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SHORT COMMUNICATION
Compartmentalization of calcium entry pathways in mouse rods

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Abstract

Photoreceptor metabolism, gene expression and synaptic transmission take place in a highly polarized structure consisting of the ellipsoid, subellipsoid, cell body and synaptic terminal regions. Although calcium, a key second messenger, regulates cellular functions throughout the photoreceptor, the molecular mechanisms underlying local region-specific action of Ca^{2+} in photoreceptors are poorly understood. I have investigated the compartmentalization of voltage-dependent and independent Ca^{2+} channels in mouse photoreceptors. Transient receptor potential channels isoform 6 (TRPC6), a putative store-operated Ca^{2+} channel, was selectively localized to the cell body of rods. By contrast, voltage-operated Ca^{2+} channels were expressed in the synaptic terminal and in the ellipsoid/subellipsoid regions. Likewise, Ca^{2+} store transporters and channels were strongly associated with the subellipsoid region. A moderate TRPC6 signal was observed in cell bodies of bipolar, amacrine and ganglion cells, but was absent from both plexiform layers. These results suggest that Ca^{2+} entry mechanisms in mammalian photoreceptors and bipolar cells are highly compartmentalized, consistent with local, region-specific activation of Ca^{2+}-dependent processes.

Introduction

Photoreceptors are highly polarized cells, divided into two main regions: an outer segment (OS), hosting the phototransduction apparatus, and a non-OS segment responsible for energy metabolism, gene expression and synaptic transmission. Ca^{2+} influx and extrusion into the non-OS region occur mainly via L-type voltage-operated Ca^{2+} channels (VOCCs) and plasma membrane Ca^{2+} ATPases, respectively (Corey *et al.*, 1984; Krizaj & Copenhagen, 1998), with additional contributions from cGMP-gated channels and intracellular ryanodine receptor channels (Rieke & Schwartz, 1994; Krizaj *et al.*, 2003).

The non-OS region is itself further partitioned into four morphologically and functionally distinct subcompartments: the ellipsoid, the subellipsoid, the cell body and the synaptic terminal, which are packed with mitochondria, endoplasmic reticulum, cell nucleus and synaptic vesicles, respectively. In contrast to dramatic differences between Ca^{2+} entry and extrusion pathways in the OS and non-OS (Fain *et al.*, 2001; Krizaj & Copenhagen, 2002), there is little information about potential compartmentalization of Ca^{2+} signaling within different non-OS regions. In this paper I present evidence that VOCCs, Ca^{2+} stores and potential store-operated Ca^{2+} channels are distributed to different regions in a highly compartmentalized fashion.

The *transient receptor potential* (*trp*) genes encode a set of Ca^{2+}-permeable plasma membrane channels that play key roles in sensory transduction and transmission – including temperature detection, pheromone detection, touch, taste and pain, and invertebrate phototransduction (Minke & Selinger, 1996; Montell *et al.*, 2002; Lucas *et al.*, 2003). TRPs were originally identified in invertebrate photo-receptors as photochannels that open during the activation of the rhodopsin–G protein–phospholipase C–inositol triphosphate receptor (InsP$_3$ receptor) cascade (Minke & Selinger, 1996). In mammals, cloning by homology with *trp* isolated seven known transient receptor potential channels (TRPC) isoforms, which show a wide variety of activation patterns by G protein-coupled receptors, depletion of internal Ca^{2+} stores, cytoskeleton and mechanical activation via InsP$_3$ receptors and ryanodine receptors (Kiselyov *et al.*, 2000; Ma *et al.*, 2000; Lucas *et al.*, 2003; Spassova *et al.*, 2004). Although most TRPC isoforms are highly expressed in the mammalian brain (Riccio *et al.*, 2002), their localization in the mammalian retina is unknown.

This paper reports that TRPC6, a ubiquitous TRPC that can be activated by both store depletion and heteromeric G protein-coupled receptors (Hofmann *et al.*, 1999; Trebak *et al.*, 2003), is selectively localized to cell bodies of mouse rods. In contrast to the localization of TRPC6, immunostaining using pan α_1 antibodies indicates that voltage-gated Ca^{2+} channels and Ca^{2+} store transporters are concentrated in synaptic terminals and inner segment subellipsoid/ellipsoid regions but are only weakly expressed in perikarya. These results suggest that Ca^{2+} release and sequestration from the endoplasmic reticulum can be highly localized, depending on distribution of Ca^{2+} release channels and sequestration pumps.

