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TRPV1 receptors modulate retinal development

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ABSTRACT

We investigated the possible participation of TRPV1 channels in retinal apoptosis and overall development. Retinas from newborn, male albino rats were treated *in vitro* with capsazepine, a TRPV1 antagonist. The expression of cell cycle markers was not changed after TRPV1 blockade, whereas capsazepine reduced the number of apoptotic cells throughout the retina,increased ERK1/2 and p38 phosphorylation and slightly reduced JNK phosphorylation. The expression of BAD, Bcl-2, as well as integral and cleaved capsase-3 were similar in all experimental conditions. Newborn rats were kept for 2 months after receiving high doses of capsazepine. In their retinas, calbindin and parvalbumin protein levels were upregulated, but only the number of amacrine-like, parvalbumin-positive cells was increased. The numbers of calretinin, calbindin, ChAT, vimentin, PKC-alpha and GABA-positive cells were similar in both conditions. Protein expression of synapsin Ib was also increased in the retinas of capsazepine-treated rats. Calretinin, vimentin, GFAP, synapsin Ia, synaptophysin and light neurofilament protein levels were not changed when compared to control values.

Our results indicate that TRPV1 channels play a role in the control of the early apoptosis that occur during retinal development, which might be dependent on MAPK signaling. Moreover, it seems that TRPV1 function might be important for neuronal and synaptic maturation in the retina.

1. Introduction

The development of correct circuits in the nervous system includes not only cell proliferation and differentiation, but also the programmed death of neurons, glial cells and their precursors (Oppenheim, 1991; Linden et al., 1999; de la Rosa and de Pablo, 2000), as well as the establishment of connections and the removal of the connections that have low activity (Miller, 1996). In the retina, several cell-to-cell interactions, underlying synchrony of spontaneous neuronal firing ("retinal waves"; Wong, 1999), production, transport and release of growth factors (Frade et al., 1999; Ferguson and Slack, 2003), and the interaction of neurotransmitters with their receptors (Cameron et al., 1998; Linden et al., 2005) are all thought to control cell viability and differentiation. Among other mechanisms that are related to those major phenomena, calcium balance can modulate the activity of protein kinases and several other pathways that could culminate in cell division, apoptosis and synaptic plasticity (Berridge et al., 2000; Demaurex and Distelhorst, 2003; Greer and Greenberg, 2008; Lu et al., 2009). In fact, a great deal of efforts has been mobilized in order to understand the mechanisms involved in calcium homeostasis in the developing retina.

TRPV1 receptors are cationic channels that can contribute to changes in calcium balance (Caterina et al., 1997). In addition to the role of these receptors as transducers of noxious stimuli in nociceptors as well as in other central areas of the nervous system (Szolcsanyi, 2004; Montell and Caterina, 2007), the function of TRPV1 receptors has been correlated with the modulation of several other mechanisms that are also important during the development of the nervous tissue. For instance, TRPV1 activation induced apoptosis in several glioma cell lines (Contassot et al., 2004; Amantini et al., 2007) and in different areas of the nervous system (Chard et al., 1995; Shirakawa et al., 2008). Excessive TRPV1-dependent calcium influx may produce disruption of mitochondrial transmembrane potential (Dedov and Roufogalis, 2000), although different apoptotic-triggering mechanisms have been postulated, including caspases (Shirakawa et al., 2008), and p38 (Amantini et al., 2007) activation. TRPV1 receptors have also been

Abbreviations: ChAT, choline acetyltransferase; CPZ, capsazepine; DAB, 3,3′-diaminobenzidine; DAPI, diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; GABA, γ-aminobutyric acid; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; MAPKs, mitogen-associated protein kinases; NBL, retinal neuroblastic layer; NFL-l, light chain neurofilaments; NGF, nerve growth factor; PCNA, proliferating cell nuclear antigen; PB, phosphate buffer; PFA, paraformaldehyde; PKC, protein kinase C; RGC, retinal ganglion cell; SDS, sodium dodecyl sulfate; TRPV1, transient receptor potential channels subfamily vanilloid type 1; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling.

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related to cell migration (Waning et al., 2007) and to the regulation of growth cones (Goswami et al., 2007).

