Neuropharmacology 75 (2013) 380–390

Contents lists available at ScienceDirect

Neuropharmacology

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

Three Ca^{2+} channel inhibitors in combination limit chronic secondary degeneration following neurotrauma

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ARTICLE INFO

Article history:
Received 8 April 2013
Received in revised form
24 July 2013
Accepted 30 July 2013

Keywords:
Secondary degeneration
Ca^{2+} channel inhibitors
Myelin
Myelin compaction
Neurotrauma
Visual system
Node/paranode complex
Oxidative stress

ABSTRACT

Following neurotrauma, cells beyond the initial trauma site undergo secondary degeneration, with excess Ca^{2+} a likely trigger for loss of neurons, compact myelin and function. Treatment using inhibitors of specific Ca^{2+} channels has shown promise in preclinical studies, but clinical trials have been disappointing and combinatorial approaches are needed. We assessed efficacy of multiple combinations of three Ca^{2+} channel inhibitors at reducing secondary degeneration following partial optic nerve transection in rat. We used lomerizine to inhibit voltage gated Ca^{2+} channels; oxidised adenosine-triphosphate (oxATP) to inhibit purinergic $P2X_7$ receptors and/or 2-[7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-1,2,3,4-tetrahydro quinoxalin-1-yl]acetic acid (INQ) to inhibit Ca^{2+} permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Only the three Ca^{2+} channel inhibitors delivered in combination significantly preserved visual function, as assessed using the optokinetic nystagmus visual reflex, at 3 months after injury. Preservation of retinal ganglion cells was partial and is unlikely to have accounted for differential effects on function. A range of the Ca^{2+} channel inhibitor combinations prevented swelling of optic nerve vulnerable to secondary degeneration. Each of the treatments involving lomerizine significantly increased the proportion of axons with normal compact myelin. Nevertheless, limiting decompaction of myelin was not sufficient for preservation of function in our model. Multiple combinations of Ca^{2+} channel inhibitors reduced formation of atypical node/paranode complexes; outcomes were not associated with preservation of visual function. However, prevention of lengthening of the paranodal gap that was only achieved by treatment with the three Ca^{2+} channel inhibitors in combination was an important additional effect that likely contributed to the associated preservation of the optokinetic reflex using this combinatorial treatment strategy.

1. Introduction

Following traumatic injury to the central nervous system (CNS), cells in the lesion site invariably die rapidly, and their function is lost (Norenberg et al., 2004). Nearby and remote neurons and glia initially spared by the lesion are vulnerable to bystander damage, known as secondary degeneration, resulting in further loss of neurons, myelin and function (Crowe et al., 1997; Levkovitch-Verbin et al., 2003; Khodorov, 2004; Farkas and Povlishock, 2007; Lasiene et al., 2008; Arvanian et al., 2009). Secondary degeneration is initiated by a cascade of reactive metabolic events including glutamate-excitotoxicity, Ca^{2+} overload, excess free radical formation, oxidative stress and reduced adenosine triphosphate (ATP) production, which lead to cell death (Camello-Almaraz et al., 2006; Giaume et al., 2007; Fitzgerald et al., 2010; Lau and Tymianski,

Abbreviations: INQ, 2-[7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-1,2,3,4-tetrahydro quinoxalin-1-yl]acetic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATP, adenosine triphosphate; i.p., intraperitoneally; Lom, lomerizine; NMDA, N-methyl-D-aspartic acid; OCT, optical cutting temperature; OPCs, oligodendrocyte precursor cells; ON, optic nerve; oxATP, oxidised adenosine-triphosphate; PFA, paraformaldehyde; PT, partial transection; PBS, phosphate buffered saline; RGC, retinal ganglion cell; SEM, standard error of the mean; TEM, transmission electron microscopy; VGCCs, voltage-gated calcium channels.

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http://dx.doi.org/10.1016/j.neuropharm.2013.07.034

2010; Peng and Jou, 2010). Myelinating oligodendrocytes and oligodendrocyte precursor cells (OPCs) are particularly sensitive to excitotoxic insult and oxidative stress, due to their high lipid content and low levels of antioxidants, with sensitivity dependent on maturation status (Back et al., 1998; Juurlink et al., 1998; Micu et al., 2006; Saggu et al., 2010). The compact layers of myelin are vulnerable to reactive oxygen species and lipid peroxidation, which is elevated during secondary degeneration (McTigue and Tripathi, 2008), and associated with myelin decompaction (Payne et al., 2011, 2012). Rescuing intact, but vulnerable, tissue from secondary death is recognised as the only feasible way to minimise adverse sequelae and improve long term functional outcomes after CNS trauma (Crowe et al., 1997; Blair et al., 2005).

