TRPV1 receptors modulate retinal development


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Received 29 November 2010
Received in revised form 19 January 2011
Accepted 8 March 2011

**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 caspase-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, 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 occurs 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.

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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 (Frate 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 glial cell lines (Contassot et al., 2004; Amanzini 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 (Amanzini et al., 2007) activation.

TRPV1 receptors have also been
related to cell death (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 capsaipenne, 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 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% CO2–95% air atmosphere and a media containing Basal Eagle Medium (Invitrogen, Gaithersburg, MD), with 1% glucose (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-PCK-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 Ksh7 as a marker for mitotic cells (Ibera 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 (Pastels 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 0.1 M 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 GPAP blotting analysis, we performed several treatments 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 GPAP protein load without quenching the signal (data not shown).

Proteins were electrophoresis transferred 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 0.025% 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 antibodies against goat, rabbit, guinea pig or mouse IgG (1:200; Jackson Labs, West Grove, PA, USA) 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 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).
Data from five sections were averaged and considered as a single eye result. Retinal length analyzed in each experiment is described in the figure and table legends. Mean values were compared using paired Student’s t-test.

Morphometric analysis of the retinal tissue was performed in transverse sections of central retinal areas, away 0.5–1 mm from the optic nerve. Sections were stained with Neurotrace. Maximal vertical length (ranging from photoreceptor outer processes and the vitreal margin) and the length of specific layers were measured, and the acquired data were plotted (five measurements for each eye; n = 4 animals for each condition). Mean values were compared using paired Student’s t-test.

2.8. Image acquisition

Sections were analyzed on a Zeiss LSM 510 confocal microscope, and in a Nikon E1000 upright microscope coupled to a Nikon DCM1200 digital camera. Figures were mounted with Adobe Photoshop CS. Manipulation of the images was restricted to threshold and brightness adjustments of the whole image.

3. Results

3.1. TRPV1 blockade during retinal histogenesis does not alter cell proliferation

We have employed the analysis of PCNA and Ki67 in order to evaluate the possible effects of TRPV1 blockade upon cell proliferation (Fig. 1). The number of Ki67-positive cells was similar in control and capsazepine-treated retinal explants (6.9 ± 0.8 cells in control retinas; 7.4 ± 1.0 cells after 10 μM capsazepine; p > 0.05). Mean PCNA protein content was statistically similar after capsazepine 10 μM incubation (89.6 ± 10.8% of control values; p > 0.05).

3.2. TRPV1 blockade during retinal histogenesis reduces apoptosis

We have used TUNEL in order to evaluate apoptosis in the retinal layers. Incubation of retinal explants of P4 animals with capsazepine (1 and 10 μM) during 6 h did not change the number of apoptotic cells in the retina (data in Fig. 2A). However, retinal incubation for 12 h (Fig. 2B) in the presence of capsazepine reduced the number of apoptotic cells in the NBL and in the INL (6.0 ± 1.1 cells in control retinas, 2.4 ± 0.6 cells after capsazepine 1 μM and 3.0 ± 0.4 cells after capsazepine 10 μM; p < 0.05 for both conditions). Retinal incubation with 10 μM capsazepine also reduced the number of apoptotic neurons in the GCL (3.4 ± 0.7 cells in control retinas and 1.7 ± 0.5 cells after capsazepine 10 μM; p < 0.05).

![Fig. 1. Evaluation of cell cycle markers in retinal explants of P4 rats. Retinas were incubated with vehicle or with 10 μM capsazepine. The retinas were cultured during 12 h and they were then prepared for immunoblotting or immunohistochemistry. (A) Ki67-positive cells were found in the retinal neuroblastic layer. Cells were counted in fields of 210 μm of linear central retina (five images for each eye; n = 4 animals for each condition). (B) PCNA protein expression. Optical density was analyzed with ImageJ software. Averages were compared with Student’s t-test for pairwise comparisons (n = 6). No significant differences were found. Bars represent standard error of mean.](image-url)
3.3. Caspase-3 and related proteins are not involved in the effects of TRPV1 blockade

We have also evaluated the expression of pro- and anti-apoptotic proteins in retinal explants after capsazepine treatment (Fig. 3). Retinal explants from P4 animals were cultured in the presence of capsazepine (1 and 10 μM) during 12 h and were processed for immunoblotting. The expression of the pro-apoptotic protein BAD, the anti-apoptotic protein Bcl-2, as well as integral (32 kDa) and cleaved caspase-3 (19 and 19 kDa) were similar in all experimental conditions when compared to controls (p > 0.05).