Methods

Immunocytochemistry and confocal microscopy

The experiments follow the procedures outlined by the US National Institutes of Health and were approved by the Committee on Animal Research at the University of California San Francisco. The experimental protocols are detailed in Krizaj *et al.* (2004). Briefly, the

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animals were anesthetized and killed via CO_2 asphyxiation followed by cervical dislocation. Retinal eyecups were fixed by immersion in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) for 60–90 min, rinsed and cryoprotected in 30% sucrose overnight. Retinal sections were incubated with primary antibodies overnight at 4 °C. For double labeling experiments, a mixture of primary antibodies was applied followed by a mixture of secondary antibodies including Alexa^{TM} 488- and Alexa^{TM} 594-nm goat anti-rabbit and anti-mouse conjugates (Invitrogen, Carlsbad, CA, USA). After incubation, sections were washed in phosphate buffer and mounted with Vectashield (Vector, Burlingame, CA, USA). The rabbit polyclonal antibody against TRPC6 and synthetic neutralizing peptides were obtained from Chemicon (Temecula, CA, USA) or from Alomone labs (Jerusalem, Israel). Specificity of TRPC antibodies was tested with neutralizing peptides, the sequences of which were derived from the cytoplasmic, NH_3-terminal domain of the rat TRPC6 proteins. Negative controls were constructed by omitting the primary antibody. The rabbit polyclonal RyR2 (ryanodine receptor isoform 2) antibody (1 : 100) was a gift from Dr Tony Lai (University of Wales College of Medicine, Cardiff, UK), mouse monoclonal antibodies SERCA2 (sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase; 1 : 100), calbindin (1 : 500), the pan α_1 antibody (1 : 100) and the guinea-pig VGLUT1 (vesicular glutamate transporter isoform 1) antibody (1 : 1000) were purchased from Chemicon. Rabbit polyclonal SERCA2 antibody (1 : 100–1 : 300) was obtained from Bethyl Laboratories (Montgomery, TX, USA). Immunofluorescent and bright-field Nomarski fields of view were obtained using a confocal microscope (Zeiss LSM 5; Thornwood, NY, USA) at 5% power for the 488-nm argon line and 100% power for the helium–neon line. The scans were collected sequentially to minimize spectral bleedthrough.

Western blot analysis

Cytoplasmic and nuclear proteins were prepared with NE-PER extraction reagents (Pierce Biotechnology, Rockford, IL, USA) as described in Krizaj et al. (2004). Fifteen to 20 μg of protein extracts was resolved on NOVEX-NuPAGE gels and transferred to PVDF membranes (Invitrogen). After 15–30 min of incubation with the StartingBlock^{TM} blocking buffer (Pierce Biotechnology), the membranes were incubated overnight in the primary antibody at 4 °C. Following washing, the blots were incubated with appropriate antiperoxidase-conjugated secondary antibodies at room temperature for 2–3 h.

PCR analysis

RT-PCR was performed using the Qiagen OneStep RT-PCR Kit (Qiagen, Valencia, CA, USA) using 1 μg of RNA under the parameters: 30 min at 50 °C for reverse transcriptase and generation of cDNA template followed by 15 min at 95 °C to activate the HotStarTaq DNA Polymerase (Qiagen). This was followed by 40 cycles of: 30 s denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C. A final extension step of 72 °C was included to ensure double stranded DNA with 3′A overhangs. PCR products were analysed using a 1.5% ethidium bromide agarose gel. The oligonucleotide primers used for TRPC6 analysis were: sense, AAAGATATCTTCAAATTCATGGTC (bases 2181–2204); antisense, CACGTCCGCATCCTCAATTTC (bases 2484–2308) (Garcia & Schilling, 1997).

