Examining protection from anoxic depolarization by the drugs dibucaine and carbetapentane using whole-cell recording from CA1 neurons

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Running head: Inhibiting anoxic depolarization

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ABSTRACT

As an immediate consequence of stroke onset, failure of the Na⁺/K⁺ATPase pump evokes a propagating anoxic depolarization (AD) across gray matter. Acute neuronal swelling and dendritic beading arise within seconds in the future ischemic core, imaged as changes in light transmittance (ΔLT). AD is itself not a target for drug-based reduction of stroke injury because it is generated in the first minutes of stroke onset. Peri-infarct depolarizations (PIDs) are milder AD–like events that recur during the hours following AD and contribute to infarct expansion. Inhibiting PIDs with drugs could limit expansion. Two types of drugs, ‘caines’ and sigma-1 receptor ligands, have been found to inhibit AD onset (and may also oppose PID initiation) yet their underlying actions have not been examined.

Imaging ΔLT in the CA1 region simultaneously with whole-cell current-clamp recording from CA1pyramidal neurons reveal that the elevated LT front and onset of the AD are coincident. Either dibucaine or carbetapentane pretreatment significantly delays AD onset without affecting resting membrane potential or neuronal input resistance. Dibucaine decreases excitability by raising spike threshold and decreasing action potential (AP) frequency, whereas carbetapentane eliminates the fast afterhyperpolarization, while accentuating the slow afterhyperpolarization to reduce AP frequency. Orthodromic and antidromic APs are eliminated by dibucaine within 15 min, but not by carbetapentane. Thus, both drugs reduce cortical excitability at the level of the single pyramidal neuron, but through strikingly different mechanisms. In vivo both drugs would likely inhibit recurring PIDs in the expanding penumbra, and so potentially could reduce developing neuronal damage over many hours post-stroke when PIDs occur.

List of Abbreviations:

AD anoxic depolarization

AP action potential
52 CP carbetapentane
53 DAP depolarizing afterpotential
54 Dib dibucaine
55 fAHP fast afterhyperpolarization
56 GC granule cell
57 ΔLT change in light transmittance
58 OGD oxygen-glucose deprivation
59 SD spreading depolarization
60 sAHP slow afterhyperpolarization
61 σR sigma receptor
INTRODUCTION

Cortical neurons undergo a propagating wave of depolarization within 1-2 minutes following focal stroke onset, leading to formation of an ischemic core over hours. The extent of this ‘ischemic’ or ‘anoxic’ depolarization (AD) represents the most reliable determinant of ensuing brain damage (Kaminogo et al. 1998). As the earliest upstream event leading to necrosis in the core, AD blockade is not considered a clinical target for improving stroke outcome because AD is over long before the patient reaches the ER and treatment can commence. However, in the hours following stroke onset recurrent peri-infarct depolarizations (PIDs) spread from the edge of the ischemic core out into the penumbra (Nedergaard and Astrup 1986; Nedergaard and Hansen 1993). Here ATP levels fall ~50% compared to 75-90% in the core (Folbergrova et al. 1992), so these events are AD-like but are able to repolarize. Their recurrence further depletes energy reserves of penumbral neurons over many hours and likely promotes dysfunction. PIDs increase both growth rate (Hartings et al. 2003) and volume of the core in rodent models (Busch et al. 1996; Takano et al. 1996; Back et al. 1996). They can be imaged coursing along the surface of mouse neocortex following focal stroke (Farkas et al. 2008). Clinically, PIDs are recorded from the cortical surface of patients in the hours and days following head trauma (Fabricius et al. 2006) or stroke (Dohmen et al. 2008). Thus a surprisingly prolonged ‘window of opportunity’ is available for suppressing these recurring events.

During AD, extracellular $K^+$ suddenly climbs to 50-60 mM, whereas extracellular $Na^+$ (from 130 to ~50 mM) and $Ca^{2+}$ (from ~2 mM to 0.1 mM) decrease precipitously (Lipton 1999). These ionic movements toward ionic equilibrium are initially through voltage-gated channels and possibly through pannexin-1 hemichannels (Thompson et al. 2006). Rupturing of neurons would also support such change over the longer term. Reducing extracellular $Na^+$ from ~145 mM to 28 mM does not significantly alter AD onset (Tanaka et al. 1997), although AD amplitude is reduced in low extracellular $Na^+$ (Yamamoto et al. 1997). Glutamate receptor activation is not required for the generation or propagation of cortical AD in adult rodent slices (Tanaka et al. 1997; Muller and Somjen 2000; Jarvis et al. 2001; Anderson et al. 2005).
and in vivo (Hernandez-Caceres et al. 1987; Nellgard and Wieloch 1992; Lipton 1999; Murphy et al. 2008). There is no evidence that any transmitter agonist or antagonist affects AD onset, probably because synapses fail before AD onset. AD is also not stopped by blocking of synaptic transmission with low calcium (Obeidat et al. 2000; Obrenovitch et al. 2000; Jarvis et al. 2001); or with K+ or Ca2+ channel blockers (Yamamoto et al. 1997). On the other hand, numerous studies have found that the ‘caine’ family of Na+ channel blockers significantly delay AD onset (Weber and Taylor 1994; Urenjak and Obrenovitch 1996; Taylor et al. 1999). This is also true for some sigma receptor (σR) ligands (Anderson et al. 2005). Like the caines, certain σR ligands have been proposed to be neuroprotective in models of stroke and Alzheimer’s disease (Maurice and Su 2009). Neither type of drug has been examined with intracellular recording to observe drug effects at the level of the cortical neuron.

A reasonable assumption is that a drug blocking or delaying AD onset will also inhibit PID onset in vivo. However PIDs are difficult to distinguish from spreading depression (SD) in vivo. A PID that propagates into uncompromised tissue is essentially an SD event. Although not measured to date, small amounts of both drugs likely cross the blood-brain barrier because dibucaine is neurotoxic at higher concentrations in patients while carbetapentane is quite lipophilic (log P value of 4.2). Here we examine how dibucaine, a potent Na+ channel blocker (Appendix 1) and AD inhibitor works at the level of the single pyramidal neuron to reduce susceptibility to AD (and probably to PIDs as well). We compare dibucaine’s effects to carbetapentane (a σR ligand that also delays AD onset) to try to identify a common mechanism of AD inhibition. (For more information on both compounds, see Appendix 1).

METHODS

HIPPOCAMPAL SLICE PREPARATION

Male Sprague-Dawley rats, 21-28 days old (Charles River, St. Constant, PQ) were cared for in accordance with the Canadian Council on Animal Care. They were housed in a controlled environment (22 ± 1°C, 12 h light: 12 h dark) with Purina rat chow and water supplied ad libitum. A rat was placed in a
rodent restrainer and guillotined. The brain was excised within one minute and placed in ice-cold oxygenated (95% O₂ / 5% CO₂) high sucrose artificial cerebrospinal fluid (aCSF). Hippocampal slices (Fig. 1A) were cut in the coronal plane (400 µm) using a vibrating blade microtome (Leica VT1000S, Wetzlar, Germany). Slices were transferred to a net submerged in a beaker of regular aCSF gassed with O₂/CO₂ at 22°C and slowly warmed to 31°C over 1 hour for electrophysiological recording and imaging. Experiments involving AD were performed at 35°C.

**EXPERIMENTAL SOLUTIONS**

Control aCSF contained (in mM): NaCl 120, KCl 3.3, NaHCO₃ 26, MgSO₄ 1.3, NaH₂PO₄ 1.2, D-glucose 11, and CaCl₂ 1.8 (pH 7.3-7.4; 295 milliosmoses). High sucrose aCSF included 240 mM sucrose instead of NaCl. Prior to electrophysiological recording, individual slices were transferred to a submersion chamber (RC-26, Warner Instruments, Hamden, CT) and held down with a slice anchor (SHD-26H, Warner Instruments). Constant aCSF flow of 1-2 ml/min was maintained throughout the experiments. Ischemia was simulated by either oxygen/glucose deprivation (OGD) or by addition of the Na⁺/K⁺ ATPase inhibitor ouabain (100 µM). Both evoked similar AD with respect to changes in LT and electrophysiology (Jarvis et al. 2001). For OGD, aCSF glucose was replaced with equimolar NaCl and the 95% O₂/5% CO₂ mixture gassing the aCSF was replaced with 95% N₂/5% CO₂. Dibucaine hydrochloride or the high affinity σR1 ligand carbetapentane (citrate salt) from Sigma-Aldrich were dissolved in double distilled water and applied by bath.