3.4. Phosphorylation of MAPK proteins is acutely affected by capsazepine treatment

We then tested the activation of some mitogen-associated protein kinases (MAPK) involved in retinal explants acutely treated with the TRPV1 antagonist capsazepine (1 and 10 μM) for 12 h (Fig. 4). ERK1/2 phosphorylation was markedly increased in capsazepine-treated retinas (293.9 ± 2.3% of control values with 1 μM capsazepine, and 531.6 ± 29.3% of control values 10 μM capsazepine; p < 0.001). p38 phosphorylation was augmented after capsazepine treatment (118.3 ± 3.1% of control values with 1 μM capsazepine, and 123.0 ± 1.2% of control values with 10 μM capsazepine; p < 0.05). On the other hand, JNK phosphorylation was...
Fig. 5. TRPV1 blockade modulates the phosphorylation of some important proteins involved in apoptosis and cell survival. Retinal explants of P4 rats were incubated with vehicle or with capsazone 1 and 10 µM for 12 h and were then prepared for Western blots. Immunoblottings of phospho-p38, phospho-ERK1/2, and phospho-JNK. The expression of β-actin was used as internal control. The approximate molecular weights of bands are indicated. One-way ANOVA with Tukey's post-hoc test was used (n = 4 for each condition). Means were considered significantly different when p-value was lower than 0.05. * indicates p < 0.05 in comparison with control values.

3.5. TRPV1 blockade in newborn rats does not alter the structure of the retina of adult animals

The effects of long-term treatment with capsazone in newborn rats (single daily dose: 40 mg/kg; IP) for 3 days on the retinal structure of adult animals. This morphometric analysis is shown in Fig. 5. Vertical length of total retina and of distinct layers of central, transverse retinal sections was not significantly affected by the treatment.

3.6. Capsazepine treatment in newborn rats increases the number of amacrine-like parvalbumin-positive cells in the adult retina

In order to determine if TRPV1 blockade for 3 days (single daily dose: 40 mg/kg; IP) in pre-weaning rats caused any significant change in the numbers of specific cell types of the adult retina, we have analyzed the expression of several cell markers by immunoperoxidase and by immunofluorescence in central retinal sections. These data are summarized in Table 2. Parvalbumin cell counts indicated that parvalbumin amacrine-like cells (Fig. 6A and B) were increased in capsazone-treated rats (208.4 ± 5.5 cells in control retinas and 228.0 ± 6.9 in retinas from capsazone-treated rats; 1 mm of linear retina; p < 0.05). We did not find any difference between control and capsazone-treated cell counts of different cell types revealed by immunohistochemistry for calretinin (Fig. 6C and D), calbindin (Fig. 6E and F), ChAT (Fig. 6G and H), vimentin (Fig. 6I and J), PKC-alpha (Fig. 7A and B), and GABA (Fig. 7C and D). GFAP immunohistochemistry was also performed in order to analyze if capsazone treatment caused any detectable Müller cell reaction. As it can be seen, capsazone signal for GFAP was similar in comparison with control retinas (Fig. 6K and L).

3.7. TRPV1 blockade during retinal histogenesis increases protein expression of some cell-specific and of synaptic markers in the adult retina

Calbindin and parvalbumin protein levels were higher in retinas from rats treated with capsazone (single daily dose: 40 mg/kg; IP) for 3 days during early life (Fig. 8; 220.10 ± 42.3% of control values for calbindin and 261.0 ± 42.3% of control values for parvalbumin; p < 0.001). Protein expression of synapsinIIb was also increased in the retinas of capsazone-treated rats (169.3 ± 18.8% of control values, p < 0.05). Calretinin, vimentin, GFAP, synapsin Ia, synaptophasin, and of light chain neurofilaments (NFL-L) protein levels were not changed when compared to control values (p > 0.05).