Results and discussion

Figure 1A illustrates RT-PCR results using specific primers for TRPC6 on the whole neural retina. The product for TRPC6 was amplified at the correct 327 base pairs. To test for the specificity of the TRPC6 antibody, homogenates from mouse retinas were analysed by Western blotting with a polyclonal antibody raised against amino acids 24–38 from mouse TRPC6. In the control, the antibody recognized a single band close to the predicted molecular weight of ~97 kDa (Fig. 1B). Two bands may correspond to the two splice variants that have been described, one of which may be non-functional as a protein (Zhang & Saffen, 2001).
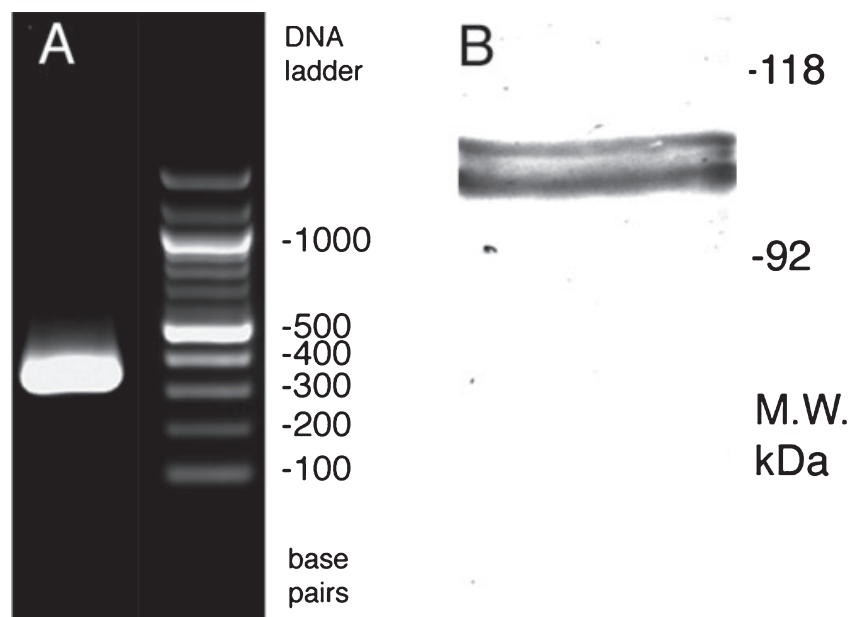

FIG. 1. (A) RT-PCR analysis of TRPC6 in the mouse retina on a 1.5% ethidium bromide agarose gel. Left lane: TRPC6, 327 bases. Right lane: molecular weight marker. (B) Western blot of TRPC6 in the mouse retina. Total protein lysate (25 μg protein per line) was blotted with a TRPC6 antibody, which recognized a protein of ~97 kDa. The positions of molecular weight (M.W) markers are depicted on the right.
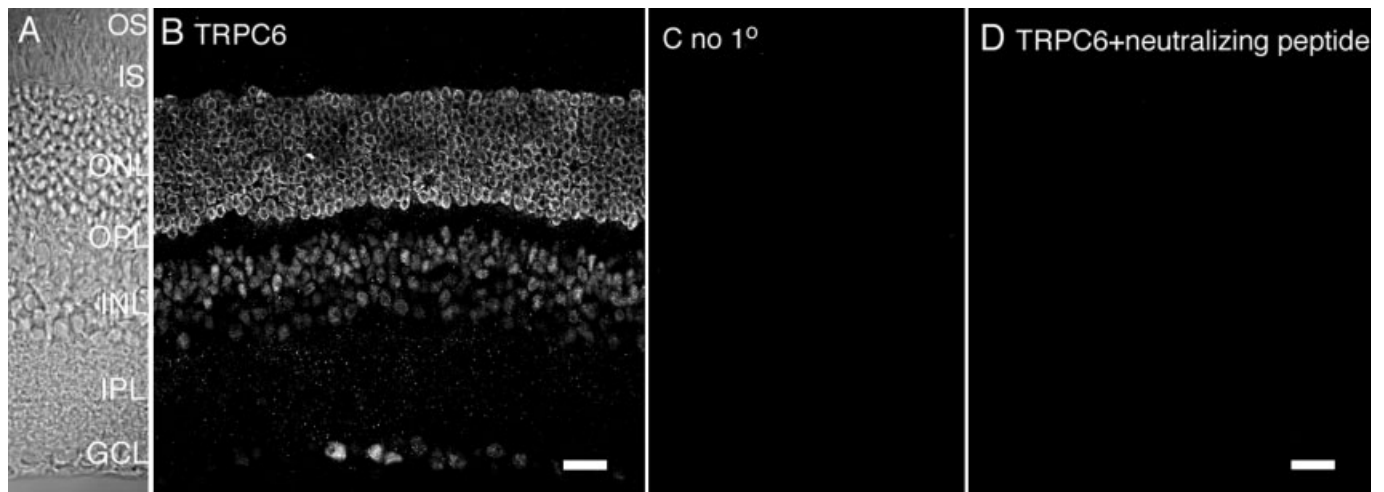
FIG. 2. TRPC6 is expressed in cell bodies of photoreceptors and bipolar cells. (A) Nomarski image of a vertical frozen section through mouse retina. (B) Confocal fluorescence photomicrograph of mouse retinal section labeled for TRPC6. The antibody strongly labels cell bodies of rods in the ONL. Moderate TRPC6 signal is also seen in bipolar cell bodies and in a subset of perikarya in the proximal INL and in GCL. (C) No TRPC6 signal is observed in the absence of the primary antibody. (D) Specific neutralizing peptide blocks TRPC6 immunostaining. Scale bars, 20 μm.

