Neuropharmacology 56 (2009) 1088–1096

Contents lists available at ScienceDirect

Neuropharmacology

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

Ischaemia differentially regulates $GABA_B$ receptor subunits in organotypic hippocampal slice cultures

Helena Cimarosti [1], Sriharsha Kantamneni [1], Jeremy M. Henley*

Department of Anatomy, MRC Centre for Synaptic Plasticity, School of Medical Sciences, University Walk, University of Bristol, Bristol, BS8 1TD, UK

ARTICLE INFO

Article history:
Received 13 October 2008
Received in revised form
19 February 2009
Accepted 18 March 2009

Keywords:
Cerebral ischaemia
$GABA_B$ receptors
Baclofen
Organotypic hippocampal slice cultures
Oxygen–glucose deprivation
NMDA-induced excitotoxicity

ABSTRACT

Reduced synaptic inhibition due to dysfunction of ionotropic $GABA_A$ receptors has been proposed as one factor in cerebral ischaemia-induced excitotoxic cell death. However, the participation of the inhibitory metabotropic $GABA_B$ receptors in these pathological processes has not been extensively investigated. We used oxygen–glucose deprivation (OGD) and NMDA-induced excitotoxicity as models to investigate whether ischaemia-like challenges alter the protein levels of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits in rat organotypic hippocampal slice cultures. Twenty-four hours after the insult both OGD and NMDA produced a marked decrease in the total levels of $GABA_{B2}$ (~75%), while there was no significant change in the levels of $GABA_{B1}$ after OGD, but an increase after NMDA treatment (~100%). The $GABA_B$ receptor agonist baclofen (100 μM) was neuroprotective following OGD or NMDA treatment if added before or during the insult. $GABA_B$ receptors comprise heterodimers of $GABA_{B1}$ and $GABA_{B2}$ subunits and our results suggest that the separate subunits are independently regulated in response to extreme neuronal stress. However, because $GABA_{B2}$ is required for functional surface expression, down-regulation of this subunit removes an important inhibitory feedback mechanism under pathological conditions.

1. Introduction

Cerebral ischaemia/stroke is a major cause of death and severe long-term disability due to excessive neuronal excitation and consequent cell death (Choi, 1992). The involvement of excitatory glutamate receptors in ischaemic cell death has been extensively investigated but the contributions of inhibitory gamma-amino-butyric acid (GABA) receptors are less well established (Schwartz-Bloom and Sah, 2001). GABA acts at three main classes of receptors in the mammalian brain, the $GABA_A$, $GABA_B$ and $GABA_C$ receptors. Both $GABA_A$ and $GABA_C$ receptors are ligand gated Cl^--permeable ion channels, while $GABA_B$ receptors are G-protein-coupled receptors that exert much longer lasting synaptic inhibition. $GABA_B$ receptors require two subunits, $GABA_{B1}$ and $GABA_{B2}$, to form a functional receptor and they are present at both post- and presynaptic compartments. $GABA_B$ receptors act presynaptically by inhibiting Ca^+ channels, and postsynaptically by activating inwardly rectifying K^+ channels (GIRKs). Changes in the number, activity and/or localisation of $GABA_B$ receptors can dramatically alter the level of synaptic inhibition (Bettler and Tiao, 2006). Thus, $GABA_B$ receptors seem likely candidates to play a role in balancing excessive glutamatergic excitation that occurs during ischaemia. For example, activation of presynaptic $GABA_B$ receptors that can down-regulate glutamate release might provide a mechanism to counteract excitotoxic neuronal cell death.

The $GABA_B$ receptor agonist baclofen inhibits glutamate release (Huston et al., 1995) and has been reported to be neuroprotective *in vivo* (Babcock et al., 2002; Jackson-Friedman et al., 1997; Lal et al., 1995; Ouyang et al., 2007) and in organotypic hippocampal slice cultures during oxygen–glucose deprivation (OGD) (Dave et al., 2005). It has also been reported that both $GABA_A$ and $GABA_B$ receptor agonists can protect neurons against death induced by ischaemia/reperfusion *in vivo* via a mechanism involving inhibition of NMDA receptor-mediated nitric oxide (NO) production by neuronal NO synthase (nNOS) (Zhou et al., 2008). Interestingly, $GABA_B$ receptors are stable at the plasma membrane showing little basal down-regulation or baclofen induced internalisation making them an attractive therapeutic target (Fairfax et al., 2004). Indeed, baclofen has been used for many years primarily as a muscle relaxant to treat spasticity (Bowery, 2006).

