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Vinpocetine regulates cation channel permeability of inner retinal neurons in the ischaemic retina ☆

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ABSTRACT

Vinpocetine is a natural drug which exerts neuroprotective effects in ischaemia of the brain through actions on cation channels, glutamate receptors and other pathways. This study investigated the effect of vinpocetine on cation channel permeability of inner retinal neurons after acute retinal metabolic insult. We focused on amacrine and ganglion cells immunoreactive for calretinin or parvalbumin due to their previously documented susceptibility to ischaemia. Using the probe, 1-amino-4-guanidobutane (AGB), we observed increased cation channel permeability across amacrine and ganglion cells under ischaemia and hypoglycaemia but not anoxia. Calretinin and parvalbumin immunoreactivity was also reduced during ischaemia and hypoglyacemia but not anoxia. Vinpocetine decreased AGB entry into ischaemic and hypoglycaemic ganglion cells indicating that the drug can modulate unregulated cation entry. In addition, vinpocetine prevented the loss of calretinin and parvalbumin immunoreactivity following ischaemia suggesting it may indirectly regulate intracellular calcium. Vinpocetine also reduced AGB permeability in selected amacrine and ganglion cell populations following *N*-methyl-D-aspartate (NMDA) but not kainate activation suggesting that vinpocetine's regulation of cation channel permeability may partly involve NMDA sensitive glutamate receptors.

1. Introduction

Ischaemia is a significant contributor to major retinal diseases that lead to irreversible blindness including glaucoma, diabetic retinopathy and retinal and choroidal vessel occlusions (Bresnick et al., 1975; Kuehn et al., 2005; Osborne et al., 2004). During ischaemic insult, energy metabolism is halted resulting in a complex cascade of events which ultimately lead to cell death (reviewed in Lipton, 1999; Osborne et al., 2004). Loss of cellular ion homeostasis, through dysfunction of membrane pumps, channels and transporters, plays a significant role in ischaemic pathogenesis (Osborne et al. 2004, Kintner et al., 2007). Specifically, depletion of ATP in ischaemia stalls membrane pumps including the Na^+/K^+ ATPase pump which leads to persistent membrane depolarisation and unregulated reversal of ion channels (Osborne et al., 2004). Abnormal ion flux in ischaemia has been associated with multiple cell death mechanisms including free radical formation (Kourie, 1998; Liu et al., 2012), mitochondrial dysfunction (Liu et al., 2009), glutamate excitotoxicity (Robin and Kalloniatis, 1992; Szydlowska and Tymianski, 2010) and activation of apoptotic pathways (Franco et al., 2006; Yu et al., 2001).

Inner retinal neurons, particularly ganglion cells, appear to highly susceptible to ischaemic cell death (Chidlow and Osborne, 2003; Goto et al., 2002; Kergoat et al., 2006; Lafuente et al., 2002; Mukaida et al., 2004). Recent studies indicate that some amacrine and ganglion cells are more sensitive to ischaemic damage than others based on their glutamate receptor profile (Dijk and Kamphuis, 2004a,b; Osborne et al., 2004, 1995; Osborne and Larsen, 1996; Schmidt et al., 2004; Sun et al., 2007a,b). Overactivation of glutamate receptors particularly the *N-methyl-D-aspartate* (NMDA) receptor is implicated in cell death in ischaemia and related retinal diseases (Choi, 1992; Ju and Kim, 2011; Kalloniatis et al., 2013; Romano et al., 1998; Sun et al., 2003). The exact pathogenesis of glutamate receptor activation in ischaemia is not known but loss of ion homeostasis has been previously

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emphasised (Kostandy, 2012; Napper and Kalloniatis, 1999; Napper et al., 1999, 2001; Sun et al., 2007b). Specifically, ionotropic glutamate receptors are ligand gated cation channels and therefore unregulated activation leads to influx of cations including Na^+, K^+ and Ca^{2+}. Ca^{2+} improperly activates multiple cellular pathways including glutamate release which propagates the excitotoxic signal (Szydlowska and Tymianski, 2010). The role of Ca^{2+} in ischaemia is also emphasised by reduced of calcium binding protein expression in inner retinal neurons in numerous ischaemia models (Dijk and Kamphuis, 2004a,b; Gunn et al., 2011; Kim et al., 2010; Kwon et al., 2005; Osborne et al., 1995; Osborne and Larsen, 1996; Osborne et al., 2002). Thus, regulating cation homeostasis with attention to ionotropic glutamate receptors should be considered in therapeutic strategies targeted at retinal ischaemia.

Vinpocetine is a herbal supplement that exhibits neuroprotective effects in ischaemia of the brain (Bonoczk et al., 2000; Jincai et al., 2013; Sauer et al., 1988; Zhou et al., 2003) and ischaemic related conditions including diabetic retinopathy (Szobor and Klein, 1976) and macular degeneration (Avetisov et al., 2007a; Avetisov et al., 2007b; Vegh et al., 2006). Multiple mechanisms have been attributed to the actions of vinpocetine (Vas and Gulyas, 2005). In metabolic studies, vinpocetine increases glucose availability of the ischaemic retina (Acosta ML personal communication) and enhances glucose availability in ischaemic post-stroke patients (Bonoczk et al., 2000; Bonoczk et al., 2002; Gulyas et al., 2001; Gulyas et al., 1999; Szilagyi et al., 2005; Vegh et al., 2006), possibly by reducing calcium influx through voltage dependent calcium channels (Tretter and Adam-Vizi, 1998; Zelles et al., 2001). Other studies show that vinpocetine inhibits Na^+ dependent voltage channels and increases the availability of ATP (Erdo et al., 1996; Kakihana et al., 1982; Molnar and Erdo, 1995; Shibota et al., 1982; Sitges et al., 2005; Sitges and Nekrassov, 1999; Tretter and Adam-Vizi, 1998; Urenjak and Obrenovitch, 1996).