The antibody raised against TRPC6 labeled the nuclear layers of the mouse retina, with the most prominent signal in the outer nuclear layer (ONL). As illustrated in Fig. 2, the TRPC6 signal in the outer retina was confined to cell bodies of photoreceptors. TRPC6 was absent from both ellipsoid region and synaptic terminals. A moderate TRPC signal was observed in cell bodies of bipolar cells and in a subset of neurons in the amacrine and ganglion cell layers (Fig. 2B), but was absent from the inner plexiform layer (IPL) neuropil. As opposed to the sharply delimited localization to rod perikarya, TRPC6 staining in the inner nuclear layer (INL) and ganglion cell layer (GCL) appeared to be diffuse, potentially due to cytoplasmic localization (Fig. 2B; e.g. Inoue *et al*., 2001).

No signal was observed when control retinal sections were incubated with the secondary antibody alone (Fig. 2C), suggesting that the primary antibody recognized a specific retinal epitope. To determine further the specificity of the antibody, retinal sections were co-incubated with a specific neutralizing peptide, the sequence of which was derived from the cytoplasmic, NH_3-terminal domain of the rat TRPC6. Co-incubation with the neutralizing peptide prevented binding of the primary antibody to the tissue (Fig. 2D). Taken together, these results suggest that the TRPC6 antibody recognizes a specific antigen in the mouse retina with molecular weight close to that of recombinant TRPC6.

To obtain a more comprehensive view of Ca^{2+} influx pathways in mouse photoreceptors, retinal sections were double-immunostained for TRPC6 and a monoclonal pan α_1 antibody, which recognizes all α_1 subunits of L-type Ca^{2+} channels. As seen in Fig. 3B, puncta immunoreactive for the pan α_1 subunit were found at the positions occupied by synaptic terminals in the outer plexiform layer (OPL) (arrowheads). Similar punctate staining in the rodent OPL was shown previously to represent clustering of voltage-activated Ca^{2+} channels containing the α_{1F} subunit near the active zones (Morgans, 2001). Unlike α_{1F}, which appears to be expressed in synaptic terminals of rodent rods (Morgans, 2001), the pan α_1 subunit antibody also immunolabeled the subellipsoid/ellipsoid space localized between the outer segment and the cell body (arrows in Fig. 3B). Little pan α_1 subunit expression, and consequently little co-localization with TRPC6 (Fig. 2D), was seen in the cell body at moderate confocal gains. These results suggest that Ca^{2+} influx mechanisms in mamma-

lian photoreceptors are compartmentalized: whereas Ca^{2+} entry into the synaptic terminal and subellipsoid/ellipsoids occurs via different VOCC subtypes, Ca^{2+} entry into the cell body may occur via store-operated and/or receptor-operated cation channels.