In the retina of rat pups, the activation of TRPV1 channels with capsaicin, the prototypic TRPV1 receptor agonist, caused cell death in both ganglion cell layer (GCL) and inner nuclear layer (INL), which was not observed in the adult retina (Ritter and Dinh, 1992). It was shown later that capsaicin-induced neuronal degeneration in newborn rats is mainly apoptotic (Sugimoto et al., 1998). However, it remains unclear if cell death caused by capsaicin in the retina was due to simple excitotoxicity caused by capsaicin and subsequent excessive calcium influx, or if TRPV1 channels actually play a role in the normal development of the retinal tissue. Corroborating with this idea, it was demonstrated that synaptic activation of TRPV1 receptors can modulate the release of several neurotransmitters (Sikand and Premkumar, 2007; Xing and Li, 2007; Medvedeva et al., 2008), which are all known to be important cues for the guidance of cell fate in the developing retina (Linden et al., 2005).

We have previously demonstrated that TRPV1 receptors are expressed since the early steps of retinal development, and that those receptors are mainly found in retinal ganglion cells (RGCs) and in amacrine-like cells, as well as in the retinal neuroblastic layer (NBL, Leonelli et al., 2009). The presence of endogenous ligands of the TRPV1 receptors has been described in the retina, such as anandamide (Nucci et al., 2007) and leukotriene B4 (Reinboth et al., 1995), but the participation of such compounds in the TRPV1 tone during development remains elusive. We have investigated here the possible participation of TRPV1 channels in several developmental processes in the retina, such as cell cycling, division, and apoptosis. We have also evaluated the expression of several specific cell markers in retinas from newborn and adult rats which have received capsazepine, a specific TRPV1 antagonist, during early life. The rationale for this evaluation was to obtain information on the general pattern of retinal differentiation.

2. Materials and methods

2.1. Animals

Experiments were carried out with suckling and adult rats (*Rattus norvegicus*). The animals were kept on a 12:12 h light/dark cycle with lights on at 07:00 a.m. Postnatal day 4 (P4) rats were killed with an overdose of ketamine (30 mg/100 g of body weight, i.m.; Parke-Davis, Ann Arbor, MI) and xylazine (2 mg/100 g, i.m.; West Haven, CT) between 08:00 a.m. and 10:00 a.m. Adult rats were anesthetized with a single dose of ketamine (5 mg/100 g, i.m.) and xylazine (1 mg/100 g, i.m.) before surgical procedures. All experiments were conducted in accordance with guidelines of the NIH and the Institute of Biomedical Sciences of the University of São Paulo.

2.2. Retinal explants

Animals were decapitated after anesthesia and their retinas were dissected under immersion on Krebs-Henseleit solution with glucose 10 mM. Retinal explants were placed on culture plates with the ganglion cell layer facing down, and maintained at 37 °C in a controlled humidified atmosphere of 5% CO_2–95% air atmosphere and a media containing Basal Eagle Medium (Invitrogen, Gaithersburg, MD), with 1% glutamine (Invitrogen), 5% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The explants were then maintained up to 2 days in culture.

2.3. Drugs and antibodies

Stock solutions of the TRPV1 antagonist capsazepine (Tocris; Baldwin, MO, cat #0464) were made in DMSO to a concentration of 25 μM, and were then maintained at low temperatures (−80 °C). Final dilutions were made immediately before use in 0.9% saline. Control solution (vehicle) consisted of 0.9% saline in the presence of 0.1% DMSO.

Several antibodies were used in this study. They are summarized in Table 1. An anti-PKC-alpha antibody was used in order to identify rod bipolar cells (Haverkamp et al., 2003). We used an antibody against the proliferating cell nuclear antigen (PCNA) in order to evaluate proliferating cells in the S-phase (Moldovan et al., 2007), and an antibody against Ki67 as a marker for mitotic cells (Ikeda et al., 2005). Amacrine and displaced amacrine cells were identified with an antibody against choline acetyltransferase (ChAT, Voigt, 1986). An antibody against neurofilaments of low molecular weight (68 kDa) was used in order to estimate the density of ganglion cell axons. Calretinin was used to identify amacrine cells and RGCs (Pasteels et al., 1990; Mojumder et al., 2008).

Some retinas were also counterstained for nuclear observation with DAPI (1:50,000) or propidium iodide (1 μM in PB). Some retinas were also counterstained with Neurotrace 640/660 (deep red fluorescent Nissl stain; Molecular Probes; Carlsbad, CA) for neuronal identification (Bareyre et al., 2005).

