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Carbonic anhydrase inhibition by acetazolamide reduces *in vitro* epileptiform synchronization

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ABSTRACT

Depolarizing $GABA_A$ receptor-mediated currents are contributed by HCO_3^- efflux, and play a role in initiating ictal-like epileptiform events in several cortical structures supporting the view that $GABA_A$ receptor signaling actively participates to epileptiform synchronization. We employed here field potential recordings to analyze the effects of the carbonic anhydrase inhibitor acetazolamide (10 μM) on the epileptiform activity generated *in vitro* by piriform and entorhinal cortices (PC and EC, respectively) during application of the K^+ channel blocker 4-aminopyridine (4AP, 50 μM). Under these experimental conditions ictal- and interictal-like discharges along with high-frequency oscillations (ripples: 80–200 Hz, fast ripples: 250–500 Hz) occurred in these two regions. In both PC and EC, acetazolamide: (i) reduced the duration and the interval of occurrence of ictal discharges along with the associated ripples and fast ripples; (ii) decreased the interval of occurrence of interictal discharges and the rates of associated fast ripples; and (iii) diminished the duration and amplitude of pharmacologically isolated GABAergic events while increasing their interval of occurrence. Our results indicate that acetazolamide effectively controls 4AP-induced epileptiform synchronization in PC and EC. We propose that this action may rest on decreased $GABA_A$ receptor-mediated HCO_3^- efflux leading to diminished depolarization of principal cells and, perhaps, of interneurons.

1. Introduction

The role of $GABA_A$ receptor-mediated signaling in epilepsy has been questioned for a long time. Early work in the *in vitro* hippocampal slice preparation demonstrated that interictal epileptiform activity results from weakened inhibition (Schwartzkroin and Prince, 1978, 1980; Johnston and Brown, 1981) but later, prolonged epileptiform discharges were identified during experimental procedures that enhance GABAergic signaling such as the K^+ channel blocker 4-aminopyridine (4AP) or Mg^{2+} free medium (Avoli, 1990; see for review Avoli and de Curtis, 2011), thus underscoring different and possibly conflicting roles of $GABA_A$ receptor-mediated inhibition in epileptiform synchronization.

$GABA_A$ receptor activation opens channels that are permeable to Cl^- and to a lesser extent to HCO_3^-. In adulthood the $GABA_A$ receptor-mediated current is hyperpolarizing thanks to the activity of a cation chloride cotransporter that maintains intracellular $[Cl^-]$ low (Farrant and Kaila, 2007; Blaesse et al., 2009). Another prerequisite for hyperpolarization is that at resting membrane potential the $GABA_A$ receptor-mediated HCO_3^- inward current is not larger than the Cl^- outward current (Kaila, 1994; Rivera et al., 2005). Although, GABA principally induces hyperpolarizing inhibition in postsynaptic neurons, it can also exert depolarizations (Andersen et al., 1980; Alger and Nicoll, 1982). Relevant to the field of epilepsy research, $GABA_A$ receptor-mediated signaling has been shown to be involved in the initiation and maintenance of ictal discharges (see for review Avoli and de Curtis, 2011). Moreover, several studies have suggested that "depolarizing GABA" is contributed by HCO_3^- efflux (Grover et al., 1993; Perez Velazquez, 2003; Ruusuvuori et al., 2004). Specifically, prolonged activation of $GABA_A$ receptors should lead to an excessive load of Cl^- into the postsynaptic neuron resulting in degradation of the Cl^- driving force (Staley et al., 1995). Under these conditions, HCO_3^- current would become dominant thus depolarizing the postsynaptic neuron since the intracellular HCO_3^- is consistently replenished by the carbonic anhydrase activity (Kaila et al., 1997; Voipio and Kaila, 2000).

Networks of inhibitory GABAergic interneurons can promote synchronicity in many brain structures due to their high connectivity ratio (Jefferys et al., 2012a). This synchronous GABAergic

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signaling has also been proposed to play a role in the generation of high frequency oscillations (HFOs) that are recorded in the EEG of epileptic patients and in animal models of temporal lobe epilepsy (see for review Jefferys et al., 2012b). HFOs can also be recorded *in vitro* in brain structures such as the piriform cortex (PC) and the entorhinal cortex (EC) (Avoli et al., 2013; Hamidi et al., 2014; Herrington et al., 2014) during application of 4AP that is known to enhance both excitatory and inhibitory transmitter release.