**SINGLE CELL ELECTROPHYSIOLOGY**

The micropipette solution contained in (mM): K⁺Gluconate (130), KCl (10), MgCl₂ (1.1), ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (5), HEPES (10), Na⁺ATP (2) and CaCl₂ (0.1). The pH was adjusted to 7.3 with KOH and osmolality was 270 milliosmoses. In one group of experiments 10 or 100 µM dibucaine was added to micropipette solution, replacing equimolar K⁺ gluconate.
CA1 pyramidal cells or granule cell neurons (Fig. 1A) were ‘blind’ patched with micropipettes pulled from borosilicate glass capillaries (1.2 mm ID) to a resistance of 3-6 MΩ. All recordings were performed in whole-cell current clamp using ‘bridge’ mode of an Axoclamp 2A amplifier, sampled using a Digidata 1322A A/D converter (Molecular Devices, Sunnyvale, CA). An external Bessel filter (LPF 202a; Molecular Devices) provided low pass filtering at 10 Hz. Pipette junction potentials were corrected just prior to achieving whole-cell mode. Series resistance was compensated by 70%. Seal resistance was estimated at >1 gigaohm. Bath application of a drug was initiated only after a stable membrane potential was obtained and <5 minutes had passed to allow for dialyzing the pipette solution into the cell. Neuronal recordings were acquired before and following 30 minutes of drug administration by bath at 31°C. Initial control values of several electrophysiological parameters were pooled and compared with the post-30 minute values. Data were analyzed off-line with Clampex software (version 10.1). Then temperature was slowly increased to 35°C over 10 minutes for a total of 40 minutes of drug pretreatment before inducing simulated ischemia with oxygen glucose deprivation (OGD). Several properties of the induced AD were measured (Fig. 1B).

The current pulse injection protocol was 9 sequential one-second current steps of 100 pA ranging between -500 and 300 pA and occurring every 6.5 seconds. Trains of action potentials were elicited at depolarizing current levels and estimates of whole cell input resistance obtained from the slope of the V-I curve at negative current pulse levels using Clampfit software.

Orthodromic responses by CA1 neurons were evoked by stimulating Schaffer collaterals of CA3 axons in stratum radiatum (Fig. 1A) using a concentric bipolar electrode (Rhodes Electronics, Woodland Hills, CA) with a shaft diameter of 0.25 mm. An antidromic response was elicited by stimulating the alveus adjacent to the recorded CA1 neuron. Stimulation (0.5 ms in duration; 0.1 Hz) was at the minimal strength to evoke a single action potential which was monitored until failure or until the 30 minute drug treatment elapsed. After orthodromic failure, EPSP amplitude continued to be monitored at the same stimulus strength and frequency.
IMAGING CHANGES IN LIGHT TRANSMITTANCE

Changes in LT (ΔLT) of the slice were monitored in real time during OGD. Cell swelling was imaged as an increase in LT during AD initiation/propagation in cortical brain slices. Dendritic damage was imaged as decreased LT in the wake of the AD front that followed the LT increase (Obeidat and Andrew 1998). This LT reduction results from light scattering caused by dendritic beads forming within minutes of AD onset in brain slices (Polischuk et al. 1998; Jarvis et al. 1999; Andrew et al. 2002). This coincides with comparable beading during AD in the intact mouse neocortex detected as a reduction in light reflectance (Murphy et al. 2008). The slice was illuminated using a broadband, voltage-regulated halogen light source (Fig. 2A) on an upright light microscope. The light traversed a band-pass filter that transmitted red and near-infrared light (690 - 1000 nm). Video frames were acquired using a 12-bit digital camera (Hamamatsu C4742-95) set to an exposure time of 0.05 s. Images were acquired at 32 frames per second and averaged using Imaging Workbench (IW6) software (Indec Biosystems, Santa Clara, CA). To synchronize imaging with electrophysiology, Clampex software triggered the IW6 image acquisition. The first averaged image in a series served as a control (T₀), which was subtracted from each subsequent experimental image of that series (T). The resulting series of subtracted images revealed changes in LT over time (using the formula ΔT/T₀ = (T – T₀)/T) which was displayed using a pseudo color intensity scale (Fig 2A). Zones of interest were selected to quantify and graphically display the ΔLT data off-line. Graphing and statistical analyses of data were carried out using SigmaPlot for Windows (Jandel Scientific) or Microsoft Excel. Images were imported and figures were prepared using CorelDraw graphics.

STATISTICAL ANALYSIS

Neurons were analyzed if they displayed stable resting membrane potentials of at least -55 mV and if the series resistance and capacitive currents could be sufficiently compensated. Recordings were terminated if access resistance increased to where it could not be compensated using the bridge balance. Statistical significance was determined by unpaired t-tests and all data presented as means +/- standard
deviation. Control data were pooled prior to drug application and compared with a 30 minute pretreatment of each concentration and drug type. The significance criterion was set at $p<0.05$.

RESULTS

SIMULTANEOUS CURRENT CLAMPING AND ΔLT IMAGING DURING OGD

Within 6 min of oxygen and glucose deprivation (OGD), a front of elevated LT coursed through the gray matter of the CA1 region (Fig. 2A), propagating at a rate of 2-4 mm/min. AD onset time in the CA1 region was measured as the time point when the LT front passed by the recording electrode (Fig. 2B,C; 4:37 min), an average of 5:20 ± 1:21 min after switching to OGD (n=41 slices). Under whole-cell current-clamp the recorded AD waveform was subdivided into 3 components: a small but prolonged early depolarization, an ensuing rapid depolarization (the fast AD) coincident with the LT front and a later slow depolarization that approached zero mV (Fig. 1B). CA1 cells consistently underwent fast AD (mean amplitude 28.8 ± 6.2 mV), coinciding with the LT front passing the micropipette (n=13, Table 1), with a slope of 2.7 ± 1.5 mV/s, and a peak potential of -9.4 ± 4.6 mV. The slower, late depolarization (also called the persistent depolarization), had a slope of 0.28 ± 0.16 mV/s and took an average of 46.4 ± 31.3 s before reaching zero mV. Six additional recordings were obtained without ATP in the pipette (to eliminate the possibility of this additional energy source interfering with the AD waveform). The absence of ATP in the pipette had no discernable effect on AD (not shown).

Following AD, membrane potential remained near zero mV while the slice evolved optical signs of damage (Fig. 2B, last panel). There was no evidence of repolarization as monitored for up to 30 minutes post–OGD. There was also a continued decrease in LT within CA1 dendritic regions over the minutes following AD propagation, with no sign of recovery after return to control aCSF. The reduction in LT in these regions is partly the result of dendritic beading that scatters light despite maintained slice swelling (see Discussion). Once AD propagated, the LT decrease was inevitable, although some neurons could return to near baseline membrane potential if OGD was immediately terminated upon AD initiation (not shown).
BATH PRETREATMENT WITH DIBUCAINE OR CARBETAPENTANE (CP)

An important initial component of AD is a massive influx of Na⁺ into neurons, so various attempts to inhibit AD have involved the use of sodium channel blockers. Dibucaine has proven to be the most effective ‘caine’ tested in our laboratory based on imaging ΔLT (Douglas et al. 2011). As demonstrated with other caines (Creveling et al. 1983), bath-applied dibucaine is thought to slowly enter neurons through briefly opened Na⁺ channels and then bind to the channel on the intracellular side to block the pore. In the current study, hippocampal slices pre-exposed to 40 minutes of 10 µM dibucaine displayed significantly delayed AD onset from 5:20 ± 1:21 min to 9:18 ± 1:51 min (p<0.001) in 9 slices tested (Fig. 3A, C). Pretreatment with 1.0 µM dibucaine caused no delay in this CA1 study (Fig. 3C), but was significantly effective on AD onset in neocortical slices (Douglas et al. 2011).