2.4. Immunoblotting analysis

Retinas (1 for each condition in adults, $n = 4$; pools of 3 for each condition in newborn rats, $n = 4$) were rapidly isolated and transferred to a tube containing 80 μL extraction buffer (100 mM Trizma, 1% SDS, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA and 10 mM sodium orthovanadate). They were homogenized using an ultrasonic processor (Sonics & Materials, Newtown, PA, USA), and boiled for 10 min. The extracts were then centrifuged at 12,000 rpm at 4 °C for 20 min to remove insoluble material. Protein determination in the supernatants was performed with the Bradford dye method using the Bio-Rad reagent 20.

The whole extracts were treated with Laemmli sample buffer containing dithiothreitol and boiled for 5 min before loading onto 8% or 10% SDS–PAGE in a Bio-Rad miniature slab gel apparatus. Similar-sized aliquots (between 60 and 80 μg) were subjected to SDS–PAGE. Specifically for GFAP blotting analysis, we performed several tests in order to determine the protein load to be applied in the gel (O'Callaghan et al., 1999). We found that 30 μg per lane was sufficient to detect differences of GFAP protein load without quenching the signal (data not shown).

Proteins were electrotransfered from the gel to nitrocellulose membranes for 1.5 h at 120 V (DC) in a Bio-Rad miniature transfer apparatus. Non-specific protein binding to the nitrocellulose membrane was reduced by preincubation for 2 h at 22 °C in blocking buffer (5% non-fat dry milk, 10 mM Trizma, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose membranes were incubated overnight at 4 °C with antibodies diluted in blocking buffer with 3% non-fat dry milk, and then washed for 30 min. The membranes were subsequently incubated with a peroxidase-conjugated secondary antibody for 1 h, and processed for enhanced chemiluminescence to visualize the immunoreactive bands.

2.5. Immunohistochemistry

The animals were perfused through the left cardiac ventricle with phosphate buffered saline at 37 °C and 2% paraformaldehyde in cold 0.1 M phosphate buffer (PB), pH 7.4. Eyes were dissected out and postfixed for 2 h. After this period, they were kept in a cryoprotective 30% buffered sucrose solution in PB for at least 4 h until sectioning. Transverse sectioning of the retinas (12 μm) was performed by embedding with OCT compound and cutting them on a cryostat.

All retinal sections were blocked for 2 h in a solution containing 5% normal goat serum, 1% bovine serum albumin, and 0.3% Triton X-100 in PB. Primary antibodies were incubated overnight at 22 °C, in the concentrations depicted in Table 1, diluted in PB and 0.3% Triton X-100.

After several washes in PB, retinal sections were incubated with tetramethylrhodamine isothiocyanate or fluorescein isothiocyanate-conjugated donkey antisera against goat, rabbit, guinea pig or mouse IgG (1:200; Jackson Labs, West Grove, PA) diluted in PB containing 0.3 Triton X-100 for 2 h at room temperature. Negative controls consisted of the omission of primary antibodies, and no staining was observed in these cases. After washing, the tissue was mounted using VectaShield (Vector Laboratories, Burlingame, CA).

Alternatively, slides were prepared for DAB reaction, as described elsewhere (Leonelli et al., 2005). Briefly, after primary antibody incubation, slides were washed in PB, and were incubated for 2 h with biotinylated antibodies against goat, rabbit, guinea pig or mouse IgG generated in donkey (Jackson Labs., West Grove, PA, USA) diluted 1:200 in PB containing 0.3% Triton X-100. The sections were washed again in PB and finally incubated for 1 h with the avidin-biotin-peroxidase complex (ABC Elite; Vector Labs., Burlingame, CA, USA). Sections were reacted with 0.05% 3,3′-diaminobenzidine and a 0.01% solution of hydrogen peroxide in PB, followed by intensification with 0.05% osmium tetroxide in water. Sections were dehydrated, cleared, and coverslipped with Permount (Fisher, Pittsburgh, PA, USA).

2.6. TUNEL assay

We used TUNEL assay (terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling; Roche Molecular Biochemicals, Mannheim, Germany) in order to detect apoptosis in the retina of preweaning rats ($n = 5$ for each condition). Animals were perfused as described previously. Retinal sections were obtained on a cryostat and the assay was performed according to the manufacturer. Slides were then coverslipped with Vectashield and observed under confocal microscopy.

2.7. Specific cell-subtype quantification and morphometric analysis

Specific cell-subtype quantification was performed as described elsewhere (Kitaoka et al., 2006). Briefly, retinas were prepared for immunohistochemistry and reacted for fluorescence or DAB methods. Regions away 0.5–2 mm from the optic nerve were analyzed (five images for each eye; $n = 4$ animals for each condition).