To gain insight into the potential roles of $GABA_B$ receptors in ischaemia we investigated changes in $GABA_{B1}$ and $GABA_{B2}$ protein levels in organotypic hippocampal slice cultures exposed to OGD or NMDA-induced excitotoxicity, two models widely used to elicit neuronal cell death. In addition, we tested the neuroprotective

* Corresponding author. Tel.: +44 117 9546 449; fax: +44 117 9291 687.
E-mail address: j.m.henley@bristol.ac.uk (J.M. Henley).
[1] These authors contributed equally to this work.

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doi:10.1016/j.neuropharm.2009.03.007

effects of baclofen in these models. Our data suggest that the individual subunits are differentially regulated following ischaemic insult. The total levels of $GABA_{B2}$ are dramatically reduced whereas $GABA_{B1}$ levels remain unchanged. Surprisingly, we also found that although baclofen was neuroprotective it did not prevent the ischaemia-induced reduction in $GABA_{B2}$.

2. Methods

Animal care and all experimental procedures were conducted in accordance with British animal protection legislation and the experimental protocols were approved by the British National Committee for Ethics in Animal Research.

2.1. Organotypic slice cultures of rat hippocampus

Organotypic hippocampal slice cultures were prepared using the method of Stoppini and colleagues (Stoppini et al., 1991). Transverse hippocampal slices (400 μm) from 7-day-old male Wistar rats were cut using a McIlwain tissue chopper and transferred to Hank's balanced salt solution (HBSS). Six slices were placed on each Millicell culture insert in 6-well culture plates together with 1 ml of culture medium per well. Culture medium (pH 7.3) consisted of minimum essential medium 50%, horse serum 25% and HBSS 25% supplemented with glucose 36 mM, HEPES 25 mM, $NaHCO_3$ 4 mM, and penicillin/streptomycin 1%. The cultures were incubated at 37 °C in an atmosphere of 5% CO_2 for 14 days *in vitro* (DIV) prior to use. Medium was changed every 3 days.

2.2. OGD and NMDA treatment

On DIV 14, the cultures were exposed to OGD (Cimarosti et al., 2001). Each insert was washed twice with OGD medium (pH 7.2) composed of $CaCl_2$ 1.26 mM, KCl 5.36 mM, NaCl 136.89 mM, KH_2PO_4 0.44 mM, Na_2HPO_4 0.34 mM, $MgCl_2$ 0.49 mM, $MgSO_4$ 0.44 mM, HEPES 25 mM, $NaHCO_3$ 4 mM and penicillin/streptomycin 1%. To deplete glucose from intracellular stores and extracellular space, the inserts were incubated in 1 ml of OGD medium for 10 min. Following this period, the medium was exchanged for OGD medium previously bubbled with N_2/CO_2 (95%/5%) for 10 min. The slice cultures were then transferred to an anaerobic chamber at 37 °C with N_2-enriched atmosphere where they were maintained for 45 min. After OGD, the slices were removed from the chamber, washed twice with HBSS, returned to culture medium, and incubated for a further 24 h. Where appropriate baclofen or AP5 was incorporated in culture medium and in OGD medium during the periods indicated.

For assessments of NMDA-induced cell death, on DIV 14 the cultures were exposed to NMDA 50 μM for 45 min (in the same culture medium; 37 °C atmosphere air with 5% CO_2). After NMDA exposure, the inserts were washed twice with HBSS and returned to culture medium for 24 h before being assessed for cell death. Baclofen or AP5 was incorporated in culture medium with or without NMDA during the periods indicated.

Slices in the control groups were treated in parallel to slices in the OGD or NMDA groups, and incubated for 45 min, but were washed and maintained in culture medium and exposed to 37 °C atmosphere air with 5% CO_2.

2.3. Assessment of cell death due to OGD or NMDA excitotoxicity

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Cimarosti et al., 2001). PI is excluded from healthy cells, but following loss of membrane integrity it enters cells, binds to DNA and becomes highly fluorescent. Twenty-four hours after OGD or NMDA-induced excitotoxicity, PI 7.5 μM was added to cultures and incubated for 1 h. Cultures were observed with an inverted microscope (Nikon Eclipse TE 300) fitted with a standard rhodamine filter set. Images were captured and analysed using Scion Image software. The intensity of PI fluorescence in the selected region of interest, (CA1, CA2 + CA3 or DG (dentate gyrus)), was used as an index of cell death. The pixel intensity and area in which PI fluorescence was detectable above background level was determined using the 'density slice' function of the software. The mean percentage values of fluorescence in the slices treated with test compounds (baclofen or AP5) were calculated and compared to standard damage (Cimarosti et al., 2001). Standard damage was obtained as the mean of the intensity of PI fluorescence in the organotypic slices subjected to OGD or NMDA with no added drug.