Carbetapentane (CP), a σ1R ligand, was previously tested in our lab and found to delay the onset of AD. We re-affirmed that pretreatment of slices for 40 minutes with 10 or 30 µM CP significantly delayed AD. Onset times were 7:12 ± 1:46 (n=13, p<0.05) and 10:24 ± 1:34 (n=6, p<0.001) respectively, from a control value of 5:20 ± 1:21 min (Fig. 3C). Increasing CP pretreatment to 100 µM for 40 min eliminated the evoked CA1 population spike (not shown), so a 20 min exposure was used. AD was significantly delayed by 100 µM CP (8:32 ± 0:47 min, n = 4) compared to control (5:20 ± 1:21 min, n=25), but less so than 30 µM CP (10:24 ± 1:34, n=6) because of the reduced pretreatment time (Fig 3C).

DIBUCAINE INTRODUCTION INTRANEURONALLY

Single CA1 Pyramidal Cells. Dibucaine’s primary pharmacological effects have been attributed to its binding within the voltage-gated sodium channel, but it is also has poorly defined effects on the membrane itself. As an alternative to bath exposure, we directly introduced 10 or 100 µM dibucaine through the recording pipette. This tested if the AD recorded in the single neuron exposed to the drug could be altered as the LT front passed through neighboring neurons and glia not exposed to the drug. Dibucaine acting within the recorded cell alone could not slow or stop the fast AD as measured either
electrophysiologically or as the LT wave front. This is not surprising because AD is a population event. However, either concentration of dibucaine clearly slowed depolarization of the recorded cell itself, particularly during the late AD (Fig. 4A-C). Ten µM dibucaine increased the late AD duration by over 50% to 73.2 ± 37.2 seconds (p<0.05, n = 11), while 100 µM dibucaine increased it by over 250% to 124.5 ± 50.9 s (p<0.001, n = 14) (Fig. 4B-C, Table 1). Dibucaine at 100 µM also reduced the mean amplitude, peak potential and slope of the fast AD as well as the slope of the late AD (Table 1). But inevitably the neuron completely depolarized.

**Single Granule Cells.** AD propagation is also imaged in the dentate gyrus (not shown). To examine if dibucaine’s intracellular effects upon AD were specific to CA1 pyramidal cells, granule cells of the dentate gyrus were recorded without (Fig. 5A) and with (Fig. 5B) 10 µM dibucaine in the pipette. This concentration reduced the fast AD amplitude from 23.3 ± 8.2 mV in 10 untreated granule cells to 15.8 ± 5.3 mV (p<0.01, n = 13, Table 2). Moreover, it reduced the peak potential of the fast AD from -11.4 ± 6.0 mV in untreated granule cells to -23.3 ± 6.3 mV (p<0.01). Importantly, the latency to reach zero mV following AD increased to 132.7 ± 63.7 s compared to 44.2 ± 27.1 s (p<0.001) in untreated granule cells (Table 2). Thus, 10 µM dibucaine pretreatment slowed AD progression within individual granule cells in a manner similar to CA1 neurons. As expected, simply attempting to block AD in the single neuron did not affect AD within the population. The remnant of the fast AD recorded in these neurons (and CA1 cells) may actually be driven by the rise in extracellular [K⁺] from AD generated in neighboring neurons.

**DIBUCAINE AND OUABAIN-INDUCED AD**

Adding 100 µM ouabain to aCSF initiated an event similar to OGD-induced AD (Fig. 6A). Ouabain directly inhibits the Na⁺/K⁺ ATPase by binding to the pump on the extracellular side of the plasma membrane. Latency to AD onset induced by ouabain (6:21 ± 0:3 min, n=17) was not statistically different than slices exposed to OGD (5:20 ± 1:2 min, n=11). Ouabain induced a slightly steeper fast AD (slope of 3.9 ± 1.3 mV/s) compared to OGD (2.7 ± 1.5 mV/s). As well, ouabain increased the peak
potential (-4.0 ± 1.8 mV) compared to OGD (-9.4 ± 4.6 mV) (p<0.05, Table 3; Fig 6A). Therefore, ouabain-induced AD was qualitatively similar to that induced by OGD, but progressed slightly faster and was marginally more depolarizing.

Ten μM dibucaine pretreatment for 40 min prevented AD in all 6 slices exposed to 10 minutes of 100 μM ouabain. This was more dramatic than the simple delay in onset induced by OGD. However with ouabain, the membrane potential did slowly depolarize from a resting level of around -65 mV to a mean level of -26.0 ± 5.50 mV by 10 minutes of ouabain (Table 3; Fig 6B). So compared to complete depolarization induced by ouabain alone (Fig. 6A), 10 μm dibucaine inhibited the fast AD such that the CA1 neuron only partially depolarized and then remained at about -40 mV for the duration of the recording, essentially blocking a full-blown AD.

**DRUG EFFECTS UPON CA1 INTRINSIC AND SYNAPTIC PROPERTIES**

**Bath-Applied Dibucaine Effects**

To help characterize how dibucaine may delay AD onset and progression, single CA1 neurons were whole-cell patched under current-clamp mode to measure changes in their intrinsic and synaptic electrophysiological properties. An AP train evoked by a depolarizing pulse lasting one second revealed changes in AP threshold and waveform depending on the drug and its concentration (Fig. 7A, B). Neither resting membrane potential nor whole-cell input resistance were altered after 30 minutes of bath pretreatment with either 1 or 10 μM dibucaine (Table 4). During depolarizing current injection, 1 μM dibucaine did not alter the fast afterhyperpolarization (fAHP), AP threshold, AP frequency or AP duration. A surprisingly small reduction in AP amplitude (p<0.05) was noted however, in 9 CA1 cells examined. Increasing dibucaine concentration to 10 μM, enhanced the fAHP amplitude (measured relative to the AP threshold) from -3.4 ± 1.9 mV (n = 45, control aCSF) to -10.8 ± 3.7 mV (n = 20, 10 μM dibucaine, p<0.001) (Fig. 8A, Table 4). The slow afterhyperpolarization (sAHP) of the last AP in the train increased from -5.8 ± 1.8 mV to -15.8 ± 5.0 mV (p<0.01, Fig. 8B). Ten μM dibucaine pretreatment also
increased AP threshold, shifting it from -47.5 ± 4.4 mV (n = 45, control aCSF) to -37.7 ± 4.5 mV (n = 20, p<0.001) (Figs. 7B, 9A, Table 4). Corresponding reductions in AP frequency at all 3 current injection levels (Figs. 9B, Table 4) were likely caused by the increase in AP threshold as well as the increase in both the fast and slow AHP amplitudes.

AP duration measured at half-AP amplitude was unchanged by both 1 and 10 µm dibucaine after the first AP in the train (Table 4). However, after the last AP in the train (measured at the highest current injection level), AP duration (measured at half-AP amplitude) increased from 1.2 ± 0.2 ms to 2.0 ± 1.7 ms (n=9, p<0.01) (Table 5). This was perhaps facilitated by Na⁺ channel inactivation by the drug, as dibucaine is known to induce a use-dependent block by binding inactivated Na⁺ channels.

