10 mM in 0.1 N HCl) were added to low-[Mg$^{2+}$] ACSF before the experiment started. To keep 300 μM CTZ in solution, final DMSO content was raised to 0.5% in the superfusing ACSF solution. Application of the solvent alone had no effect on SLE duration and dynamics (Lasztóczí et al. 2006). All solutions were continuously bubbled with 5% CO$_2$-95% O$_2$ gas mixture. All drugs and other chemicals were purchased from Tocris (Bristol, UK) or Sigma-Aldrich (Budapest, Hungary). Drugs were applied for three (GYKI-52466 and GYKI-52466 + CTZ) or four (CTZ, 4-AP, and CTZ + 4-AP) SLEs or an equivalent time period extrapolated from the control inter-SLE interval. This application protocol was chosen on the basis of our previous findings (Lasztóczí et al. 2006; Nyikos et al. 2003), showing no change in SLE durations and inter-SLE intervals for the first eight consecutive SLEs (see Fig. 1 of Nyikos et al. 2003).

Statistical significance of the differences was assessed by one-sample or independent Student’s t-test, applied to data normalized to control values, with P < 0.05 taken as significant. If not indicated in the text differently, data are expressed as mean ± SE. For data analysis, plotting and preparation of the figures, Clampfit 9 (Axon Instruments), Origin 6.1 (OriginLab, Northampton, MA), and CorelDraw 9 (Corel, Dallas, TX) software was used. To obtain time-frequency plots, wavelet transformation was done in Matlab 6.1 environment (The MathWorks, Natick, MA) as described earlier (Lasztóczí et al. 2004; Nyikos et al. 2003). In this study, wavelet transformation was performed by 100 logarithmically equidistant frequencies from 0.5 to 50 Hz using scripts provided by Lajos Nyikos. Power spectra were calculated from the same 200-s segments, by applying Fourier transformation, with a resolution of 0.15 Hz (Clampfit 9, Axon Instruments). Control power spectra of 200-s-long segments recorded under normal ACSF perfusion were subtracted from each spectrum. Spectra were plotted from 0.3 to 25 Hz.

RESULTS

The first SLE appeared within 741 ± 294 (SD) s (n = 40 slices from 24 animals) after switching the perfusing solution to low-[Mg$^{2+}$] ACSF. A further two SLEs were recorded to obtain a control period, during which the SLE durations and intervals were 93 ± 22 and 542 ± 184 s, respectively. The dynamical pattern of recurrent SLEs (Fig. 1A) was similar to that observed earlier (Anderson et al. 1986; Lasztóczí et al. 2004, 2006; Nyikos et al. 2003). Few preictal paroxysmal spikes (PSs; white arrowheads in Fig. 1B1) preceded the SLE onset (black arrowhead in Fig. 1B1). The SLE started with tonic-like discharges transforming into clonic-like burst activity. The approximate point of the tonic-to-clonic transition is indicated by the arrow on the top trace of Fig. 1A and is also shown on an expanded time scale in Fig. 1B3. Relatively fast oscillation of a simple waveform in the tonic phase (Fig. 1B2) occupying the first 29 ± 7.8 s of the SLE (mean ± SD; n = 40 slices from 24 animals) was disrupted at the tonic-to-clonic transition (Fig. 1B3), followed by the appearance of intermittent bursting activity in the clonic phase (Fig. 1B4). Time-frequency plot of the SLE in Fig. 1A below the trace showed a distinct continuous light band declining from 10–20 to 2–5 Hz, breaking up at the tonic-to-clonic transition (white arrow) identified on the basis of the waveform transition in field potential recording (c.f. positions of black and white arrows in Fig. 1A).