Redistribution of Ca^{2+} is thought to be a key trigger of secondary degeneration (Lobsiger and Cleveland, 2007; Knoferle et al., 2010; Wells et al., 2012). Normal intracellular Ca^{2+} homeostasis is impaired following injury, with increased concentration of cytosolic Ca^{2+} through Ca^{2+} influx from extracellular pools, leading to further elevations *via* release from intracellular stores (Paschen, 2001; Syntichaki and Tavernarakis, 2003; Weber, 2012). Voltage-gated Ca^{2+} channels (VGCCs) are able to facilitate both extracellular Ca^{2+} influx and intracellular release of Ca^{2+} into the cytosol and are considered critical for the initial Ca^{2+} signalling observed after neurotrauma (Imaizumi et al., 1999; Weber, 2012). Purinergic $P2X_7$ receptors may also contribute to excess Ca^{2+} influx as they are upregulated following injury in response to released ATP (Franke et al., 2004; Hamilton et al., 2008). $P2X_7$, AMPA and N-methyl-D-aspartic acid (NMDA) receptor activation in OPCs and oligodendrocytes has been implicated in various forms of white matter injury (Li et al., 2000; Stevens et al., 2002; Karadottir et al., 2005; Salter and Fern, 2005; Matute et al., 2007; Pitt et al., 2010), associated with disruptions to axoglial junction and node and paranode domains, resulting in failure of saltatory conduction (Fu et al., 2009; Rosenbluth, 2009; Buttermore et al., 2011).

The effects of inhibition of Ca^{2+} channels implicated in secondary degeneration have been assessed in preclinical studies. Lomerizine dihydrochloride (lomerizine) is a relatively CNS specific L- and T-type VGCC inhibitor already in use as an anti-migraine agent (Hara et al., 1999; Toriu et al., 2000; Tamaki et al., 2003). Lomerizine protects RGCs in animal models of ischaemia, hypoxia and secondary death (Hara et al., 1999; Toriu et al., 2000; Karim et al., 2006), limiting elevations in cytosolic Ca^{2+} concentrations (Yamada et al., 2006). We have previously shown that lomerizine protects retinal ganglion cell (RGC) axons and somata vulnerable to secondary degeneration *in vivo* following partial optic nerve (ON) transection, but has limited effects on visual function (Fitzgerald et al., 2009a; Selt et al., 2010). Similarly, blockade of ATP-gated $P2X_7$ receptors *via* the irreversible, non-competitive antagonist oxATP limits Ca^{2+} flux in several models of CNS dysfunction and protects against secondary damage following spinal cord injury (Wang et al., 2004; Choi et al., 2007; Kennedy, 2007; Matute et al., 2007). The novel Ca^{2+}-permeable AMPA receptor competitive antagonist INQ has the advantage of high solubility and has shown therapeutic potential in preclinical studies of ischaemia (Takahashi et al., 2002). INQ may also prevent activation of the NMDA receptor and VGCCs (Miller, 1991; Wong and Kemp, 1991) and reversal of the Na^{+}–Ca^{2+} exchanger (Takahashi et al., 2002).

However, clinical trials with these and other single Ca^{2+} channel inhibitors for treatment of CNS injury have been disappointing (Takahashi et al., 2002; Muir, 2006; Klein and Engelhard, 2010), and while combinatorial treatments are now widely acknowledged as necessary to successfully combat secondary degeneration following neurotrauma (Coleman, 2005; Miller and Mi, 2007), few studies addressing effects of combinations of Ca^{2+} channel inhibitors have been reported. Here we have used the partial ON transection model of secondary degeneration *in vivo* (Levkovitch-Verbin et al., 2003; Fitzgerald et al., 2009a) and assessed the effects of three Ca^{2+} channel inhibitors in combination. We used combinations of lomerizine, oxATP and/or INQ, assessing visual function, neuroprotection, myelin compaction, and structure of node of Ranvier/paranode complexes, at 3 months after injury.