To further investigate drug actions, single APs were synaptically evoked in CA1 neurons by orthodromic stimulation of the CA3 Schaffer collaterals. Antidromic activation was also evoked by stimulating the alveus region. One µM dibucaine was not tested further as it was ineffective at this concentration. Orthodromic APs were lost an average of 7.4 ± 2.4 min (n=8) after 10 µm dibucaine exposure measured at 0.5 V above the of the initial voltage strength required to evoke a typical response (p<0.01, Fig. 10A, left). AP threshold continued to increase over the following 5-10 minutes until the evoked spike failed. Evoked EPSPs continually decreased in amplitude over the 30 minute treatment, from a maximum of 14.8 ± 5.0 mV at initial AP loss to 6.3 ± 5.0 mV (p<0.05, n=8). Antidromic spikes were more resistant to block, taking an average of 15.0 ± 14.6 min to fail at the initial stimulus strength (p<0.01, Fig. 10A, right). It remained possible to evoke an antidromic AP by increasing the stimulus voltage (not shown).

After 30 minutes of pretreatment with 10 µM dibucaine, antidromic APs displayed slightly enhanced depolarizing after-potentials (DAP) at 3 and 5 ms (Fig. 10B, C). However as recorded in 24 CA1 neurons, this enhancement was not statistically significant compared to drug-free aCSF.
Bath-Applied Carbetapentane Effects

CP also reduced excitability in CA1 pyramidal neurons, although in markedly different ways than dibucaine. AP frequency was significantly reduced in 10 and 30 µM CP (p<0.05, Table 4) and in 100 µM CP (p<0.001) as shown in Figure 7A and C. Unlike dibucaine, this slower AP discharge was not the result of an increase in AP threshold at 10 or 100 µM CP (Fig. 8A). AP duration, as measured at half AP amplitude, did increase in both 30 and 100 µM CP to 1.4 ± 0.9 ms and 2.0 ± 2.6 ms respectively (n=5) from a control value of 1.0 ± 0.1 ms (p<0.01) as shown in Table 4. The threshold of the last AP in the train increased following 30 minutes pretreatment with 30 µM CP from -41.2 ± 4.0 mV to -34.3 ± 4.3 mV or with 10 µM CP pretreatment to -31.1 ± 4.0 (Fig. 9A, Table 5). The fAHP was notably eliminated in the first AP of the train in all three concentrations, apparently facilitating spike train discharge (Table 4). Conversely, by the last AP in the train, the slow AHP amplitude increased to -11.2 ± 2.9 mV (p<0.01) in 10 µM CP and -10.6 ± 2.3 mV in 30 µM CP (n=11, p<0.01) from a control value of -5.8 ± 1.8 mV (n=25) (Fig. 8B, Table 5). The last AP in the train was not measured with 100 µM CP because only a single AP or AP doublet could be evoked.

When single APs were evoked by either antidromic or orthodromic stimulation, DAP amplitude (measured at 3 and 5 ms) was enhanced following pretreatment with 30 µM CP to 18.7 ± 5.3 mV and 15.5 ± 2.8 mV (p<0.01) respectively, from control values of 11.3 ± 2.5 mV and 10.9 ± 2.5 mV (Fig. 10B, C). One hundred µM CP enhanced the DAP to 31.9 ± 7.7 and 21.8 ± 3.9 mV as measured in 6 CA1 cells (p<0.01, Fig. 10C). At 100 µm CP, orthodromic AP’s failed at ~30 minutes. Unlike dibucaine, AP’s could still be evoked at higher voltages, as tested in 3 CA1 cells (data not shown). Furthermore, antidromic stimulation (at baseline strength) could generate APs for the entire 30 minutes of CP exposure in 5 of 6 cells with 30 µM CP and 1 of 3 cells with 100 µM CP (data not shown). This suggests that the CP was altering spike-related currents more in the soma-dendritic region than the in the axon.
Combined Bath-Applied Effects of Dibucaine and Carbetapentane

Our observations that dibucaine and CP each reduced the excitability of CA1 neurons through different mechanisms prompted us to search for additive effects of both drugs upon the excitability of single neurons. Following 30 minute pretreatment with 1 µM dibucaine and 10 µM CP, the intrinsic electrophysiological properties of 14 CA1 cells (Table 4) were not significantly different than pretreatment with 10 µM CP alone. The exception was the fAHP, which was preserved in 8 of 14 cells (Table 4). The inclusion of 3 µM dibucaine with 30 µM CP raised the AP threshold of the first AP in the train to -38.8 ± 4.6 mV compared to -49.1 ± 2.8 mV with 30 µM alone (p<0.01, Fig. 9A, Table 4). The increased AP threshold observed at these drug concentrations caused an expected reduction in AP frequency as compared to 30 µM CP alone (p<0.001, Fig. 9B, Table 4). The effect of pretreatment with dibucaine and CP on intrinsic CA1 properties, therefore appeared to be additive.

DISCUSSION

Anoxic depolarization is imaged as a focally initiating, and then propagating, increase in light transmittance (Obeidat and Andrew 1998; Somjen 2001; Jarvis et al. 2001; Anderson et al. 2005). Extracellular recording simultaneously with ΔLT imaging reveals a wave front of elevated LT corresponding with a sudden negative shift in extracellular DC potential that represents a depolarization by the neuronal population (Muller and Somjen 1999; Jarvis et al. 2001). We recorded AD in single pyramidal neurons while simultaneously imaging ΔLT within the coronal cerebral slice, to study AD initiation and potential recovery. Combining these techniques also helps clarify previous experiments concerning AD delay induced by either dibucaine or carbetapentane. Either drug that delayed AD at lower concentrations also preserved synaptic function based on evoked population responses, suggesting that AD could be inhibited without undermining normal cortical function (Douglas et al. 2011). Here we looked at the level of the single neuron to determine how these drugs may be protective by delaying AD onset and, by extension, inhibiting recurring PIDs. Delaying AD or PID onset protects because gray
matter spends less time post-depolarization exposed to OGD which is when neurons become irreversibly damaged.

**DRUG EFFECTS ON AD INITIATION**

*Bath Pretreatment with Dibucaine.* Previous experiments in our lab showed that 1 µM dibucaine pretreatment delayed AD, while at the same time preserved evoked synaptic function in both cortical and hippocampal neurons (Anderson et al. 2005). The present experiment did not detect a delay in AD with 1 µM dibucaine, probably due to the shorter pretreatment time of 30 minutes. Ten µM dibucaine pretreatment however, was found to significantly delay AD in the present experiment.

With 10 µM dibucaine, the evoked CA1 field potential was reduced by 80% (Douglas et al. 2011). Our current intracellular work revealed that both the orthodromic and antidromic AP’s were blocked and that evoked EPSP amplitude was significantly reduced during the 30 minute pretreatment. It is likely that dibucaine’s inhibition of AD onset at 10 µM results in part from suppression of AP firing by blocking both the sustained and the slow Na⁺ currents. OGD itself blocks the fast Na⁺ current thereby dampening AP discharge and thus synaptic transmission. Additional intracellular parameters were measured to better delineate how reducing pyramidal cell excitability may delay AD, thus providing neuroprotection.

Dibucaine’s best documented effect is its action on the local anesthetic binding site within voltage-gated Na⁺ channels (Ragsdale et al. 1994; Kuroda et al. 2000), although direct measurements of the various Na⁺ currents reduced by dibucaine have not been carried out. Lidocaine is a similar (but less potent) ‘caine’ that reduces the voltage-activated Na⁺ current in isolated pyramidal neurons (Kaneda et al. 1989).

*Intracellular Introduction of Dibucaine.* Dibucaine slowed AD progression when introduced into the single pyramidal neuron or granule cell, even as surrounding neurons go through the standard, faster AD. The fact that dibucaine was able to reduce the fast AD amplitude while delaying the late depolarization...
indicates that the single neuron itself can dictate much of the intracellular AD waveform. However, a
remnant of the fast AD was always recorded. Note that a fast AD is also recorded in astrocytes (not
shown) suggesting that the fast AD is partly generated by sudden efflux of K\(^+\) by the surrounding neurons
synchronously undergoing AD. The current responsible for the fast AD involves Na\(^+\), Cl\(^-\) and Ca\(^{2+}\) influx
and K\(^+\) efflux (Czeh et al. 1993) but is also driven passively by the sudden increase in K\(^+\) to ~50mM. The
late depolarization has been attributed to inward Na\(^+\) and Ca\(^{2+}\) currents (Yamamoto et al. 1997).