2.4. Western blots and densitometry

For Western blot analysis, slices were added to Tris–HCl (pH 7.4) 50 mM, NaCl 150 mM, EDTA 1 mM, SDS 0.1%, Triton X-100 1% and mammalian protease inhibitor 1%, and homogenized on ice. The homogenates were sonicated for 10–15 s at 4 °C and the protein concentration determined. Samples were heated for 5 min at 37 °C with β-mercaptoethanol 5%, and subjected to SDS-PAGE (8%) loaded at 15 μg protein/lane. Proteins were blotted onto Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA) and probed with appropriate primary antibodies after blocking with non-fat dry milk. The primary antibodies used were: rabbit polyclonal anti-$GABA_{B1a,b}$ antibody (1 μg/ml, Santa Cruz Biotechnology), guinea pig polyclonal anti-$GABA_{B2}$ (1:1000, Chemicon), and mouse monoclonal anti-β-actin (1:5000, Sigma Chemicals). The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:10 000, Sigma Chemicals) for 1 h followed by substrate incubation with BM Chemiluminescence Blotting Substrate (POD, Roche Molecular Biochemicals) or SuperSignal West Femto (Pierce). The chemiluminescence signal was detected on Hyperfilm HP (Amersham Biosciences). The band intensity was quantified by densitometry using ImageJ (NIH). OGD and NMDA groups were compared to control groups, which were designated as 100%, on the same Western blot to avoid any differences in signal intensity due to exposure times. All the blots were then re-probed with β-actin antibody as an internal control to ensure equal protein loading in all lanes.

2.5. Statistical analysis

Data are presented as mean ± SEM (standard error of the mean) of the indicated number of independent experiments. One-way analysis of variance (ANOVA), followed by Duncan's multiple-range method, was applied to the means to determine significant differences between experimental groups. p Values < 0.05 were considered statistically significant.

3. Results

3.1. Dose–response effect of baclofen present during oxygen–glucose deprivation (OGD)

Organotypic hippocampal slice cultures were subjected to OGD for 45 min in the absence or presence of baclofen. In the CA1 region of OGD slices, baclofen at concentrations of 5, 50, 100 and 200 μM decreased cell death assessed by the incorporation of propidium iodide (PI) by 37 ± 7%, 36 ± 10%, 34 ± 7% and 49 ± 10%, respectively, compared to the CA1 region of untreated OGD slices (Fig. 1B). Similar neuroprotection was observed in the CA2 + CA3 region, where baclofen at the concentrations of 5, 50 and 100 μM decreased PI uptake by 30 ± 10%, 27 ± 10% and 38 ± 8%, respectively, whereas the concentration of 200 μM conferred higher levels of neuroprotection (71 ± 11%, Fig. 1C). In the DG baclofen decreased the PI uptake by 32 ± 7% at 100 μM and by 33 ± 15% at 200 μM (Fig. 1D). To validate the OGD protocol as a model to assess neuroprotection, we tested the NMDA receptor antagonist AP5, an established neuroprotective agent (Fatokun et al., 2008). AP5 (50 μM) added during exposure to OGD decreased PI incorporation by ~95% in all regions analysed (see Fig. 3) indicating that nearly all OGD-invoked cell death is mediated by NMDA receptor activation.

3.2. $GABA_B$ receptors levels after OGD are not affected by baclofen treatment

We next investigated the effects of OGD on $GABA_B$ receptor levels in control and baclofen-treated slices. Slices analysed 24 h after OGD showed dramatically decreased levels of $GABA_{B2}$. Levels of $GABA_{B1}$, however, remained unchanged by OGD (Fig. 2A and B). Interestingly, inclusion of baclofen (100 μM) during the 45 min OGD did not alter the profiles of $GABA_B$ receptor subunits (Fig. 2A and B, see Fig. 4). To determine whether the OGD effect on $GABA_B$ receptor levels was an immediate or delayed response to cellular stress, we analysed slices collected immediately after (0 min), 60 and 120 min after OGD. The levels of $GABA_{B2}$ were decreased at 0 min and decreased further at 60 min and 120 min time points (Fig. 2C and E). In contrast, $GABA_{B1}$ did not decrease rather there was a trend towards increased levels following OGD, and the changes were statistically significant at 120 min (Fig. 2C and D).

We next tested the time window for neuroprotection by baclofen. Baclofen (100 μM) was added either 45 min before, during or immediately after OGD (Fig. 3). While not as effective as AP5,