Neuropharmacology 56 (2009) 198–207

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Post-hypoxic changes in rat cortical neuron $GABA_A$ receptor function require L-type voltage-gated calcium channel activation

Liping Wang [a,b,c], L. John Greenfield, Jr. [a,b,c,*]

[a] Department of Neurology, University of Toledo College of Medicine, Health Science Campus, Toledo, OH 43614-2598, USA
[b] Department of Physiology and Pharmacology, University of Toledo College of Medicine, Health Science Campus, Toledo, OH 43614-2598, USA
[c] Neuroscience and Neurological Disorders Track, Biomedical Sciences Graduate Program, University of Toledo College of Medicine, Health Science Campus, Toledo, OH 43614-2598, USA

ARTICLE INFO

Article history:
Received 1 May 2008
Received in revised form 26 June 2008
Accepted 5 July 2008

Keywords:
Patch-clamp
Reversal potential
Gramicidin
Nitrendipine
Benzodiazepine
Zolpidem

ABSTRACT

Hypoxia modifies $GABA_A$ receptor ($GABA_AR$) function and can cause seizures, encephalopathy or myoclonus. To characterize the effects of hypoxia on neuronal $GABA_ARs$, we subjected rat cortical neurons to 1% O_2 for 2, 4 or 8 h, followed by recovery times of 0–96 h, and used whole-cell and perforated patch-clamp recording to assess $GABA_AR$ currents and pharmacology. Hypoxic exposure for 4 h caused downregulation of maximal GABA current immediately following hypoxia and after 48 h recovery without changing the EC_{50} for GABA. Two- and eight-hour hypoxic exposures had inconsistent effects on $GABA_AR$ currents. Maximal diazepam potentiation was increased immediately following 4 h hypoxia, while potentiation by zolpidem was increased after 48 h recovery. Pentobarbital enhancement and zinc inhibition of GABA currents were unchanged. Hypoxia also caused a depolarizing shift in the reversal potential of GABA-induced Cl^- currents after 24 h recovery. The L-type voltage-gated calcium channel (L-VGCC) blocker, nitrendipine, during hypoxia or control treatment prevented the reduction in $GABA_AR$ currents, and increased control currents over baseline. Nitrendipine also prevented the increase in zolpidem potentiation 48 h after hypoxia, and blocked the depolarizing shift in Cl^- reversal potential 24 h after hypoxia. The effects of hypoxia on maximal $GABA_AR$ currents, zolpidem pharmacology and Cl^- reversal potential thus require depolarization-induced calcium entry via L-VGCCs, and constitutive L-VGCC activity appears to reduce maximal $GABA_AR$ currents in control neurons via a calcium-dependent process. Calcium-dependent modulation of $GABA_AR$ currents via L-VGCCs may be a fundamental regulatory mechanism for GABA receptor function.

1. Introduction

$GABA_A$ receptors ($GABA_ARs$) mediate fast inhibitory synaptic neurotransmission in the central nervous system. They are members of the ligand-gated ion channel family, composed of five subunits from multiple subunit subtypes (α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ and ρ1–ρ3) (Macdonald and Olsen, 1994). The binding of γ-aminobutyric acid (GABA) regulates gating of the chloride ion channel, and a number of positive and negative allosteric regulators, including the benzodiazepines (BZ), barbiturates, neurosteroids, Zn^{2+} and other divalent cations, interact with $GABA_ARs$ at binding sites formed by the presence of specific subunit subtypes in the holoreceptor (Korpi et al., 2002). The BZ full agonist diazepam binds to $GABA_ARs$ containing α1, α2, α3 or α5 subunits, combined with β and γ2 subunit (Korpi et al., 2002). High affinity zolpidem binding is conferred by the presence of an α1 subunit, combined with β and γ subunits. Presence of α2 or α3 subtype results in receptors with moderate zolpidem affinity (MacDonald and Kapur, 1999). Zn^{2+} inhibits GABA-evoked currents with higher potency at receptors containing an α4, α5 or α6 and lacking an α1 and/or a γ subunit (Macdonald et al., 1996). The pharmacological and biophysical properties of $GABA_ARs$ thus depend on subunit composition (Sieghart et al., 1999).

Both excitatory and inhibitory neurotransmission are affected during and after hypoxia, leading to excitotoxicity (Yue et al., 1997). Modification of $GABA_A$ receptor function has been implicated in a range of hypoxia-related pathologies, including encephalopathy (Low et al., 1985), seizures (Bergamasco et al., 1984) and myoclonus (Hallett, 2000). GABA-mediated currents were reduced in CA1 pyramidal neurons in hippocampal slices exposed to hypoxia both in vivo and in vitro (Xu and Pulsinelli, 1994; Congar et al., 1995). Decreased $GABA_AR$ current in cultured hippocampal neurons

* Corresponding author. Department of Neurology, University of Toledo College of Medicine, 3000 Arlington Avenue, Mail Stop 1195, Toledo, OH 43614-2598, USA. Tel.: +1 419 383 3856; fax: +1 419 383 3093.
E-mail address: john.greenfield@utoledo.edu (L.J. Greenfield Jr.).