The current study examines the effects of vinpocetine on retinal neurons during acute ischaemia. We focus on amacrine and ganglion cells immunoreactive for calretinin and parvalbumin based on the sensitivity of these proteins to ischaemic insult (Dijk and Kamphuis, 2004a, b; Gunn et al., 2011; Kim et al., 2010; Kwon et al., 2005; Osborne et al., 1995; Osborne and Larsen, 1996; Osborne et al., 2002). The effect of ischaemia on these cells will be deduced by analyzing changes in the cation entry through cation channels as abnormal ion homeostasis is associated with ischaemic damage. To assess cation channels, we will use 1-amino-4-guanidobutane (AGB), a sensitive probe for determining cation channel permeability in the retina (Marc, 1999a,b). The advantages of AGB in studying neural networks has been extensively discussed elsewhere (Kalloniatis et al., 2013; Marc, 1999a,b; Marc et al., 2005; Nivison-Smith et al., 2013b; Sun and Kalloniatis, 2006). Specifically, increased AGB entry and therefore increased cation channel permeability is an early marker for impeding cell death secondary to retinal damage (Acosta et al., 2005; Chua et al., 2009; de Souza et al., 2012a; Fu and Sretavan, 2012; Yu et al., 2007; Zhu et al., 2013). In ischaemia, increased AGB permeability is observed in inner retinal neurons 48 h post insult (Sun et al., 2007a; Sun et al., 2007b) however we will extend on this by examining retina immediately following acute ischaemia and with vinpocetine treatment. In addition, we assess vinpocetine action following glutamate receptor activation with NMDA and kainate to determine if its effects are related to ionotropic glutamate receptor function.

2. Materials and methods

2.1. Ethics

All experimental protocols and intervention procedures were approved by the University of Auckland Animal Ethics committee and were in adherence with the statement for The Use of Animals in Ophthalmic and Visual Research, Association for Research in Vision and Ophthalmology (ARVO).

2.2. Animal manipulation

Six week-old adult Sprague Dawley rats were deeply anesthetised using domitor (5 mg/kg; Phoenix Pharm, Auckland, New Zealand) and ketamine (60 mg/kg; Phoenix Pharm, Auckland, New Zealand) before eye enucleation. Tissue processing was conducted as outlined previously (Acosta et al., 2008; Acosta et al., 2007; Chua et al., 2009; Nivison-Smith et al., 2013b; Sun and Kalloniatis, 2006). Briefly, the posterior eye cup was separated from the anterior segment and placed on a 0.8 μm pore filter paper (Gelman Sciences, Ann Arbor, MI, USA). The pigmented epithelium, choroid and sclera were removed and the retina incubated under relevant *ex vivo* conditions.

2.3. Ex vivo retinal ischaemia models

An in-house laboratory system was used for the synchronized incubation of retinal samples in normal, ischaemic, anoxic or hypoglycaemic conditions. Details of the incubation conditions are outlined in Table 1. For each condition, known volumes of buffer was placed in petri dishes and the petri dish lids were perforated and inserted with a thin plastic capillary tube connected to a pressurised controlled gas tank to supply the dish with a gas mixture (see Table 1 for gas composition). A tube adaptor was used to allow gas delivery to several dishes simultaneously. Buffers were incubated with the gas mixture at 37 °C for at least 1 h before retinal tissue was placed in the buffer and incubated for 35 min. To activate glutamate receptors, retinae were incubated in normoxia with 5 mM NMDA or 200 μM kainate – concentrations shown to achieve maximal NMDA/kainate receptor activation (Marc, 1999a; Sun et al., 2003). The incubation characteristics when using glutamate analogs in this study are different to previous AGB studies and allow for glutamate receptor desensitization (Jones et al., 1997). Müller cell, photoreceptor or random inner retinal neuronal AGB labelling was not observed suggesting agonist doses did not induce toxicity (Marc, 1999a,b; Nivison-Smith et al., 2013b; Sun and Kalloniatis, 2006).

For vinpocetine studies, vinpocetine (14-ethoxycarbonyl-(3α,16α-ethyl)-14,15-eburnamine; Sigma–Aldrich, St. Louis, MI) was prepared as a concentrated stock solution in dimethyl sulfoxide (DMSO) before diluting to 100 μM in the relevant normal or modified physiological buffers, correcting for osmolarity with an equimolar reduction in NaCl. Vinpocetine dosage was based within concentrations shown to have a protective effect against different insults on the brain (4–400 μM) and ischaemic insult on the retina (10–100 μM; Bonoczk et al., 2000; Erdo et al., 1990). A pilot study with an AGB incubation of ischaemic tissue was also performed with different doses of vinpocetine (10–100 μM) and determined effects were best seen at the high dosage. A total of 6 retinal samples were incubated for each condition, with or without vinpocetine.

2.4. Assessment of cation channel permeability

Retinal tissue was transferred immediately from the treatment chamber to new petri dishes containing the same treatment gas mixture and buffer but with the addition of 25 mM AGB. An equimolar reduction of NaCl in the AGB buffer was made to correct for osmolarity. Tissue was incubated for 5 min before fixation in 4% (w/v) paraformaldehyde, 0.01% (w/v) glutaraldehyde in 0.1 M phosphate buffer for 30 min.