To date the role played by the $GABA_A$ receptor-mediated HCO_3^- efflux in epileptiform synchronization and in particular its participation to ictogenesis remains unclear. Therefore, in this study we investigated the effects induced by the carbonic anhydrase inhibitor acetazolamide on the epileptiform discharges and associated HFO that are generated by rat olfactory (PC) and limbic (EC) cortical networks maintained *in vitro* during 4AP treatment.

2. Methods

2.1. Brain slice preparation and maintenance

Male, adult Sprague-Dawley rats (250–275 g) were decapitated under isoflurane anesthesia according to the procedures established by the Canadian Council of Animal Care. The brain was quickly removed and placed in cold (1–3 °C), oxygenated artificial cerebrospinal fluid (ACSF) with the following composition (mM): 124 NaCl, 2 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 1.25 KH_2PO_4, 26 $NaHCO_3$, 10 D-glucose. Horizontal brain slices (450 μm) containing PC and EC were cut from this brain block using a vibratome. Slices were then transferred to an interface tissue chamber where they were superfused with ACSF and humidified gas (95% O_2, 5% CO_2) at a temperature of 31–32 °C and a pH of 7.4. In nominally CO_2/HCO_3^--free N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-buffered medium, NaHCO3 was replaced by 20 mM HEPES, and the solution was gassed with 100% O_2; in these experiments the pH was adjusted to 7.4 with NaOH. 4AP (50 μM), acetazolamide (ACTZ, 10 μM), benzolamide (BA, 10 μM), 3, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP, 10 μM), 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 10 μM) and picrotoxin (PTX, 100 μM) were bath applied. Chemicals were acquired from Sigma–Aldrich Canada (Oakville, Ontario, Canada) except for BA that was generously provided by Dr. Kai Kaila at the University of Helsinki (Helsinki, Finland).

2.2. Electrophysiological recordings

Field potential recordings, which were sampled at 5000 Hz were obtained 50 min after the application of 4AP with ACSF-filled, glass pipettes (1B150F-4; World Precision Instruments, Sarasota, Florida, USA; tip diameter <10 μm, resistance 5–10 MΩ) that were connected to high-impedance amplifiers. After 25–40 min recordings in the presence of 4AP, any given drug was applied and recordings were continued for 60–90 min. The recording electrodes were positioned in the deep layers of the posterior PC and of the lateral EC. Field potential signals were fed to a computer interface (Digidata 1322A, Molecular Devices, and Palo Alto, CA, USA), acquired and stored using the PCLAMP 9.2 software (Molecular Devices). Subsequent data analyses were performed with CLAMPFIT 9.2 (Molecular Devices).

2.3. Detection of high-frequency oscillatory events

Time-periods containing ictal and interictal discharges recorded from the PC and EC were extracted. To identify oscillations in each frequency range (80–200 Hz and 250–500 Hz), a multi-parametric algorithm was employed using routines based on standardized functions (Matlab Signal Processing Toolbox) as described in detail in Salami et al. (2012) and Hamidi et al. (2014).

To be considered as HFOs, oscillatory events in each frequency range had to show at least four consecutive cycles, having amplitude of 3 SD above the mean of the reference period. The time lag between two consecutive cycles had to be between 5 and 12.5 ms for ripples (80–200 Hz) and between 2 and 4 ms for fast ripples (250–500 Hz). Oscillatory events containing overlapping ripples and fast ripples were excluded from the analysis (Bénar et al., 2010).

2.4. Statistical analysis

We used CLAMPFIT 9.2 (Molecular Devices) for offline analysis of the duration and interval of occurrence of ictal and interictal discharges. To segregate ictal from interictal discharges, we applied the k-means clustering algorithm with squared euclidean distances on the duration of all recorded events. Appropriate clustering of data was obtained with two groups, with a cutoff at 3 s. Therefore, all events ≥3 s were considered as ictal activity, whereas those lasting <3 s were considered as interictal events (The distribution of the event duration is summarized in Fig. 2A). These values are similar to those "arbitrarily" chosen by Traub et al. (1996). The same cutoff at 3 s was also used to segregate ictal and interictal discharges after application of ACTZ. The duration of both ictal and interictal discharges were defined as the time between the first deflection of the discharge from baseline to its return to baseline. The interval of occurrence of both ictal and interictal discharges were defined as the time between the onsets of two consecutive discharges. Amplitude of epileptiform events was measured from peak to peak. Since the kurtosis and skewness measures showed that values were not normally distributed, we transformed the raw data to Z-scores and performed One-way ANOVAs followed by Tukey post-hoc tests to identify differences between experimental conditions (i.g., 4AP alone, and 4AP+ ACTZ) in each region (PC and EC) regarding the rate and duration of ictal events and the rate, duration and amplitude of interictal discharges and isolated GABAergic events.