*Bath Pretreatment with Carbetapentane.* The mode of action on CNS neurons of \(\sigma\)R ligands had not
been examined in detail. Our study supports previous limited data showing no change in CA1 membrane
potential, cell input resistance or action potential threshold during 50 min of exposure to 100 \(\mu\)M
dextromethorphan (Wong et al. 1988) or to the \(\sigma\)R ligand OPC-24439 (Ishihara et al. 1999). Our CP
pretreatment reduced CA1 excitability, but unlike dibucaine, CP did not increase the AP threshold. It did
increase the sAHP, which likely helped slow the firing rate. \(\sigma\)Rs have been localized to plasma
membrane, mitochondrial membrane and dendrites (Alonso et al. 2000), and no staining has been
observed on the axons of presynaptic neurons (Alonso et al. 2000). So AD delay by CP likely involves
the CA1 soma/dendritic region. The unchanged antidromic AP evoked in our study confirms the lack of
a \(\sigma\)R ligand effect at the level of the axon.

Sigma receptor ligands have been shown to inhibit voltage-activated K\(^+\) current in tumor cells
(Wilke et al. 1999) and in oocytes expressing voltage-gated K\(^+\) channels and sigma receptors (Aydar et al.
2002). Specific potassium channel effects observed in cardiac cells include decreased delayed outwardly
rectifying potassium current, BK current and M current (Zhang and Cuevas 2005). Calcium-activated
potassium channels such as large conductance (BK), small conductances (SK) and intermediate
conductance (IK) underlie the AHP in neurons. CP enhanced the sAHP in our study which would also
explain the spike frequency adaptation that we observed (Sah 1996). The spike broadening by CP is also
suggestive of BK channel block through its role in spike repolarization.
Some sigma receptor ligands (including CP) are neuroprotective in cortical neurons (Hayashi et al. 2000; Katnik et al. 2006), where the authors measured Ca\(^{2+}\) increase under simulated ischemia. CP attenuated the increase in intracellular calcium caused by sodium azide (which blocks aerobic metabolism) and glucose deprivation in cultured cortical neurons. This was attributed to direct preservation of intracellular Ca\(^{2+}\) homeostasis. The IC\(_{50}\) value (13 \(\mu\)M CP) required to attenuate Ca\(^{2+}\) increases caused by simulated ischemia in cultured neurons corresponds to values obtained in our experiments showing AD delay in brain slices.

**Dibucaine Effects on Ouabain-induced AD.** Low concentrations of dibucaine have been shown to block AD during 10 minutes of ouabain exposure (Douglas et al. 2011) but it was unclear if the drug maintains neurons at a normal resting potential. Our current clamp recordings with dibucaine show that there is a small but steady depolarization that would not be detected with extracellular DC recording. Thus, the lack of a DC shift makes it appear that AD was fully blocked (Douglas et al. 2011), when in fact a slow depolarization was on-going, which would reduce the evoked population spike. Ouabain-induced AD is more easily blocked with equivalent drug concentrations than OGD-induced AD (Anderson et al. 2005).

Comparing dibucaine’s role in AD delay as a reduction in metabolic load seems to contradict its ability to delay AD induced by ouabain, which is independent of tissue ATP levels (Anderson et al. 2005). It seems likely that dibucaine protects from ouabain and OGD in a similar fashion by inhibiting currents associated with the AD that open as a result of Na\(^+/K^+\) pump failure.

**ACTIONS OF DIBUCAINE AND CARBETAPENTANE**

Our findings show a reduced neuronal excitability induced by both dibucaine and carbetapentane as indicated by the reduction in AP frequency in response to current injection. Identifying the exact underlying mechanism however, requires voltage clamp work utilizing various channel blockers. Clearly, dibucaine reduces excitability by increasing AP threshold through the blockade of AP-associated sodium channels and CP reduces excitability by increasing the slow AHP. Subtle calcium or potassium channel
effects however, cannot be excluded as both can be inhibited by local anesthetics (Oda et al. 1992; Hirota et al. 1997). While both drugs delayed AD through different mechanisms of reduced cortical excitability, it is unlikely that combining both drugs would delay AD any further without compromising slice health.

CA1 NEURON RECOVERY FROM OGD

The ability of single CA1 neuron to recover membrane potential after AD depends on several factors. As in our study, Tanaka et al. (1997) did not observe recovery by adult CA1 neurons in submerged slices. However, Czeh et al. (1993) demonstrated repeatable ‘hypoxic spreading depression’ (HSD) events within slices in an interface chamber. Interface slices have the advantage of a rapid diffusion rate from the oxygen-rich atmosphere compared to submerged slices, which draw oxygen from the aCSF. More importantly, by lowering O₂ but not glucose, neurons still have a residual energy source and are thus not truly ischemic. We could quickly reintroduce aCSF immediately following the fast AD by observing the approaching AD front. Such neurons were able to recover full membrane potential after AD had passed the recording electrode, if the aCSF was immediately returned. Accurate recording of whole-cell input resistance measured during the recovery period was difficult due to movement of the slice as it swells. However in the cells that did not display a large increase in access resistance, whole cell input resistance levels were similar to levels measured prior to OGD exposure (data not shown). This indicates that an additional period of tens of seconds is required for permanent depolarization and dysfunction.

In our experiments that tested recovery of the orthodromic response in CA1 neurons, full AP’s could not be evoked at any time within the recovery interval (up to 40 min). EPSPs could eventually be evoked, but at reduced amplitude compared to pre-OGD. In contrast, antidromic APs could be re-activated once the neuron repolarized enough to restore Na⁺ channel activation. Perhaps the lasting effects of AD cause irreparable harm to dendritic spines while preserving AP discharge. In support, no slice showed a return to LT baseline in dendritic regions in recovered slices, further indicating synaptic damage from continuing dendritic beading. Since the current space clamp is incomplete past the proximal
dendrites (Brown et al. 1990), it is possible that distal dendrites remain susceptible to OGD and continue to bead, while the cell body recovers to some degree. Also, while dendritic beading has shown recovery following brief ischemia (Murphy et al. 2008), CA1 population recovery following AD in slices, is more problematic.

CONCLUSIONS

This study shows that at low millimolar concentrations, dibucaine (a Na\(^+\) channel blocker) and carbetapentane (a sigma receptor ligand) maintain whole-cell input resistance and membrane potential of CA1 pyramidal neurons. Therefore the drugs do not seem to affect the baseline excitability of the neurons. However they both reduce AP discharge, albeit in distinctly different ways, which helps explain their ability to inhibit AD onset. Reducing spike firing means lowered synaptic communication. Synaptic release/reuptake is a major expenditure of energy in the brain, yet preservation of ATP levels does not seem to be the key protective mechanism because both drugs also delay AD induced by ouabain where the ATP levels do not drop. There are likely other ways that both drug types slow AD onset but we are still limited in our understanding of the exact mechanisms driving AD (and thus PIDs). For example, we know that the caines block the fast Na\(^+\) channel driving the action potential, but it is not clear how effectively they bind Na\(^+\) channels driving the sustained and slow Na\(^+\) channels during OGD (Appendix 1). Indeed, it is not possible to pharmacologically isolate these channels to ascertain their contribution to the AD.

Nevertheless, our results show that the two drugs affect single pyramidal cells in distinctly different ways. It is likely that the specific pharmacophore for the caines that elicits AD inhibition and the pharmacophore for sigma receptor ligands are different. Theoretically, drug design techniques can identify these pharmacophores and incorporate their properties into a single drug that might inhibit AD in the sub-micromolar range.


Czech G, Aitken PG and Somjen GG. Membrane currents in CA1 pyramidal cells during spreading depression (SD) and SD-like hypoxic depolarization. *Brain Res* 632: 195-208, 1993.