4. Discussion

4.1. TRPV1 controls retinal apoptosis during development: possible roles in undifferentiated and differentiated cells

We found that TRPV1 blockade during the early steps of retinal development reduced apoptosis. The very beginning of normal
development of the retinal tissue is characterized by intense proliferation and apoptosis (Oppenheim, 1991; de la Rosa and de Pablo, 2000). Extracellular molecules can signal for the start of the apoptotic process. Neurotransmitters can also mobilize calcium and modulate intracellular pathways which are calcium-dependent (Linden et al., 2005). Effects of calcium transients in cells depend on their functional state, as well as on the amount and timing of the calcium changes (Berridge et al., 2000; Hajnoczky et al., 2000).

TRPV1 receptors can mobilize calcium (Caterina et al., 1997) and their activity is correlated with apoptosis in both mitotic cells or in differentiated cells. TRPV1 activation caused apoptosis in cells derived from neuroblastoma (Maccarrone et al., 2000; Lam et al., 2007), lymphoma (Maccarrone et al., 2000), glioma (Contassot et al., 2004; Amantini et al., 2007), as well as in several other neuronal tissues such as dorsal root ganglion neurons (Chard et al., 1995), brain cortex (Shirakawa et al., 2008), and in the retina (Ritter and Dinh, 1990; Leonelli et al., 2010).

TRPV1 antagonism reduced apoptosis in the same retinal layers where the expression of TRPV1 has been described before (Leonelli et al., 2009). Moreover, capsaicin caused marked cell death in the INL and in the GCL of preweaning rats (Ritter and Dinh, 1990). One possible explanation is that TRPV1 cause large calcium influx in the cell, which is largely buffered by mitochondria, and the resulting mitochondrial calcium influx caused disruption of the mitochondrial transmembrane potential (Dedov and Roufogalis, 2000). These

![Fig. 6. Transverse sections of adult rat retinas labeled with peroxidase-DAB methods for parvalbumin (A and B), calretinin (C and D), calbindin (E and F), ChAT (G and H), vimentin (I and J), and GFAP (K and L). P4 rats received single, daily doses of vehicle (saline with DMSO 0.1%) or capsazepine (40 mg/kg; IP) for 3 days, and were kept for 2 months. Labels indicate the approximate location of the outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). Scale bars = 50 μm.](image)

![Fig. 7. Transverse sections of adult rat retinas prepared for immunofluorescence, labeled for PKC-alpha (A and B; green) or GABA (C and D; green), and fluorescent-Nissl (red). P4 rats received single, daily doses of vehicle (A and C; saline with DMSO 0.1%) or capsazepine (B and D; 40 mg/kg; IP) for 3 days, and were kept for 2 months. Labels indicate the approximate location of the outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)](image)

![Fig. 8. Comparative analyses of protein expression of several cell markers in the adult rat retina. P4 rats received single, daily doses of vehicle (saline with DMSO 0.1%) or capsazepine (40 mg/kg; IP) for 3 days, and were kept for 2 months. Estimated molecular weight for each protein is indicated. Measurements of optical densities were performed using ImageJ software. Student’s t-test for pairwise comparisons test was used (n = 4 for each condition). Means were considered significantly different when p-value was lower than 0.05. β-actin was used as internal control.](image)
factors are known to release important mitochondrial factors to the cytosol and to induce caspase-mediated apoptosis (Demaurex and Distelhorst, 2003). However, our data imply that caspases are not involved in TRPV1 control of retinal apoptosis. This was also observed in SH-SYSY human neuroblastoma cells, where apoptosis caused by TRPV1 activation was not correlated with caspase activity (Davies et al., 2010).

Several neurotransmitters can regulate apoptosis in the developing retina (Linden et al., 2005). Glutamate has main excitotoxic effects, and the activation of glutamate receptor is correlated with cell death in the developing INL (Rocha et al., 1999). It was recently shown that TRPV1 activation and subsequent calcium influx can lead to release of neurotransmitters (Lam et al., 2007), including glutamate (Marinelli et al., 2002; Palazzo et al., 2002; Musella et al., 2009), which could be a possible explanation for capsazepine reduction of apoptosis. Our data imply, therefore, that calcium signals mediated by TRPV1 receptors might be important initial triggers of the apoptosis that occurs in the developing retina.