In a number of tissues Ca^{2+} entry through TRPC6 is activated by depletion of intracellular Ca^{2+} stores (Trebak *et al*., 2003). Immuno-labeling for SERCA2 shows that this ubiquitous Ca^{2+} sequestration transporter (Wuytack *et al*., 2002) is expressed in the subellipsoid space and in photoreceptor terminals, as seen by exclusion of the SERCA2-immunopositive signal in the OPL from the horizontal cell marker calbindin (Fig. 3I). Similarly, incubation with antibodies raised against the ryanodine receptor isoform 2 (Fig. 3K and L) suggests these Ca^{2+} store channels are localized preferentially to the subellipsoid/ellipsoid regions and the OPL neuropil. Although both RyR2 and SERCA2 antibodies labeled the inner segment region, the co-localization was not complete and their expression in the ONL was faint (Fig. 3I–L).

To evaluate further the compartmental expression of TRPC6, retinal sections were double-immunolabeled for TRPC6 and the synaptic markers PSD-95, SV2, bassoon and the vesicular glutamate transporter VGLUT-1. VGLUT-1 is expressed in synaptic terminals of photoreceptors (Fig. 3E), consistent with their expression in synaptic vesicles that fill rod terminals (Dick *et al*., 2003). No PSD-95, SV2, bassoon (data not shown) or VGLUT-1 (Fig. 3E) co-localization with TRPC6 signal was detected in rod terminals, nor was TRPC expressed in radial processes of the Müller glia, which traverse the ONL and are immunopositive for glutamine synthetase (Fig. 3H).

The data presented here suggest three new findings related to Ca^{2+} regulation in mammalian rods. (1) TRPC6, a putative store-operated and/or receptor-operated Ca^{2+} channel (Trebak *et al*., 2003), is strongly expressed in rod perikarya but is absent from other IS regions. (2) Rods may express two different VOCC α subunits localized to the synaptic terminal and ellipsoid/subellipsoid, respectively. Although the subunit expressed in the synaptic terminals of rat rods was shown to be α_{1F} (Morgans, 2001; Berntson *et al*., 2003), the identity of the α subunit located distally within the IS is not known. (3) The key Ca^{2+} store-related proteins, ryanodine receptor isoform 2 and the SERCA2 transporter are expressed in the subellipsoid/ellipsoid region, which contains most of the cells' endoplasmic reticulum (Mercurio &
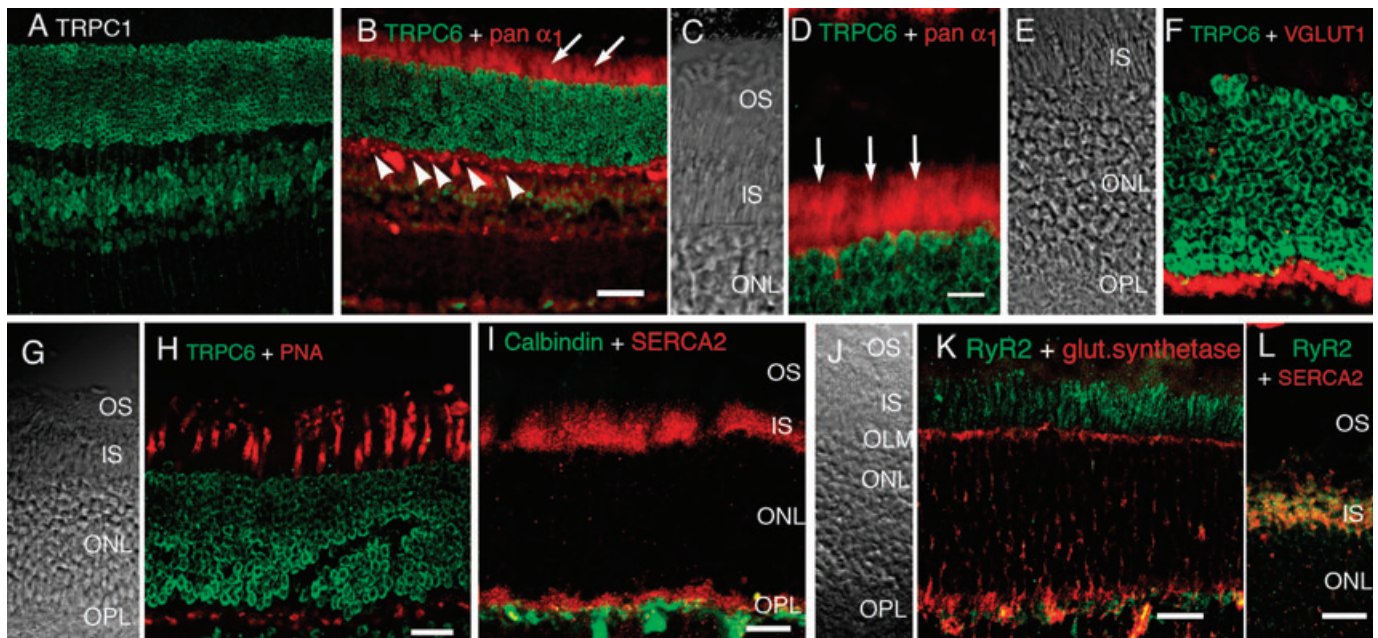
FIG. 3. Compartmentalization of Ca²⁺ entry pathways in mouse rods. (A) TRPC6 expression in the mouse retina. (B) Double labeling for TRPC6 and pan α_1 subunit of L-type voltage-operated Ca²⁺ channels. Ca²⁺ channels are mainly expressed in punctate fashion within synaptic terminals of photoreceptors (arrowheads) and in subellipsoid/ellipsoid regions distal to the ONL (arrows). Scale bar, 20 μm. (C and D) High-resolution detail from B; scale bar, 10 μm. (E and F) Double labeling for TRPC6 and VGLUT-1 shows no co-localization in the synaptic region. (G and H) labeling for TRPC6 and the cone marker peanut agglutinin (PNA) lectin. (I) Co-localization of SERCA2 and the horizontal cell marker calbindin. SERCA2 is expressed in the IS ellipsoid/subellipsoid regions and in the OPL. (J) Nomarski image. (K) Double-labeling of the outer retina for ryanodine receptor isoform 2 (RyR2) and glutamine synthetase. RyR2 is expressed in ellipsoid/subellipsoid regions of the IS, whereas glutamine synthetase is localized to the outer limiting membrane and throughout radial processes of the Müller glia within the ONL. Scale bar, 20 μm. (L) Double labeling for RyR2 and SERCA2 shows partial co-localization in the ellipsoid/subellipsoid region of the IS. Scale bar, 20 μm.