0028-3908/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2008.07.004

subjected to experimental ischemia was attributed to depletion of ATP and increased intracellular Ca^{2+} (Harata et al., 1997). Within 1 h after cerebral ischemia, GABA-gated $^{36}Cl^-$ flux dramatically decreased in gerbil neurons (Verheul et al., 1993). Intracellular Cl^- in rat hippocampal slices was increased early after ischemia, resulting in reduced Cl^- driving force and $GABA_A$ responses (Inglefield and Schwartz-Bloom, 1998).

Our previous studies demonstrated that maximal GABA currents in NTera2 (NT2-N) neuronal cells changed in a biphasic manner after 8 h exposure to 1% O_2. Maximal currents were significantly increased immediately following 8 h hypoxia, but were dramatically reduced after 48 h recovery, and then returned to baseline after 96 h recovery. $GABA_A$ receptor α1, α5, β2 and γ2 subunit mRNAs were downregulated after hypoxia (Gao et al., 2004). Since NT2-N cells are derived from a human tumor cell line, they may not completely reflect the behavior of brain-derived neurons. Here we investigated the effects of different durations of hypoxia and normoxic recovery on $GABA_AR$ currents and pharmacology in rat cortical neurons in primary culture. Hypoxic exposure reduced GABA currents immediately following hypoxia and after 48 h recovery, altered benzodiazepine pharmacology and shifted the chloride equilibrium potential. These post-hypoxic changes could be prevented by the voltage-gated calcium channel (L-VGCC) blocker, nitrendipine, during hypoxic treatment.

2. Methods

2.1. Cortical neuron culture

Cortices were dissected from E18 fetal Sprague–Dawley rats after euthanasia of the dam by a protocol approved by University of Toledo College of Medicine, IACUC. Primary culture of cortical neurons was performed according to a procedure modified from standard techniques (Banker and Cowan, 1977; Ransom et al., 1977; Porter et al., 1997). Dissected cortices were transferred to 0.25% Trypsin–ethylene diaminotetraacetic acid (EDTA) for 10 min at 37 °C, washed three times with Spiner's modification of Eagle's minimum essential medium (SMEM), and then dispersed by repeated trituration. The cell suspension was diluted with SMEM plus fetal bovine serum (5%) and horse serum (5%) to a final concentration of $3–5 \times 10^5$ cells/ml and plated onto a poly-D-lysine coated 35 mm plastic culture dishes. To inhibit proliferation of non-neuronal cells at 3 days in vitro (DIV), half of the medium was replaced with SMEM with horse serum (SMEM/HS) containing 5-fluoro-2′-deoxyuridine (FUDR) and uridine. Half of the medium was exchanged for fresh SMEM/HS at least twice a week (Porter et al., 1997). Cells were cultured for 13–15 DIV before use in the experiments below.

2.2. Hypoxia and reoxygenation

Neurons were examined microscopically to confirm viability before starting the experiment. Deoxygenated culture medium (SMEM/HS) was prepared by bubbling for 10 min with 95% N_2/5% CO_2 at 37 °C. SMEM/HS was replaced with deoxygenated SMEM/HS and then placed in an O_2- and CO_2-controlled incubator (Innova CO-48, New Brunswick Scientific Co., Inc.) pre-equilibrated to 1% O_2, 5% CO_2 at 37 °C for 2, 4 or 8 h. After hypoxic exposure, the medium was replaced with fresh aerated SMEM/HS and neurons were either studied immediately or returned to the normoxic incubator (95% air/5% CO_2, 37 °C) for 24 or 48 h prior to recording. For the "0 h" recovery time point, neurons were recorded within 1–2 h of termination of hypoxia; for 24 and 48 h recovery times, recordings occurred within ±2 h of the stated time. For L-VGCC antagonist experiments, SMEM/HS was replaced with deoxygenated SMEM/HS containing 3 μM nitrendipine and kept in the hypoxic incubator (1% O_2, 5% CO_2 at 37 °C) for 4 h. After hypoxic exposure, the medium was replaced with fresh aerated SMEM/HS without nitrendipine and cells were returned to the normoxic incubator. Control experiments were similarly handled but maintained in a normoxic environment and solutions at all times.