After the first three control SLEs had been registered, CTZ (100 μM) was added to the perfusing solution (n = 9 slices from 9 animals) for the time of the next four SLEs (1,295 ± 262 s). In seven of nine slices, recurrent SLEs were preserved throughout CTZ application, however, SLEs were lengthened in a self-similar manner (Fig. 2A). In two of nine slices, the third or the fourth SLE under CTZ application did not end for >60 min, even though the CTZ application was discontinued (Fig. 2B). This CTZ-induced activity appeared as an endless continuation of the clonic phase of the last SLE (Fig. 2E). Time-frequency plots showed that on CTZ application the frequency decay in the tonic phase slowed down thereby lengthening the tonic phase (Fig. 2A and B). The duration of the tonic phase increased somewhat faster than the duration of the SLE (Fig. 3), reaching significant level at the second SLE of CTZ application (P < 0.05; one-sample t-test). Both measures further increased until the fourth SLE of CTZ application (tonic phase duration: 161 ± 18% of control; SLE length: 151 ± 15% of control). The comparison of frequency spectrum of SLEs under control conditions and CTZ application did not disclose major alterations except for an increase of power in
the 1- to 15-Hz range in some cases (Fig. 2A, right). To estimate the dose dependence of CTZ action, effects of 30 and 300 μM CTZ were also studied. CTZ (30 μM) had no significant effects on the duration of either the tonic phase or the SLE (104 ± 20 and 93 ± 14% of the control, respectively; n = 4 slices from 2 animals, 1-sample t-test). CTZ (300 μM; n = 8) caused a significant increase in the tonic phase duration immediately after the onset of the first SLE in CTZ (218 ± 32%; n = 8 slices from 4 animals, one-sample t-test) and induced endless epileptiform activity in four of eight slices, with tonic- (3 slices) or clonic-like (1 slice) activities being dominant. In the other half of slices, SLEs were prolonged (to 238–793% of the control). Preictal PS activity was not affected by 30 or 100 μM CTZ, but SLEs lacking preictal PSs were occasionally observed in the presence of 300 μM CTZ.

To reveal if CTZ would affect SLE duration through inhibition of GABA_A receptor function (Deng and Chen 2003), CTZ (100 μM) was also applied under AMPA receptor blockade using the allosteric inhibitor of AMPA receptor function GYKI-52466 (100 μM). Opposite to the effect of CTZ applied alone, the duration of the SLEs and of their tonic phase were decreased (37 ± 4.2 and 47 ± 4.2% of the control level, respectively; 3rd SLE of drug application; P < 0.05; one-sample t-test; n = 4 slices from 3 animals). In addition, the preictal PS activity disappeared under the combined application of 100 μM CTZ and 100 μM GYKI-52466. Similarly, GYKI-52466 (100 μM) decreased the duration of SLEs and of their tonic phase (43 ± 4.8 and 49 ± 4.9% of the control, respectively; 3rd SLE of drug application; P < 0.05; one-sample t-test; n = 4 slices from 4 animals) and blocked preictal PSs (Laszto´czi et al. 2006). Effects of GYKI-52466 (Laszto´czi et al. 2006) and CTZ with GYKI-52466 (this study) were indistinguishable (P > 0.05, independent t-test), indicating that CTZ acts through a GYKI-52466-sensitive component process. Possibly mediated through the inhibition of presynaptic K^+ ion channels (Ishikawa and Takahashi 2001), the CTZ-induced increase of presynaptic Glu release (Diamond and Jahr 1995; Ishikawa and Takahashi 2001) could also prolong SLEs in a GYKI-52466-sensitive manner. To see how the CTZ-induced Glu release would affect SLE dynamics, 4-AP (50 μM), a
compound known to increase Glu release without affecting AMPA receptor desensitization, was compared using the CTZ application protocol. The first SLE under 4-AP application started like a control SLE with tonic phase unchanged (96 ± 8.9% of the control; P > 0.05; one-sample t-test; n = 5 slices from 4 animals) followed by a clonic phase activity (top trace of Fig 4 and 4B; n = 5 slices from 4 animals). After 3–5 min, the clonic phase activity smoothly transformed into frequently recurring (interburst intervals of 0.5–20 s), irregularly paced intermittent bursts activity (see representative example in Fig. 4C). Time-frequency plots indicated that this activity contained intermittent bursts activity (see representative example in Fig. 4C). Accordingly, the power spectra showed the highest power at low frequencies (0.5–1 Hz), with the power monotonically decreasing toward the higher frequencies. When CTZ (100 μM) was added together with 30 μM 4-AP (n = 6 slices from 5 animals; Fig. 5), the frequency decay of the tonic phase was slowed, and the average duration of the tonic phase increased (189 ± 19.9%, P < 0.05; one-sample t-test). After the tonic-to-clonic transition, this SLE evolved first into a prolonged clonic phase (Fig. 5B), and (after 3–5 min) smoothly but incessantly transformed into a sustained synchronized activity represented on Fig. 5C. This synchronized activity was of tonic-like character, with episodes of simple-waveform oscillations building up and fading away and appeared in wavelet plots as long, horizontally oriented white bands at 2–5 Hz (Fig. 5C). Interestingly, this frequency corresponded to the frequency at which the tonic-to-clonic transition occurred in the SLE (Fig. 5, B vs. C). The tonic nature of this activity was also supported by the power spectra (Fig. 5C) that disclosed distinct peaks in the 2 to 5 Hz range of frequencies.

