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Sigma-1 receptor stimulation attenuates calcium influx through activated L-type Voltage Gated Calcium Channels in purified retinal ganglion cells

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ABSTRACT

Sigma-1 receptors (σ-1rs) exert neuroprotective effects on retinal ganglion cells (RGCs) both *in vivo* and *in vitro*. This receptor has unique properties through its actions on several voltage-gated and ligand-gated channels. The purpose of this study was to investigate the role that σ-1rs play in regulating cell calcium dynamics through activated L-type Voltage Gated Calcium Channels (L-type VGCCs) in purified RGCs. RGCs were isolated from P3-P7 Sprague–Dawley rats and purified by sequential immunopanning using a Thy1.1 antibody. Calcium imaging was used to measure changes in intracellular calcium after depolarizing the cells with potassium chloride (KCl) in the presence or absence of two σ-1r agonists [(+)-SKF10047 and (+)-Pentazocine], one σ-1r antagonist (BD1047), and one L-type VGCC antagonist (Verapamil). Finally, co-localization studies were completed to assess the proximity of σ-1r with L-type VGCCs in purified RGCs. VGCCs were activated using KCl (20 mM). Pre-treatment with a known L-type VGCC blocker demonstrated a 57% decrease of calcium ion influx through activated VGCCs. Calcium imaging results also demonstrated that σ-1r agonists, (+)-N-allylnormetazocine hydrochloride [(+)-SKF10047] and (+)-Pentazocine, inhibited calcium ion influx through activated VGCCs. Antagonist treatment using BD1047 demonstrated a potentiation of calcium ion influx through activated VGCCs and abolished all inhibitory effects of the σ-1r agonists on VGCCs, implying that these ligands were acting through the σ-1r. An L-type VGCC blocker (Verapamil) also inhibited KCl activated VGCCs and when combined with the σ-1r agonists there was not a further decline in calcium entry suggesting similar mechanisms. Lastly, co-localization studies demonstrated that σ-1rs and L-type VGCCs are co-localized in purified RGCs. Taken together, these results indicated that σ-1r agonists can inhibit KCl induced calcium ion influx through activated L-type VGCCs in purified RGCs. This is the first report of attenuation of L-type VGCC signaling through the activation of σ-1rs in purified RGCs. The ability of σ-1rs to co-localize with L-type VGCCs in purified RGCs implied that these two proteins are in close proximity to each other and that such interactions regulate L-type VGCCs.

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1. Introduction

The sigma-1 receptor (σ-1r) is a 26 kDa protein that was once categorized as an opioid receptor (Martin et al., 1976; Hanner et al., 1996). However, further studies have demonstrated that this receptor is a unique non-opioid receptor with separate pharmacological properties including the ability to bind to benzomorphans (e.g. (+)-pentazocine and (+)-SKF10047), steroids (e.g. progesterone and testosterone) and psychotropic drugs (e.g. haloperidol) (Hayashi and Su, 2005). There appear to be several subtypes of sigma receptors (σ-1r and σ-2r, and σ-3r) (Quirion et al., 1992; Myers et al., 1994), but σ-1r is the only receptor that has been

Abbreviations: KCl, Potassium Chloride; σ-1r, sigma-1 receptor; VGCCs, Voltage Gated Calcium Channels; L-type VGCCs, L-type Voltage Gated Calcium Channels; RGCs, Retinal Ganglion Cells; ER, endoplasmic reticulum; TIFRM, Total Internal Reflection Fluorescence Microscopy; EFF, evanescent field fluorescence; $[Ca^{2+}]_i$, cytoplasmic calcium concentration.

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cloned and characterized in several animal species (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1997, 1998). The endogenous ligand and function of σ-1r is not yet known. This receptor has been shown to be predominantly localized on the endoplasmic reticulum (ER), with two transmembrane spanning regions, that possess the ability to translocate to the plasma membrane upon agonist stimulation or under prolonged stress (Hayashi and Su, 2003; Aydar et al., 2002). The translocation of σ-1r to the plasma membrane is thought to be the mechanism underlying the ability of an ER protein like σ-1r to modulate and regulate many different ion channels on the plasma membrane including voltage-gated and ligand-gated Ca^{2+}, K^{+}, Na^{+}, Cl^{-}, and SK ion channels (Hayashi and Su, 2003; Aydar et al., 2002; Maurice and Su, 2009; Su et al., 2009).

A number of studies have suggested that σ-1r ligands regulate the activity of voltage gated calcium channels (VGCCs). For instance, Zhang and Cuevas (2002) demonstrated in parasympathetic and sympathetic primary neuronal cultures that σ-1r ligand compounds can facilitate calcium current inactivation of L-, N-, P/Q-, and R-type VGCCs. In primary hippocampal neurons, Church and Fletcher (1995) also demonstrated that σ-1r ligands block multiple subtypes of VGCC currents. Hayashi et al. (2000) and Tchedre et al. (2008) demonstrated in neuronal cell lines that σ-1r agonist stimulation also diminished calcium ion influx through activated VGCCs.

To date, there have been no reported studies demonstrating σ-1r modulatory effects on any ligand gated ion channels or VGCCs in purified retinal ganglion cells (RGCs). Only one study has demonstrated an inhibitory effect of σ-1r agonist stimulation on NMDA induced calcium ion influx in retinal cross-sections (Zhang et al., 2011). Therefore, it was important to investigate the role that σ-1r and σ-1r ligands play in regulating calcium ion influx in purified RGCs.

