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Activation of the TRPV1 channel attenuates N-methyl-D-aspartic acid-induced neuronal injury in the rat retina

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Calcitonin gene-related peptide (PubChem CID: 44563440)
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Capsazepine (PubChem CID: 2733484)
Substance P (PubChem CID: 36511)

ABSTRACT

Capsaicin, a transient receptor potential vanilloid type1 (TRPV1) agonist, has been reported to protect against ischemia-reperfusion injury in various organs, including the brain, heart, and kidney, whereas activation of TRPV1 was also reported to contribute to neurodegeneration, including pressure-induced retinal ganglion cell death *in vitro*. We histologically investigated the effects of capsaicin and SA13353, TRPV1 agonists, on retinal injury induced by intravitreal N-methyl-D-aspartic acid (NMDA; 200 nmol/eye) in rats *in vivo*. Under ketamine/xylazine anesthesia, male Sprague–Dawley rats were subjected to intravitreal NMDA injection. Capsaicin (5.0 nmol/eye) was intravitreally admianeously with NMDA injection. SA13353 (10 mg/kg) was intraperitoneally administered 15 min before NMDA injection. Morphometric evaluation at 7 days after NMDA injection showed that intravitreal NMDA injection resulted in ganglion cell loss. Capsaicin and SA13353 almost completely prevented this damage. Treatment with capsazepine (TRPV1 antagonist, 0.5 nmol/eye), CGRP (8–37) (calcitonin gene-related peptide (CGRP) receptor antagonist, 0.5 pmol/eye), or RP67580 (tachykinin NK_1 receptor antagonist, 0.5 nmol/eye) almost completely negated the protective effect of capsaicin in the NMDA-injected rats. Seven days after intravitreal NMDA injection, the cell number of retinal ganglion cell was significantly smaller than in the eye that had received capsaicin in B6.Cg-TgN(Thy1-CFP)23Jrs/J transgenic mice that express the enhanced cyan fluorescent protein in retinal ganglion cells in the retina. These results suggested that activation of TRPV1 protects retinal neurons from the injury induced by intravitreal NMDA in rats *in vivo*. Activation of CGRP and tachykinin NK_1 receptors is possibly involved in underlying protective mechanisms.

1. Introduction

Retinal ganglion cell death is a characteristic of glaucoma, but the underlying mechanism is not completely understood. However, it is known that glutamate-receptor stimulation by excess glutamate during hypoxia (David et al., 1988) and ischemia-reperfusion (Louzada-Júnior et al., 1992) is toxic to neurons. Activation of the N-methyl-D-aspartic acid (NMDA) receptor, a glutamate receptor subtype (Choi, 1987, 1988), is followed by a large Ca^{2+} influx *via* NMDA receptor-operated channels. This excess intracellular Ca^{2+} is involved in predominant neuronal excitotoxicity mechanisms and is thought to be an underlying mechanism of glaucoma-induced neuronal cell death (Kuehn et al., 2005).

Capsaicin-sensitive sensory nerves are widely distributed throughout the cardiovascular system and can be found in the blood vessels, the heart, and the kidneys (Barja et al., 1983; Wharton et al., 1986). Capsaicin activates transient receptor potential vanilloid type 1 (TRPV1) (Caterina et al., 1997), a non-selective cation channel, which causes release of neurotransmitters, including calcitonin gene-related peptide (CGRP) and substance P (Hoover, 1987; Manzini et al., 1989). It was recently shown that capsaicin has protective effects against cerebral (Pegorini et al., 2005), myocardial (Wang and Wang, 2005), pulmonary (Wang et al., 2012), hepatic (Harada et al., 2002), and renal (Ueda et al., 2008) ischemia-reperfusion injuries in rats, as well as hypoxia-reoxygenation injury in cultured hippocampus slices (Guo et al., 2008). Anandamide, an endogenous agonist of TRPV1 and cannabinoid receptors, has been reported to protect against ischemia-reperfusion injury in brain, *in vivo* (Schomacher et al., 2008).

Very strong [^{3}H]-resiniferatoxin labeling has been reported in the retina (Szallasi et al., 1995), and mRNA and the TRPV1 protein are expressed in retinal ganglion cells (RGCs) (Sappington et al., 2009). Methylanandamide has also been shown to protect the retina against ischemia-reperfusion injury (Nucci et al., 2007). In contrast, capsaicin was shown to induce RGC degeneration in

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preweanling rats (Ritter and Dinh, 1990) and apoptosis *via* extracellular Ca^{2+} influx in isolated RGCs (Sappington et al., 2009). However, whether capsaicin protects against or aggravates retinal glutamate neurotoxicity *in vivo* remains uncertain.

SA13353 was originally synthesized by the research laboratory of Santen Pharmaceutical Co., Ltd. (Nara, Japan), and the compound is a potent and orally active inhibitor of TNF-α production (Murai et al., 2008). This effect of the drug was caused by the activation of TRPV1 *in vivo* (Murai et al., 2008). The binding affinity of SA13353 to TRPV1 is approximately 15 times higher than that of capsaicin (Ueda et al., 2009). SA13353 was reported to prevent the ischemic-reperfused kidney injury (Ueda et al., 2009), the lipopolysaccharide-induced acute lung injury (Tsuji et al., 2010a), and the lipopolysaccharide-induced experimental autoimmune encephalomyelitis (Tsuji et al., 2010b) in the rat or mouse.

In the present study, we first examined whether capsaicin and SA13353 protected against NMDA-induced retinal injury in the rat, *in vivo*. On the basis of these results, we also evaluated whether TRPV1, CGRP, and substance P are involved in underlying neuroprotective mechanisms.