**DISCUSSION**

Here we present data on the effects of CTZ on low [Mg2+]–induced spontaneously recurrent SLEs in juvenile (P9–P13) rat hippocampal slices. By slowing down the frequency decay of the tonic phase discharges, CTZ increased the tonic phase duration, delayed SLE termination, and occasionally made the SLEs endless. The effect of CTZ was dose dependent. Our data show that a CTZ-sensitive component process exists that is operational during SLEs, shapes their dynamics, and regulates their duration. The proepileptic effect of CTZ reported here is in line with previous observations on the induction or facilitation of epileptiform activity by this compound in vitro (Qi et al. 2006) and in vivo (Fornai et al. 2005; Qi et al. 2006; Yasuda et al. 2000).

All the known effects of CTZ may underlay such a proepileptic effect. By binding to an allosteric site of the AMPA receptor and modifying its conformational equilibrium (Kovacs et al. 2004; Nakagawa et al. 2005; Sun et al. 2002) CTZ inhibits AMPA receptor desensitization that normally occurs within a few milliseconds in response to Glu exposure (Brauner-Osborne et al. 2000; Diamond and Jahr 1995; Ishikawa and Takahashi 2001; Partin et al. 1993; Szarics et al. 1999; Yamada and Tang 1993). Other mechanisms may possibly include the direct inhibition of GABAA receptor–mediated inhibitory currents (Deng and Chen 2003) and the presynaptic enhancement of Glu release (Diamond and Jahr 1995; Ishikawa and Takahashi 2001).

Addressing the mechanism of action of CTZ, we took advantage of the persistence of low [Mg2+]–induced SLEs against AMPA receptor blockade (Lasztoczi et al. 2006), allowing us to test CTZ effects in the virtual absence of functional AMPA receptors. Assuming that the observed CTZ effects on SLEs documented here relied on a component process different from AMPA receptor desensitization, coapplication of CTZ with the AMPA receptor antagonist GYKI-52466 and GYKI-52466 alone should have been different. Contrasting this expectation, the slowing of SLE dynamics and the prolongation of SLEs by CTZ was not observed under AMPA receptor blockade that left, however, some shortened SLEs alive. These findings suggest that AMPA receptor activation is not a prerequisite of ictogenesis in the present model (Lasztoczi et al. 2006), but shapes SLE dynamics.

The absence of CTZ effect in the presence of GYKI-52466, however, does not rule out a CTZ-induced presynaptic facilitation of the Glu release (Diamond and Jahr 1995; Ishikawa and Takahashi 2001) as a potential mediator of the CTZ effects on SLE duration and dynamics. We addressed this issue by comparing the effects of CTZ and 4-AP on SLEs. Like CTZ, 4-AP enhances Glu release presynaptically (Gu et al. 2004; Ishikawa and Takahashi 2001; Qian and Saggau 1999) but does not affect AMPA receptor currents directly (Gu et al. 2004). 4-AP had no effect on the tonic phase duration, and its
prolonged application resulted in a sustained, irregular bursting activity with occasional “mini-SLEs.” The increased Glu release and neuronal excitability can result in a sustained seizure-prone state implying the existence of an endogenous seizure-suppression mechanism. Indeed, the accumulation of endogenous adenosine during seizures and SLEs (Avsar and Empson 2004; During and Spencer 1992; Lücke et al. 1996; Slezia et al. 2004) and the consequent decrease in Glu release and neuronal excitability (Malva et al. 2003; Thompson et al. 1992) have been shown to silence neuronal network (Avsar and Empson 2004; During and Spencer 1992; Lücke et al. 1996; Malva et al. 2003).