On the other hand, the expression of p75, the pro-apoptotic receptor for nerve growth factor (NGF), was mainly found in the GCL of the developing retina (Frade and Barde, 1999). p75 activation by NGF was reported to cause apoptosis in the developing GCL (Frade et al., 1996; Frade and Barde, 1999; Harada et al., 2006), and the endogenous sources of NGF that actually induce cell death are microglial cells (Frade and Barde, 1998). Several reports also indicate that NGF can sensitize TRPV1 receptors (van den Worm et al., 2004). Thus, our results suggest the inclusion of TRPV1 channels in the discussion on the control of programmed cell death in the retina, and TRPV1 may represent one of the possible links between the signals mediated by neurotrophins and by conventional neurotransmitters. In agreement with this idea, it has been shown that isolated retinal ganglion cells from rat pupies are not protected against apoptosis with the ultrapotent TRPV1 antagonist iodo-resiniferatoxin (Sappington et al., 2009). Our results also indicate that TRPV1 antagonism reduced cell death more prominently after 12 h of incubation, which could rise the possibility that signaling cascades downstream to TRPV1 are complex and possibly interact with several other, TRPV1-independent, cascades. This could slow the apoptotic process in relation to the time points tested. Taken together, the above results reinforce the hypothesis that cell death in the developing retina is modulated by complex cell to cell interactions.

4.2. TRPV1 function modulates MAPK signaling in the developing retina

Our results indicate that TRPV1 antagonism in preweaning rats modulates the phosphorylation of several MAPKs in the retina. MAPK signaling is regulated by a large number of extracellular and intracellular stimuli, which can result in the modulation of gene transcription, and translational and post-translational changes in proteins, thus controlling a plethora of cell events associated with cell division, differentiation, growing, and apoptosis (Junttila et al., 2008). JNKs are involved in regeneration and in the development of the nervous system (Haeusgen et al., 2009), where they are essential signaling molecules in processes such as migration of developing neurons (Kawauchi et al., 2003; Gdalyahu et al., 2004) and cell death (Kuan et al., 1999). During development, p38 activation has been correlated with neurite outgrowth, cell differentiation (Morooka and Nishida, 1998), and programmed cell death (Campos et al., 2006; Oliveira et al., 2008), whereas ERK1/2 phosphorylation has been correlated with proliferation (Melocha and Pouysségur, 2007) and cell survival (Junttila et al., 2008).

In this study, TRPV1 antagonism reduced JNK phosphorylation, suggesting that vanilloid tone might regulate JNK function during retinal development. This is also supported by the fact that TRPV1 activation with capsacin and resiniferatoxin increased the phosphorylation of JNK in Jurkat cells (Macho et al., 1998). On the other hand, TRPV1 activation caused apoptosis in glioma cells (Contassot et al., 2004; Amantini et al., 2007), which seems to be dependent on p38 activation (Amantini et al., 2007). Our results indicate that phosphorylation of p38 MAPK and of ERK 1/2 was increased in capsazepine-treated retinas. It was recently shown that ERK1/2 and p38 phosphorylation are dynamically modulated during development of the retina (Oliveira et al., 2008). Interestingly, p38 phosphorylation peaked at P4, an age in which cell death in the developing retina is prominent (Beazley et al., 1987); p38 activation peaks again in the adult retina, and so does ERK1/2, which suggests that the pro-apoptotic effects of p38 are marked in the early apoptotic period, and that ERK 1/2 may protect the cells in the adult retina. This is also supported by the fact that p38 and JNK-mediated cell death was blocked by the activation of ERK1/2 (Xia et al., 1995). Thus, our results are suggestive that ERK1/2 phosphorylation counterbalanced p38 activation, therefore reducing retinal apoptosis.

It is relevant that several clues point to mutual interactions between p38 and ERK1/2 MAPKs. In PC12 cells that are undergoing programmed cell death caused by NGF withdrawal, ERK1/2 function is reduced, while p38 activity is augmented (Xia et al., 1995). In Hela cells, inhibition of ERK 1/2 caused p38 activation and apoptotic-like cell death (Berra et al., 1998). Despite the fact that the gap between calcium signaling and MAPK signaling in the retina still remains to be fully elucidated, our results clearly show that MAPK signaling during retinal development is partially modulated by the TRPV1 tone, thus suggesting that ERK1/2 and p38 might be suppressed by calcium signals generated by TRPV1.