When analyzing the dynamics of HFO occurrence, in order to account for differences in duration, ictal discharges were transformed into a time scale from 0 (start of the ictal event) to 100 (end of the ictal event). The ictal period was then divided in three parts and rates of ripples and fast ripples in each region (PC and EC) were compared using non-parametric Wilcoxon signed rank tests followed by Bonferroni–Holm corrections for multiple comparisons. This allowed us to evaluate if ripples or fast ripples predominated at specific moments of the ictal event in different experimental conditions (i.e., 4AP alone, and 4AP+ ACTZ) in each region. Statistical tests were performed in Matlab R2012b (Matworks, Natick, MA) and the level of significance was set at $p < 0.05$. Results are expressed as Mean ± SEM and *n* indicates the number of slices used for analysis.

3. Results

3.1. Effects of carbonic anhydrase inhibition on 4AP-induced epileptiform activity

Field potential recordings – which were obtained simultaneously from PC and EC during 4AP application – revealed ictal and interictal discharges that occurred in both structures (Hamidi et al., 2014) (Fig. 1). Ictal discharges recorded from PC lasted 98.67 ± 12.02 s, and recurred every 169.01 ± 10.41 s (69 events, n = 10 experiments) whereas those occurring in EC lasted 132.31 ± 13.17 s, and recurred every 184.16 ± 10.58 s (60 events, n = 10 experiments). Interictal discharges recorded from PC (45 events, n = 10 experiments) and EC (50 events, n = 10 experiments) in these experiments lasted 1.88 ± 0.34 s and 2.01 ± 0.21 s, occurred every 57.5 ± 3.7 s and 49.9 ± 5.8 s, and had amplitudes of 1.28 ± 0.09 mV and 1.38 ± 0.18 mV, respectively.

As illustrated in Fig. 1, 20 min application of ACTZ (10 μM) disrupted the pattern of epileptiform discharge recorded from PC and EC under control conditions. Specifically, ACTZ (10 μM) abolished the prolonged ictal discharges seen under control conditions and disclosed a continuous pattern of synchronous epileptiform events lasting between 2 and 16 s. As illustrated in Fig. 2A ACTZ (10 μM) caused a significant change in the distribution of epileptiform discharge duration ($p < 0.0001$) in both areas. Using cutoff point between ictal and interictal discharges at 3 s, we were able to analyze the interval of occurrence of these events. In both PC and EC, ACTZ (10 μM) induced a significant decrease ($p < 0.0001$) in the interval of occurrence of ictal discharges to 53.9 ± 7.7 s and 82.5 ± 8.7 s, respectively (Fig. 2B, n = 10 experiments). To determine whether net effect of acetazolamide on epileptiform discharges is anticonvulsive, we compared the cumulative duration of epileptiform activity in time windows of 30 min before and during ACTZ (10 μM) application. Application of ACTZ (10 μM) reduced the cumulative duration of ictal discharges from 465.2 ± 41.6 s to 201.1 ± 46.8 s in PC and from 428.3 ± 80.7 s to 143.4 ± 22.1 s in EC (Fig. 2B, n = 10 experiments, $p < 0.01$). We also analyzed the effect of ACTZ (10 μM) on the interval of interictal discharges recorded from PC and EC. As illustrated in Fig. 2C, we found that application of ACTZ (10 μM) resulted in a reduction in the interval of occurrence of interictal discharges to 34.9 ± 3.2 s in PC and to 38.7 ± 2.7 s in the EC (Fig. 2C, n = 10 experiments, $p < 0.01$).

The role of carbonic anhydrase in the generation of the long-lasting ictal discharges was further confirmed by recordings performed in HCO_3^--free HEPES-buffered solution. Withdrawal of CO_2/HCO_3^- for 20 min completely blocked the ictal discharges in

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