In this study, we used purified RGC cultures to study the effects of two σ-1r agonists (SKF10047 and Pentazocine) and one σ-1r antagonist (BD1047) on activated VGCCs in purified RGCs. The goal of this study was to evaluate pharmacologically if σ-1r ligands act through the σ-1r to attenuate or potentiate calcium ion influx through activated VGCCs. Lastly, this study aims to identify the subtype of VGCC that σ-1r ligand mediated effects occur in purified RGCs.

2. Materials and methods

2.1. Primary RGC isolation

All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Texas Health Science Center. Dr. Ben Barres trained our laboratory to perform his two-step panning purified retinal ganglion cell isolation (Christopherson et al., 2005; Meyer et al., 1995; Ullian et al., 2001; Barres et al., 1988). Purity utilizing this method has been described at 96%–99.5% (Christopherson et al., 2005; Meyer et al., 1995; Ullian et al., 2001; Barres et al., 1988). We found our isolation procedure resulted in a greater than 98% purity as seen using appropriate cell markers.

Sprague Dawley rats (post-natal day 3–7) (Charler River, Wilmington, MA) were euthanized, and the retinas were placed in 4.5 units/mL of papain solution (Worthington, Lakewood, NJ) to dissociate the cells. Cells were then incubated for 10 min with a rabbit anti-macrophage antibody (Cedarlane, Burlington, Ontario, Canada). Cell suspensions were then incubated in a 150-mm petri dish coated with a goat anti-rabbit IgG (H + L chain) antibody (Jackson ImmunoResearch, West Grove, PA) for 30 min. Cells that did not adhere to the 150-mm dish were then transferred to a 100-mm dish coated with anti-Thy1.1 antibody (from hybridoma T11D7; American Type Culture Collection, Rockville, MD) for 45 min. Cells were then trypsinized off (1250 units/mL) (Sigma–Aldrich, St. Louis, MO) the petri dish and plated on coverslips coated with mouse-laminin (Trevigen Inc., Gaithersburg, MD). Cells were then cultured in a serum free defined media, DMEM (Invitrogen, Grand Island, NY) containing BDNF (50 ng/mL) (Peprotech, Rocky Hill, NJ), CNTF (10 ng/mL) (Peprotech, Rocky Hill, NJ), and forskolin (5 ng/mL) (Sigma–Aldrich, St. Louis, MO). Cells were incubated at 37 °C in a humidified atmosphere of 10% CO_2 and 90% air.

2.2. Intracellular calcium ($[Ca^{2+}]_i$) measurement

Purified RGCs were allowed to incubate in culture for 10 Days *in vitro* (D.I.V) before subjecting them to calcium imaging experiments. Intracellular calcium in purified RGCs was measured at 37 °C by the ratiometric technique using fura-2-AM (excitation at 340 nm and 380 nm, emission at 510 nm) (Invitrogen, Carlsbad, CA) as described by Prasanna et al. (2000) utilizing Nikon Eclipse TE2000-5 microscope and NIS-Elements AR3.2 software (Nikon Instruments, Melville, NY). The Grynkiewicz equation was used to convert the 340/380 ratio to internal levels of calcium in nanomolar (nM) concentrations (Grynkiewicz et al., 1985) using the kd value of 224 nM. During the calcium imaging studies, some cells were pretreated with (+)-SKF10047 (Tocris, Ellisville, MO), (+)-Pentazocine (Sigma–Aldrich, St. Louis, MO), BD1047 (Tocris, Ellisville, MO), or Verapamil (Tocris, Ellisville, MO) for 30 min before they were stimulated with potassium chloride (KCl). Peak calcium levels were calculated after KCl administration. Basal levels of cytoplasmic calcium were calculated 30 s prior to the administration of KCl. Peak cytoplasmic calcium levels were calculated after the administration of KCl.

2.3. Immunocytochemistry

Purified retinal ganglion cells were grown on coverslips and fixed with 100% methanol at −20 °C for 10 min or incubated at room temperature with paraformaldehyde for 15 min. These cells were then blocked with 5% normal donkey serum for 2 h at room temperature. Blocking solution was removed and cells were then incubated with primary antibodies: affinity-purified σ-1r monoclonal antibody (1/50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), voltage-dependent L-type-1C subunit antibody (1/100; Millipore, Temecula, CA), or *Thy1.1* monoclonal antibody (1/100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. Coverslips were then washed three times with PBS, and a 1:1000 dilution of secondary antibodies donkey anti-rabbit IgG (Alexa Fluor 648) conjugate and donkey anti-mouse IgG (Alexa Fluor 488) conjugate (Invitrogen, Carlsbad, CA) were added and incubated for 1 h in the dark. After incubation, the coverslips were washed again three times with PBS. Mounting was performed on glass slides using antifade reagent with DAPI (Prolong Gold; Invitrogen, Carlsbad, CA) and allowed to dry for 20 min in the dark. Cells were viewed on a confocal laser scanning microscope (LSM 510; Zeiss, Thornwood, NY).

2.4. Co-localization with immunofluorescence staining

Confocal microscopy was performed using a Zeiss LSM 510 META laser-scanning microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 40× objective, using the following laser wavelengths: excitation 488 nm, emission 505–530 nm or excitation 648 nm, emission 630–700 nm. Quantifications of the co-localization coefficients, derived from measured pixel overlaps between sigma-1

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