4.3. Consequences of TRPV1 blockage on cell fate

Our results indicate that the expression of calbindin and parvalbumin, as well as of the synaptic marker synaptophysin Ib, was increased in the retinas of adult animals which received capsazepine during development. These results are suggestive that the reduced apoptosis during development have a repercussion in the adult retinal organization. Synaptophysins are presynaptic proteins (Wiedenmann and Franke, 1985) that can be found in the outer and inner plexiform layers of the retina (Brandstatter et al., 1996). Calbindin is a well-known marker of horizontal cells, but it can also be expressed by amacrine and a few ganglion cells in the rat retina (Hamano et al., 1990; Pasteels et al., 1990). Our observations indicate that calbindin protein levels rise whereas the number of calbindin-positive cells remains unchanged. The exact mechanisms which control calbindin expression are not well understood. We could speculate that the reduced apoptosis during retinal development caused by capsazepine might increase the number of inputs to such cells, which may be important to the control calbindin expression (Diaz de Barboza et al., 2003).

Parvalbumin is expressed by the vast majority of amacrine cells and in some RGCs (Hamano et al., 1990). Our results depict a scenario in which TRPV1 blockade during retinal histogenesis reduced apoptosis and increased the number of parvalbumin amacrine-like cells and the content of calbindin and synapsin Ib. Nevertheless, we were not able to find any differences in the adult retinal morphology. As the increase of the number of parvalbumin-positive cells can be considered small (∼9.6%), this might not be enough to increase the retinal thickness. Moreover, our results clearly show that parvalbumin and calbindin upregulation might be due to the modulation of the expression of those proteins, and not only to an increase in the number of cells expressing them.

Amacrine cell survival is dependent on ERK 1/2 signaling (Kunzevitzky et al., 2010), but the mechanisms involved remain unclear. Several interesting findings point to ERK 1/2 modulation.
by synaptic depolarization (Fioe et al., 1993). In this context, ERK 1/2 seems to modulate synaptic plasticity (Thomas and Huganir, 2004), increasing the number of dendritic arborizations (Wu et al., 2001) and the local traffic of several important synaptic proteins (Engert and Bonhoeffer, 1999; Toni et al., 1999). However, our results indicate that TRPV1 blockade actually increased ERK 1/2 phosphorylation. TRPV1 receptors are poorly expressed in our results indicate that TRPV1 blockade actually increased ERK 1/2. TRPV1 receptors are expressed in parvalbumin-containing cells, whereas they are not expressed at all in calbindin-positive horizontal and amacrine cells (unpublished data). These cells are mainly hyperpolarizing interneurons (Versaux-Botteri et al., 1989; Cueva et al., 2002), which receive excitatory signals (Hanksin and Ikeda, 1991; Chavez and Diamond, 2008). Based on the fact that TRPV1 receptors are expressed in presynaptic terminals in the retina (Leonelli et al., 2009), and that their function has been implicated in the release of excitatory neurotransmitters (Sikand and Premkumar, 2007; Xing and Li, 2007; Medvedeva et al., 2008), we hypothesize that TRPV1 blockade during development might reduce excitatory signals to those inhibiting interneurons, thus reducing their activity and the release of inhibitory neurotransmitters. This could allow depolarizing inputs which could also act as trophic and survival factors in the retina.

4.4. Conclusions

In summary, this paper provides evidence that TRPV1 channels play a role in the control of the early apoptosis that occur during retinal development and that MAPK signaling might be involved in this process. Moreover, TRPV1-mediated signaling may be important for neuronal differentiation and synaptic maturation in the retina.

Acknowledgments

The authors would like to thank Adilson S. Alves for technical assistance. Thanks are also due to Maria Fernanda R. Graciano (University of São Paulo) for critically reading the manuscript. Grant sponsors: FAPESP and CNPq (Brazil). ML and DOM are recipients of fellowships from FAPESP.

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