2.3. Electrophysiology

2.3.1. Whole-cell patch-clamp recording

Whole-cell recordings were obtained using standard patch-clamp technique (Hamill et al., 1981) with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Cortical neurons were taken out from the incubator and the medium was replaced with external recording solution containing (in mM): 142 NaCl, 10 $CaCl_2$, 6 $MgCl_2$, 8.1 KCl, 10 glucose, 10 HEPES, 315–325 mOsm, pH 7.4. Patch-clamp electrodes of 2.5–5.0 MΩ were filled with internal solution containing (in mM): 153.3 KCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA, 4 MgATP, 305–312 mOsm, pH 7.3. Signals were digitized on line at 1000 Hz using a Digidata 1322A Data Acquisition System (Axon instruments, Foster City, CA) running Clampex 9.2 software and subsequently analyzed off-line using Clampfit 9.2 (pClamp 9.2, Axon Instruments). Patch-clamp electrodes were pulled from Fisher Micro-hematocrit capillary tubes (Fisher Scientific) using a P-97 Flaming–Brown micropipette puller (Sutter Instrument Co.). For whole-cell recordings, membrane potential was held at −70 mV. GABA and other drugs were sequentially applied in increasing concentrations using a pressurized drug application system to facilitate flow through 100 μM applicator tips. Peak $GABA_AR$ currents elicited by increasing GABA concentrations were fitted to a sigmoidal function using a four parameter logistic equation (sigmoidal *C–R*) with a variable slope: $I = (I_{max})/(1 - 10^{(\log(EC_{50} - [drug]) \times Hill\ slope)})$, where I is the peak current at a given GABA concentration, and I_{max} is the maximal $GABA_AR$ current. Curve fitting was performed using Prism 4.0 software (Graph Pad Software Inc., San Diego, CA). Maximal current and *C–R* curve fits were obtained from individual neurons, then averaged and compared by ANOVA with post-hoc *t*-tests using Bonferroni's correction for multiple comparisons. Cell capacitance was measured shortly after obtaining whole-cell configuration using the "Membrane Test" function of Clampex 9.2. The percent enhancement or inhibition produced by co-application of a modulator was determined by dividing the peak amplitude in the presence of modulator by the average of control currents elicited by GABA alone before and after co-application and multiplying by 100. Neurons with significant rundown (>20% over 10 min) were excluded from analysis.

2.3.2. Gramicidin-perforated patch recording

Gramicidin-perforated patch recordings were performed as previously reported (Gao and Greenfield, 2005) using similar methods as whole-cell recordings, except the plasma membrane was not mechanically ruptured after gigaseal formation. Gramicidin was dissolved in DMSO (10 mg/ml) and then diluted in the internal solution to a final concentration of 10 μg/ml. The electrode tip was filled with a small amount of gramicidin-free internal solution to avoid problems with seal formation. After seal formation, the progress of gramicidin Cl^- impermeable pore formation was evaluated by monitoring the gradual reduction in membrane resistance. GABA (10 μM) was applied to the cell after the membrane resistance had stabilized to 300–400 MΩ which usually took about 12–20 min. The reversal potential for GABA currents (E_{Cl}) was determined by varying holding potential of neurons from −90 to +50 mV in 20 mV increments and measuring the resulting peak - GABA-evoked currents. Linear regression was used to calculate a best-fit line for the voltage dependence of GABA currents, and the interpolated intercept of this line with the abscissa was taken as the E_{Cl} value. The slope of this line was used as the corresponding slope conductance. Perforated patch recordings were terminated by increased suction on the micropipette or when the patch spontaneously ruptured into whole-cell configuration as evidenced by large depolarizing currents due to E_{Cl} of 0 mV.

3. Results

3.1. Effects of duration of hypoxia on $GABA_AR$ currents

After 13–15 DIV, healthy cortical neurons developed long axons and dendrites. No overt changes in cortical neuron morphology were observed immediately after exposure to 1% O_2 for 2, 4 or 8 h of hypoxia or after 24 or 48 h recovery under normoxic conditions (95% air/5% CO_2, 37 °C). This was consistent with our previous studies demonstrating that 8 h hypoxic exposure on NT2-N neuronal cells did not result in significant death or injury by trypan blue staining and lactate dehydrogenase (LDH) assay. There was no difference in initial membrane potential or membrane capacitance (C_m) between control cells and cells recorded after hypoxic exposure with recovery durations up to 48 h (Table 1). Since membrane capacitance is proportional to cell membrane surface area, hypoxia did not appear to cause somatic swelling or dendrite retraction, features commonly associated with neuronal injury.

Table 1
Rat cortical neuron membrane capacitance after hypoxia

Hypoxia duration (h)	Capacitance (pF), Mean ± SEM (*n*)			
	Control	0 h Recovery	24 h Recovery	48 h Recovery
2	19.5 ± 0.46 (9)	20.0 ± 0.93 (8)	19.3 ± 0.77 (9)	18.2 ± 1.3 (9)
4	20.3 ± 0.71 (10)	20.1 ± 0.75 (9)	19.1 ± 1.07 (7)	19.3 ± 0.71 (10)
8	19.9 ± 0.88 (10)	20.2 ± 1.06 (10)	20.2 ± 0.58 (8)	17.9. ± 084 (9)
4 + NT	18.8 ± 0.67 (11)	19.1 ± 0.89 (8)	17.3 ± 0.75 (8)	18.5 ± 0.67 (9)

Membrane capacitance (pF) at control and post-hypoxic recovery times after stated durations at 1% O_2. 4 + NT: 4 h hypoxia in presence of nitrendipine (3 uM). No significant differences were observed.