2. Materials and methods

2.1. Animals

This study was conducted in accordance with the regulations set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experimental procedures conformed to the Regulations for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kitasato University.

Male Sprague–Dawley rats, weighing 230–300 g were purchased from Japan SLC, Hamamatsu, Japan. B6.Cg-TgN(Thy1-CFP) 23Jrs/J transgenic mice express the enhanced cyan fluorescent protein (ECFP) in RGCs in the retina (Feng et al., 2000; Dratviman-Storobinsky et al., 2008). Using this transgenic mouse enables us to evaluate the loss of RGCs by various hazardous stimuli including intravitreal NMDA injection easily without the retrograde labeling of RGCs. Male and female B6.Cg-TgN(Thy1-CFP)23Jrs/J mice, in which the Thy1 promoter is linked to a ECFP reporter, were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were maintained by brother–sister mating in the animal room of our university. We used the mice aged 8–16 weeks old.

The environment of the animal room was kept at 25 °C with a 12 h:12 h light-dark cycle. All animals were fed and watered *ad libitum*.

2.2. Intravitreal injection

Intravitreal injection was performed as previously described (Sakamoto et al., 2009, 2010a, 2010b, 2011). Briefly, animals were anesthetized with intraperitoneal ketamine (90 mg/kg, Daiichi-Sankyo, Tokyo, Japan) and xylazine (10 mg/kg, Tokyo Kasei, Tokyo, Japan). We confirmed that ketamine, an NMDA receptor antagonist, did not affect the NMDA-induced retinal damage at the dose used in the present study by comparing the present results with those in our previous report in which pentobarbital was used as anesthesia (Sakamoto et al., 2009). Intravitreal injection was performed with a 33-gauge needle connected to a 25-μL microsyringe (MS-N25, Ito Seisakujo, Fuji, Japan). The tip of the needle was inserted ~1 mm behind the corneal limbus. One (for mice) or 5 (for rats) microliters of the drug solution described below was administered into 1 eye, and a vehicle was administered into the contralateral eye, which served as the control. Core temperature, measured by a rectal thermometer, was maintained at 37 °C during experiments by using a heating pad and a heating lamp.

2.3. Drug preparations

Capsaicin (Wako Pure Chemical, Osaka, Japan) and RP67580 (Santa Cruz Biotechnology, Dallas, TX) were dissolved in ethanol. Capsazepine (Sigma) was dissolved in DMSO. The final ethanol and DMSO concentrations in the drug solutions were 10% and 1%, respectively. NMDA (Nacalai Tesque, Kyoto, Japan), CGRP (Peptide Institute, Minoh, Japan), CGRP (8–37) (Peptide Institute), and substance P (Peptide Institute) were completely dissolved in the vehicle solution (10% ethanol and 1% DMSO in saline). These drugs were given simultaneously with NMDA. SA13353 (a gift from Santen Pharmaceutical Co., Ltd., Osaka, Japan) was dissolved in 3% DMSO and 3% CREMOPHOR EL in saline, and intraperitoneally administered 15 min before NMDA injection.

2.4. Histological evaluation

Histological evaluation methods have been described previously (Sakamoto et al., 2009, 2010a, 2010b, 2011). Briefly, animals were euthanized with an overdose of sodium pentobarbital 7 days after intravitreal NMDA injection or the ischemic episode. Both eyes were enucleated and fixed with a Davidson solution (37.5% ethanol, 9.3% paraformaldehyde, 12.5% acetic acid) for 24 h at room temperature. Fixed eyes were dissected through the optic nerve head in the vertical meridian with a microtome blade (PATH BLADE+PRO by Kai, Matsunami Glass, Kishiwada, Japan) and embedded in paraffin after the lens had been removed. We used a microtome (HM325, Microm International, Walldorf, Germany) and a microtome blade (PATH BLADE+PRO by Kai, Matsunami Glass) to make 4-μm thickness, horizontal sections through the optic nerve head. Sections were made along the vertical meridian so that they contained the entire retina from the ora serrata in the superior hemisphere to the ora serrata in the inferior hemisphere. Sections were stained with hematoxylin and eosin and examined for morphometry. Sections from oblique regions were excluded to avoid tissue artifacts. Using a light microscope (Optiphot-2, Nicon, Tokyo, Japan), the total number of RGCs in the retinal ganglion cell layer (GCL) was manually counted in a region beginning 1 mm from the center of the optic nerve head and ending 1.25 mm from the center of the optic nerve head (for a retinal length of 0.25 mm). No attempts were made to distinguish between cell types in the GCL; displaced amacrine cells were included in RGC counts. Thickness measurements of the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), and the outer nuclear layer (ONL) were also performed. Digital photographs (digital camera [DP11, Olympus, Tokyo, Japan] connected to a light microscope) were taken so that ~0.25 mm of retina appeared in each photograph, with sections ~1 mm from the center of the optic nerve head chosen. The thickness of IPL, INL, OPL and ONL was then measured. The number of the cells in GCL and the thickness of retinal layers of the NMDA-injected eyes were normalized to those of the contralateral eye and are presented as percentages.

2.5. Retinal ganglion cell count in B6.Cg-TgN(Thy1-CFP)23Jrs/J mice

Seven days after NMDA injection, the mice were sacrificed. The eyes were enucleated and immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline for 1 h at 4 °C. Four radial relaxing incisions were made and the retina was flattened on a glass slide. The whole mount retina was sealed with fluoromount G (Southern Biotech, Birmingham, AL) and a cover glass (Matsunami Glass). Images were obtained using a confocal laser microscope