Hayashi T, Maurice T and Su TP. Ca(2+) signaling via sigma(1)-receptors: novel regulatory mechanism affecting intracellular Ca(2+) concentration. *J Pharmacol Exp Ther* 293: 788-798, 2000.


APPENDIX 1.

Dibucaine is one of the most potent local anesthetics of the caine family based on its binding to the batrachotoxin (BTX) binding site within the pore of the voltage-gated sodium channel (Creveling et al. 1983). IC$_{50}$ values are 1.4 µM for dibucaine, 5.4 µM for bupivacaine, 240 µM for lidocaine and 940 µM for benzocaine (Creveling et al. 1983). No binding occurs at the TTX binding site, nor within the scorpion toxin binding site (Postma and Catterall 1984). Initially, the uncharged caine molecule can diffuse across the nerve membrane and equilibrate with its charged form which then binds within the Na$^+$ channel near the BTX binding site on the intracellular side. During firing, caines can also enter the neuron through the open sodium channels, cumulatively binding with each depolarizing pulse (Hille 2001). This explains the use-dependent block observed: more potent anesthetic effects arise in actively firing neurons. Use-dependent block involves a tighter binding in the inactivated state than in resting or activated states of the Na$^+$ channel (Hille 2001).

There are three types of Na$^+$ channels in cortical pyramidal neurons that are likely inhibited by caines. First is the classic voltage-activated Na$^+$ current driving the AP upstroke. This fast or transient Na$^+$ current ($I_{NaF}$) is TTX-sensitive and rapidly activates with depolarization over a few milliseconds. Oxygen deprivation increases the probability of $I_{NaF}$ inactivation (O`Reilly et al. 1997) which decreases the load on the Na$^+$/K$^+$-ATPase pump to some degree, thereby lowering energy consumption.

Second is a sustained or persistent Na$^+$ current ($I_{NaP}$) representing 1–4% of total Na$^+$ current amplitude in normal conditions. It regulates spike threshold in a number of neuronal types, can generate Na$^+$-dependent plateau potentials and helps drive rhythmic membrane oscillations (see Astman et al. 2006). It is active at resting potential and resists inactivation during depolarization (Stys et al. 1993; Taylor 1993), significantly increasing intracellular Na$^+$ when activated for several seconds, as during OGD (Taylor 1993). As such, it has been considered an O$_2$ sensor in central neurons (Hammarstrom and Gage 2000). It is sensitive to TTX and QX-314, the former inducing a 5-10 mV hyperpolarization in
CA1 neurons (Xie et al. 1994). Part of the AD-delaying affect of TTX has been attributed to this increased hyperpolarization (Muller and Somjen 1999; Fung et al. 1999).

Third, a slow Na\(^+\) current \(I_{\text{NaS}}\) is activated at depolarizations beyond -40 mV and reaches maximum amplitude near zero mV. It is TTX-insensitive and activates and deactivates slowly over hundreds of milliseconds (Hoehn et al. 1993). It is activated at levels more depolarized than -40 mV and so contributes to DAPs and the depolarizing shift driving epileptiform activity by pyramidal neurons (Hoehn et al. 1993). The \(I_{\text{NaS}}\) channel may also conduct K\(^+\) (Hoehn et al. 1993) which would promote K\(^+\) efflux during AD. \(I_{\text{NaS}}\) is in cortical, striatal and pyramidal neurons so their blockade should also delay AD onset.

Dibucaine also interacts with Ca\(^{2+}\) channels (Sugiyama and Muteki 1994), as well as affecting the stability of plasma membranes (Kuroda et al. 1996), but these effects involve higher concentrations than used in this study.

Dibucaine at 10 µM elicited a significant increase in the amplitude of the CA1 fast AHP in our experiments. Local anesthetics prolong the inactivated state of the sodium channel as a result of tighter binding during this state (Kuroda et al. 2000). Prolonged inactivation could lead to increased AP duration as observed with the latter APs of a spike train compared with control CA1 neurons. What increases AP duration cannot be determined without further experiments. Aside from sodium channel alterations affecting the fAHP, the activation of voltage-gated potassium channels and Ca\(^{2+}\) activated K\(^+\) currents could also increase the fAHP as reported in pyramidal neurons (Sah 1996; Bekkers 2000). However, the caines have not been shown to affect these currents (Creveling et al. 1983).

Carbetapentane is a sigma receptor (\(\sigma\)R) ligand. It is a 26 k-Dalton protein with two known subtypes (\(\sigma1\) and \(\sigma2\)) in a single membrane spanning domain (Gundlach et al. 1986). Its function is unknown. The \(\sigma1\)R is widely located within the rat CNS particularly neuronal cell bodies and dendrites of brainstem, hypothalamus and hippocampus, but not axon fibers or terminals (Hanner et al. 1996). Intracellular location includes both the plasma membrane and intracellular compartments such as
mitochondrial membrane and endoplasmic reticulum (Alonso et al. 2000). Activation of σ1R increases delayed outwardly rectifying K⁺ currents (Wilke et al. 1999), BK channels, and the M-current within cardiac neurons (Zhang and Cuevas 2005).
FIG. 1. A: ‘Blind’ patch clamp recording from the CA1 hippocampal neurons were made in current clamp mode. Orthodromic (ortho) action potentials of CA1 neurons were activated by stimulating the stratum radiatum (RAD) near the CA3 region and antidromic (anti) activation involved stimulating the alveus. Single cell recording in the granule cell layer (GC) was in the upper blade. B: Individual components of the AD trace recorded under current clamp in the single cell. AD initiation (1) is the point where the initial slow depolarization transitions to a more rapid depolarization (min:sec). The peak potential (2) of the AD is the point where the slope of the fast AD transitions to the slope of the late depolarization (in mV). The fast AD amplitude (3) was measured between AD initiation and the peak potential (in mV). The duration of the late depolarization (4) was measured between the peak potential and the maximal depolarization (in sec).

FIG. 2. A: The equipment consisted of a broad band halogen light source focused on the slice where light was transmitted, absorbed or reflected. Transmitted light was collected by the digital camera and processed by the camera controller. Increases in light transmittance (LT) were pseudocoloured blue-green-yellow and decreases in LT were pseudocoloured magenta. B: Imaging of LT during single cell current clamp in CA1 neurons. Pseudocolored images demonstrating LT changes as a result of oxygen-glucose deprivation (OGD). The AD front was observed as a wave of increased LT propagating along the length of the CA1 region. Following in the wake of the AD front was a reduced LT within the dendritic regions (stratum oriens and stratum radiatum), the result of light scattering by beaded, injured dendrites (Obeidat & Andrew 1998; Andrew et al. 2007). Meanwhile the stratum pyramidale continued to show increases in LT denoting pyramidal soma swelling, even after aCSF re-introduction within seconds after the passing AD front. C: A current clamp recording in CA1 showed that the fast AD was concurrent with the front of elevated LT passing the recording electrode.

FIG. 3. Bath-applied drug pre-treatment for forty minutes prolongs the latency to AD onset evoked by 10 min OGD and recorded in CA1 neurons under current clamp. A, B: Representative trace of AD delay induced by of 10 µM dibucaine (dib) and 10 µM carbetapentane (CP). Note that unlike dibucaine, CP did not block action potentials (arrow) consistent with it not raising spike threshold (although amplitude was reduced). C: Mean AD onset times in drug-treated slices, measured as the increased LT front passed the
recording electrode. Significant differences compared to controls were induced by 10 µM dibucaine as well as 10 to 100 µM CP.

**FIG. 4.** CA1 recordings of AD onset during intracellular introduction of Na⁺ channel blockers compared to control A: No drug was included in the patch pipette. B, C: 10 and 100 µM dibucaine respectively reduced the fast AD (but not its onset time), thereby prolonging the late AD. Such a delay in reaching complete depolarization is considered neuroprotective.

**FIG. 5.** Granule cell recordings and AD. A: control recording and B: intracellular introduction 10 µM dibucaine. As in CA1 neurons, the AD onset time was unaffected but the fast AD was attenuated, prolonging the late AD and delaying complete depolarization.