Qi et al. (2006) suggested that the facilitation of Glu release by CTZ might be primarily responsible for the induction of epileptiform activity in hippocampal cell cultures. Facilitated Glu release, however, may not account for the major effect found with CTZ application here, i.e., the self-similar slowdown of SLE dynamics. Moreover, coapplication of CTZ with 4-AP resulted in a dramatic prolongation of the tonic phase first, followed by the development of virtually endless tonic phase-like activity, an effect markedly different from the effect of either drug applied alone. Our data suggest that CTZ acts to slow down SLE dynamics through inhibition of AMPA receptor desensitization, whereas the facilitation of Glu release may contribute to the delay or inhibition of SLE termination.

The role of AMPA receptor desensitization in shaping excitatory synaptic currents under baseline conditions is debated (Arai and Lynch 1998; Hjelmstad et al. 1999; Otis et al. 1996). On the basis of theoretical calculations and recordings of glial transporter currents, the extracellular [Glu] in response to a single presynaptic action potential may exceed 10 μM for a few milliseconds (Bergles et al. 1997; Diamond 2005). Although the amplitude and time-course of the [Glu] transient during an SLE is not known, the excessive high-frequency presynaptic activation during SLEs (Khoshhravani et al. 2005; Lasztóczi et al. 2004) may result in prolonged and increased extracellular [Glu] transients (During and Spencer 1993; Lee et al. 2002; Swann et al. 1993) compared with basal activity. AMPA receptor desensitization occurs within the subsecond range of time (Fucile et al. 2006; Häusser and Roth 1997;
Robert and Howe 2003; Sun et al. 2002; Szárics et al. 1999; Zorumski et al. 1996). The question arises how the inhibition of AMPA receptor desensitization by CTZ may perform a self-similar slow-down of SLE dynamics, a phenomenon orders of magnitude slower? Our hypothesis that gives a possible reason for the observed CTZ-sensitive change in SLE dynamics (discharge frequency, waveform, and duration) is that desensitization (Hausser and Roth 1997; Robert and Howe 2003; Zorumski et al. 1996) induced by slowly increasing extracellular [Glu] during SLEs progressively decreases the population of functional AMPA receptors (see Zorumski et al. 1996). The hypothesis allows other processes affecting Glu release and cellular excitability, including desensitization of N-methyl-D-aspartate (NMDA) receptors (Traub et al. 1994, 1996), K⁺ ion-induced depolarization block (Bragin et al. 1997), intracellular acidification (Xiong et al. 2000), upregulation of Na⁺/K⁺ pump (Konnerth et al. 1986), and progressive accumulation of endogenous adenosine (During and Spencer 1992; Etherington and Frenguelli 2004) to shape SLE dynamics.

**GRANTS**

This work was supported in part by Grants MediChem2 1/A/005/2004 National Office for Research and Technology MediChem2 (Hungary) and Transporter Explorer AKF-050068 (European Union).

**REFERENCES**


**FIG. 5.** Effects of combined application of CTZ and 4-AP on SLEs. Representative field potential recording of epileptiform activity from the CA3 s. pyramidale before, during, and after CTZ + 4-AP application (n = 6). Time of drug application is indicated above the trace. Periods (200 s) representative of a control SLE (SLE3), the 1st SLE after combined CTZ + 4-AP application (SLE4), and a prolonged discharge pattern of 4-AP application are marked below the trace and expanded in A, B, and C subplots, respectively. Corresponding time-frequency plots and power spectra are shown below traces and on right, respectively. White arrows point to tonic-to-clonic transition point in A and B. Note that combined application of CTZ with 4-AP slowed tonic phase progression and delayed tonic-to-clonic transition. Boxed parts of trace in C are further expanded below time-frequency plot and represent typical discharge pattern observed after prolonged coapplication of CTZ and 4-AP.
Adenosine: a potential mediator of seizure
During MJ and Spencer DD.
epileptic seizures.
On the cellular and network bases of
Nonsynaptic epileptogenesis in the
Eur J Neurosci
Neuroscience