2. Materials and methods

2.1. Animals

Female Piebald Virol Glaxo (PVG) adult rats (160–200 g), obtained from the Animal Resource Centre (Murdoch, Western Australia), were housed in groups of three under standard conditions including 12 h light/dark cycles and *ad libitum* access to chow and water. All procedures were approved by The University of Western Australia Animal Ethics Committee and all efforts were made to minimise animal suffering and reduce the number of animals used; alternatives to *in vivo* techniques were not suitable for a study of this nature. Anaesthesia was administered intraperitoneally (i.p.) as a combination of Xylazine (Ilium Xylazil 20, 10 mg/kg) and Ketamine (Ketamil, 50 mg/kg, Troy Laboratories). Partial transection (PT) of ON, in which RGC axons in the dorsal aspect of the ON are lesioned leaving those on the ventral side intact (but susceptible to secondary degeneration), was conducted as described previously (Fitzgerald et al., 2009a). Briefly, approximately 1 mm behind the eye, the dorsal side of the right ON was partially transected to a controlled depth of 200 μm using a diamond radial keratotomy knife (Geuder). Post-operative analgesia was administered (2.8 mg/kg carprofen, Norbrook). Controls were uninjured normal animals as sham injured animals have been shown to be no different to normals in terms of visual function, RGC numbers and other cellular parameters (Fitzgerald et al., 2009a).

2.2. Treatments

Test animals ($n = 7$–10/group, total study $n = 93$) were split into groups for testing of combinations of three Ca^{2+} channel inhibitors, lomerizine dihydrochloride (lomerizine or Lom; LKT Labs), oxATP (Sigma) and/or INQ (synthesised as described below). Choice of treatment durations and concentrations were based on previously published studies using these agents individually. Lomerizine in butter (30 mg/kg, Tamaki et al., 2003) or butter alone was administered orally to all animals, while the animals were gently held. Treatment began on the day of surgery after recovery from anaesthesia and continued twice daily for 3 months (6 days/week). oxATP (1 mM, Matute et al., 2007) and/or INQ (240 μM), based on continued delivery of the 1 μg administered as a single bolus dissolved in phosphate buffered saline (PBS), were delivered for the first 2 weeks after injury at a rate of 0.5 μL/h *via* a subcutaneously implanted, pre-loaded mini-osmotic pump (Model, 2002, Alzet) (Beazley et al., 1996; Matute, 1998), attached to a cannula targeting the dorsal aspect of the ON dura mater. Longer duration of treatment with oxATP and/or INQ was not possible due to the 2 week maximum implant time of the mini-osmotic pumps for optimal efficacy. The volumes remaining in the mini-osmotic pumps were assessed after surgical removal and found to correspond with the predicted delivered volume. Rats were housed individually to minimise disturbance by cage-mates, until pumps were surgically removed at 2 weeks after injury. Additional groups of animals were included in the study design to control for isolation, and further controls included injured animals treated with vehicle only (PBS in pumps), injured animals without pumps but treated with butter vehicle only (control for lomerizine vehicle) and completely normal animals ($n = 7$–10 for all groups). Outcomes were assessed at 3 months after injury. A separate cohort of animals, for selected treatments only ($n = 4$–5/group), was assessed for oxidative stress at 2 days after injury.

INQ was synthesised using a previously reported procedure with slight modifications to steps 2 and 3 (Shishikura et al., Yamanouchi Pharmaceuticals Co., Ltd., (2000) *1,2,3,4-Tetrahydroquinoxalinedione Derivative*, U.S. Pat. 6,096,743). The structure and purity of the final compound was confirmed by nuclear magnetic resonance (^{1}H NMR) analysis in deuterated DMSO: δ_H (500 MHz, DMSO-d6) 4.89 (s, 2H), 7.88 (s, 1H), 8.01 (s, 1H), 8.08 (s, 1H), 8.28 (s, 1H), 9.43 (s, 1H), 12.89 (s, 1H), 13.1–14.2 (bs, 1H). Modifications to Step 2 (reduction of N-(2-nitro-5-fluorophenyl) glycine ethyl ester to N-(2-amino-5-fluorophenyl)glycine ethyl ester) were tetrahydrofuran was distilled prior to use; methanol was dried over magnesium sulphate and stored over molecular sieves prior to use. The hydrogenation reaction was stirred at room temperature for 1.5 h and monitored by thin layer chromatography (diethylether: methanol 10:1). The reaction mixture was then filtered through celite and concentrated under reduced pressure. The resulting crude product was purified by loading onto a short plug of silica, the silica plug was initially washed with ethyl acetate to remove impurities and then with methanol to collect the product, N-(2-amino-5-fluorophenyl)glycine ethyl ester, in 20% yield. Modifications to step 3 (acylation of N-(2-amino-5-fluorophenyl)glycine ethyl ester to ethyl 2-(7-fluoro-2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-1-yl)acetate), were chloroform was dried as for methanol above and the crude product purified as for step 2, to collect the product, ethyl 2-(7-fluoro-2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-1-yl)acetate, in quantitative yield.