**FIG. 6.** Ouabain-induced AD (100 µM, 10 min) was elicited in all control CA1 neurons (A) whereas a 40 minute pretreatment with 10 µM dibucaine (B) attenuated AD despite ouabain treatment for the full 10 minutes. The resting potential remained at about -40 mV.

**FIG. 7.** AP discharge by CA1 neurons is attenuated by both dibucaine and carbetapentane but by different mechanisms. All recordings had resting potentials between -61 and -64 mV. A: An AP train in control aCSF during a 100 pA depolarizing current pulse lasting one second (initiated at the arrow). B: A 30 minute pretreatment with 10 µM dibucaine required a 200 pA injection to fire a maximum two APs. Dibucaine reduced AP frequency by raising the AP threshold (Table 4). C: A 30 minute pretreatment with 100 µM CP also reduced AP frequency but without a corresponding increase in threshold (Table 4). Rather, CP increased post-AP afterhyperpolarizations and slowed AP discharge rates.

**FIG. 8.** Enhancing AP afterhyperpolarization reduces firing. A: The CA1 fast afterhyperpolarization (fAHP) was measured relative to AP threshold (dashed line) the first action potential (AP) of a train evoked by a one-second depolarizing current pulse. The fAHP was measured relative to AP threshold (dashed line). The fAHP significantly increased in amplitude and duration following 10 µM dibucaine treatments. In contrast, CP at 10 to 100 µM eliminated the fAHP, resulting in a pronounced depolarizing afterpotential (DAP). B) The slow afterhyperpolarization (sAHP) that followed the first AP of a train evoked by a one-second depolarizing current pulse was also examined. Compared to control, both Dib and CP increased the sAHP amplitude and duration. A 30 µM CP exposure inhibited the fAHP enough to
reveal a DAP. Despite this, the prolonged sAHP enhancement helps account for the slowed firing displayed in Figure 7.

**FIG. 9.** A: Drug effects upon AP threshold in CA1 neurons. Pretreatment with 10 µM Dib for 30 min significantly raised AP threshold in both the first and the last AP of the train. The threshold of the first AP increased to -37.7 ± 4.5 mV compared to -47.5 ± 4.4 mV in control CA1 neurons. The last AP threshold increased to -28.3 ± 4.2 mV from -41.2 ± 4.0 mV. CP failed to raise the threshold of the first AP. However, by the last AP in the train, threshold significantly increased in 10 or 30 µM CP. This helps explain why spiking slowed during the spike train (Fig. 8). Addition of 3 µM dib to 30 µM significantly raised threshold in both the first and the last AP. B: Drug effects upon AP firing frequency. Firing was significantly reduced by exposure to 10 or 100 µM CP at all three levels of current injection (0.1, 0.2 and 0.3 nA). Likewise 10 µM dibucaine reduced firing rate. Adding 3 µM dib to 30 µM CP significantly reduced frequency compared to 30 µM alone.

**FIG. 10.** A) Upon either orthodromic (left) or antidromic (right) stimulation, 10 µM dibucaine induced spike failure. The orthodromically-evoked spike failed after an average of 7.4 ± 2.4 min, while it took about twice as long for the antidromic spike to fail (15 ± 4.6 min). This may be because presynaptic axonal APs in CA3 axon collaterals were more easily blocked than the CA1 axonal AP. B: Drug effects on the depolarizing afterpotential (DAP). Representative traces of antidromically-evoked APs in control, 10 µM dibucaine and 100 µM CP. The DAP was measured as the voltage amplitude relative to resting membrane potential at 3 and 5 ms after AP threshold. C: CP significantly increased the DAP amplitude at both 3 and 5 ms in a concentration-dependent manner at 30 and 100 µM, whereas 10 µM Dib did not (* p<0.05).
**Table 1: Anoxic depolarization properties recorded in CA1 pyramidal cells evoked by oxygen/glucose deprivation**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=17)</th>
<th>10 µM Dib (n=11)</th>
<th>100 µM Dib (n=14)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast AD ampl (mV)</td>
<td>28.8 ± 6.2</td>
<td>26.1 ± 10.2</td>
<td>18.9 ± 6.9*</td>
<td>reduced</td>
</tr>
<tr>
<td>Peak Potential (mV)</td>
<td>-9.4 ± 4.6</td>
<td>-15.4 ± 11.0</td>
<td>-23.4 ± 11.2**</td>
<td>reduced</td>
</tr>
<tr>
<td>Slope of Fast AD (mV/s)</td>
<td>2.7 ± 1.5</td>
<td>2.1 ± 1.6</td>
<td>1.5 ± 0.4*</td>
<td>reduced</td>
</tr>
<tr>
<td>Slope of Slow Depol (mV/s)</td>
<td>0.28 ± 0.16</td>
<td>0.18 ± 0.06</td>
<td>0.16 ± 0.04*</td>
<td>reduced</td>
</tr>
<tr>
<td>Late AD duration (s)</td>
<td>46.4 ± 31.3</td>
<td>73.2 ± 37.2*</td>
<td>124.5 ± 50.9**</td>
<td>increased</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p<0.05; **p<0.01. Dib = Dibucaine; Fast AD ampl = Amplitude of fast component of anoxic depolarization; Peak Potential = peak amplitude of the depolarization; Slope of Fast AD = slope of the fast component of anoxic depolarization; Slope of Slow Depol = slope of slow component of depolarization; Late AD duration = duration of the late or persistent depolarization.
Table 2: Anoxic depolarization properties recorded in granule cells evoked by oxygen/glucose deprivation

<table>
<thead>
<tr>
<th></th>
<th>Control (n=13)</th>
<th>10 µM Dib (n=10)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast AD ampl (mV)</td>
<td>23.3 ± 8.2</td>
<td>15.8 ± 5.3*</td>
<td>reduced</td>
</tr>
<tr>
<td>Peak Potential (mV)</td>
<td>-11.4 ± 6.0</td>
<td>-23.3 ± 6.3**</td>
<td>reduced</td>
</tr>
<tr>
<td>Slope of Fast AD (mV/s)</td>
<td>1.0 ± 0.6</td>
<td>0.75 ± 0.3</td>
<td>no effect</td>
</tr>
<tr>
<td>Slope of Slow Depol (mV/s)</td>
<td>0.29 ± 0.20</td>
<td>0.22 ± 0.10</td>
<td>no effect</td>
</tr>
<tr>
<td>Late AD duration (s)</td>
<td>44.2 ± 27.1</td>
<td>132.7 ± 63.7**</td>
<td>increased</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p<0.05; **p<0.01. For list of abbreviations see Table 1.
Table 3: Anoxic depolarization properties evoked by oxygen/glucose deprivation or ouabain recorded in CA1 neurons

<table>
<thead>
<tr>
<th></th>
<th>OGD (n=17)</th>
<th>Ouabain (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast AD amp (mV)</td>
<td>28.8 ± 6.2</td>
<td>29.0 ± 6.6</td>
</tr>
<tr>
<td>Peak Potential (mV)</td>
<td>-9.4 ± 4.6</td>
<td>-4 ± 1.8*</td>
</tr>
<tr>
<td>Slope of Fast AD (mV/s)</td>
<td>2.7 ± 1.5</td>
<td>3.9 ± 1.3*</td>
</tr>
<tr>
<td>Slope of Slow Depol (mV/s)</td>
<td>0.28 ± 0.16</td>
<td>0.18 ± 0.09</td>
</tr>
<tr>
<td>Late AD duration (s)</td>
<td>46.4 ± 31.3</td>
<td>25.7 ± 16.6</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p<0.05; **p<0.01. For list of abbreviations see Table 1.
Table 4: Drug effects on the intrinsic properties of CA1 neurons

<table>
<thead>
<tr>
<th></th>
<th>Control (n=45)</th>
<th>3 µM Dib (n=9)</th>
<th>10 µM CP (n=12)</th>
<th>30 µM CP (n=11)</th>
<th>100 µM CP (n=5)</th>
<th>3 µM Dib &amp; 30 µM CP (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>-62.5 ± 3.0</td>
<td>-59.7 ± 3.0</td>
<td>-62.4 ± 3.2</td>
<td>-65.6 ± 3.1</td>
<td>-64.0 ± 2.6</td>
<td>-63.5 ± 3.7</td>
</tr>
<tr>
<td>fAHP (mV)</td>
<td>-3.4 ± 1.9</td>
<td>-2.7 ± 1.7</td>
<td>absent* (11/12)</td>
<td>absent*</td>
<td>absent*</td>
<td>absent*</td>
</tr>
<tr>
<td>AP Threshold (mV)</td>
<td>-47.5 ± 4.4</td>
<td>-46.0 ± 3.8</td>
<td>-47.7 ± 2.7</td>
<td>-49.1 ± 2.8</td>
<td>-47.0 ± 3.4</td>
<td>-38.8 ± 4.6</td>
</tr>
<tr>
<td>AP freq (Hz)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.1nA)</td>
<td>12.7 ± 9.5</td>
<td>12.3 ± 9.5</td>
<td>6.8 ± 4.4*</td>
<td>6.0 ± 2.1*</td>
<td>1.2 ± 1.3**</td>
<td>0.5 ± 0.8**</td>
</tr>
<tr>
<td>(0.2nA) (Hz)</td>
<td>24.3 ± 9.5</td>
<td>34.2 ± 16.4</td>
<td>13.2 ± 4.2**</td>
<td>9.4 ± 2.5**</td>
<td>1.6 ± 0.9**</td>
<td>2.3 ± 1.8**</td>
</tr>
<tr>
<td>(0.3nA) (Hz)</td>
<td>31.5 ± 8.7</td>
<td>N/A</td>
<td>16.8 ± 5.1**</td>
<td>11.4 ± 2.8**</td>
<td>2.2 ± 1.1**</td>
<td>2.9 ± 2.3**</td>
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<tr>
<td>AP ampl (mV)</td>
<td>89.6 ± 10.0</td>
<td>70 ± 21.5*</td>
<td>82.3 ± 14.4</td>
<td>74.9 ± 15.4*</td>
<td>62.5 ± 15.0**</td>
<td>70.5 ± 10.2*</td>
</tr>
<tr>
<td>AP dur (1/2 ampl) (ms)</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.9*</td>
<td>2.0 ± 2.6**</td>
<td>1.3 ± 0.2*</td>
</tr>
<tr>
<td>Rin (MΩ)</td>
<td>47.4 ± 8.4</td>
<td>46.6 ± 1.7</td>
<td>45.8 ± 6.3</td>
<td>66.8 ± 17.2*</td>
<td>80.9 ± 16.9**</td>
<td>57.5 ± 11.5*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p<0.05; **p<0.01. Dib = dibucaine; CP = carbetapentane; RMP = resting membrane potential; fAHP = fast after hyperpolarization; AP Threshold = action potential threshold; AP freq = action potential frequency; AP ampl = action potential amplitude; AP dur = action potential duration; Rin = input resistance.
Table 5: Properties of the last action potential in the train

<table>
<thead>
<tr>
<th></th>
<th>Control (n=25)</th>
<th>10 µM Dib (n=9)</th>
<th>10 µM CP (n=12)</th>
<th>30 µM CP (n=11)</th>
<th>3 µM Dib &amp; 30 µM CP (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAHP ampl (mV)</td>
<td>-5.8 ± 1.8</td>
<td>-15.8 ± 5.0**</td>
<td>-11.2 ± 2.9*</td>
<td>-10.6 ± 2.3**</td>
<td>-17.3 ± 3.1**</td>
</tr>
<tr>
<td>AP thresh (mV)</td>
<td>-41.2 ± 4.0</td>
<td>-28.3 ± 4.2**</td>
<td>-31.1 ± 4.0**</td>
<td>-34.3 ± 4.3**</td>
<td>-32.4 ± 3.1**</td>
</tr>
<tr>
<td>AP ampl (mV)</td>
<td>69.6 ± 9.5</td>
<td>43.9 ± 12.0**</td>
<td>50.1 ± 11.5**</td>
<td>47.4 ± 7.4**</td>
<td>51.21 ± 6.7**</td>
</tr>
<tr>
<td>AP dur (1/2 ampl) (ms)</td>
<td>1.2 ± 0.2</td>
<td>2.0 ± 1.7**</td>
<td>1.4 ± 0.3</td>
<td>2.0 ± 0.5*</td>
<td>1.6 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p<0.05; **p<0.01. sAHP ampl = slow after hyperpolarization amplitude; AP thresh = action potential threshold; AP ampl = action potential amplitude; AP dur (1/2 ampl) = action potential duration at ½ of action potential amplitude.
A) Hippocampal slice preparation

B) Components of AD Trace of a CA1 pyramidal neuron

1: AD Initiation
2: Fast AD Amplitude (mV)
3: Peak Potential of Fast AD (mV)
4: Late Depolarization

opening of slow $I_{Na^+}$
opening of sustained $I_{Na^+}$
closing of fast $I_{Na^+}$

Fig. 1
Fig. 2

A) wide band light source
   infrared pass filter
   CCD

B) Dendrite Beading
   CA1 PYR
   AD front
   0:00 4:37 4:42
   4:47 4:52 9:35
   ΔLT
   0 Swelling

C) OGD
   0 mV...
   20 mV
   1 min
   4:37
   4:42
   -62 mV

Fig. 2
A) 10 μM Dibucaine

B) 10 μM Carbetapentane

C) Drug Effects Upon AD Latency in CA1

* P<0.01 compared to control

Fig. 3
Intracellular Sodium Channel Blocker Effects on CA1 AD

OGD

A) Control

0 mV

Late AD

Fast AD

B) 10 μM Dibucaine

0 mV

Late AD

Fast AD

C) 100 μM Dibucaine

0 mV

Late AD

Fast AD

20 mV

1 min

Fig. 4
Intracellular Effects of Dibucaine on Granule Cell AD

OGD

A) Control
0 mV
-66 mV

B) 10 μM Dibucaine
0 mV
-67 mV

Fig. 5
Ouabain-Induced AD in CA1 Neurons

A) Control

B) 10 μM Dibucaine
AP Trains by CA1 Neurons

A) Control

B) 10 μM Dibucaine

C) 100 μM Carbetapentane

Fig. 7
A) fAHP of First AP in Train

Control
10 μM Dib
10 μM CP
100 μM CP

AP Threshold

5 mV
3 ms

B) sAHP of Last AP in Train

Control
10 μM Dib
10 μM CP
30 μM CP

AP Threshold

5 mV
25 ms

Fig. 8
A) Drug Effects Upon AP Threshold

* p<0.05 Compared to Control

First AP

Last AP

Threshold Voltage (mV)

Control 3 μM Dib 10 μM Dib 10 μM CP 50 μM CP 3 μM Dib + 30 μM CP

45 25 9 9 12 11

45 20 9 9 12 11

-55 -45 -35 -25

B) Drug Effects on AP Firing Frequency

*p<0.05 compared to control

**p<0.05 compared to CP alone

AP Frequency (Hz)

Control 10 μM Dib 10 μM CP 30 μM CP 100 μM CP 3 μM Dib + 30 μM CP

45 14 12 11

45 14 12 11

0.0 10.0 20.0 30.0 40.0

Fig. 9
A) Orthodromic Stimulation  Antidromic Stimulation

![A) Orthodromic Stimulation  Antidromic Stimulation](image)

B) Drug Effects Upon the CA1 Antidromic Response

![B) Drug Effects Upon the CA1 Antidromic Response](image)

C) Drug Effects Upon DAP Amplitude of Orthodromic AP

![C) Drug Effects Upon DAP Amplitude of Orthodromic AP](image)

Fig. 10