Holtzman, 1982). Taken together, these results suggest that Ca²⁺ influx into mammalian photoreceptors is compartmentalized between different regions. It remains to be determined how this localized expression of Ca²⁺ channels and transporters regulates metabolism (ellipsoid), translation (subellipsoid), transcription (cell body) and synaptic release (synaptic terminal).

The precedent for TRP-mediated Ca²⁺ influx into rods was established in invertebrate photoreceptors, where TRPCs play a critical role in phototransduction (Minke & Selinger, 1996). Recently, it has been shown that TRPCs also gate Ca²⁺ influx into vertebrate neurons (Li et al., 1999) and may mediate sensory transduction (Menco et al., 2001; Lucas et al., 2003). Although there is no information on TRPC distribution in the mammalian retina, TRPC isoform 4 in the avian outer retina may be localized to either photoreceptors and/or Müller glial cell processes; by contrast, TRPC1 is almost exclusively expressed in the IPL (Crousillac et al., 2003). Recent Ca²⁺ imaging experiments suggested that depletion of intracellular Ca²⁺ stores activates entry of Ca²⁺ into cell bodies of salamander rods and cones (Szikra & Krizaj, 2004). TRPCs are the potential candidates for the store-operated Ca²⁺ influx in these cells.

Several elements potentially involved in TRPC signaling have been identified in rod ISs. These include phospholipase Cβ, ryanodine and InsP₃ receptors, as well as G protein-coupled receptors including dopamine D2/D4, metabotropic GluR8 and somatostatin 2A receptors (Krizaj & Copenhagen, 2002). TRPC6 can be activated both by depletion of intracellular Ca²⁺ stores and in a store-independent fashion via phospholipase C and diacyglycerol (Hofmann et al., 1999; Jung et al., 2002; Trebak et al., 2003). Although the spatial separation between SERCAs, ryanodine receptors and TRPC6 argues against depletion-mediated activation of TRPC6, it is possible that other

TRPC isoforms are localized to the subellipsoid region of mouse rods or that ryanodine receptors are expressed in cell bodies at low levels. Moreover, it is possible that cell bodies express InsP₃ receptors, which are known to be potential modulators of TRPC-mediated Ca²⁺ entry (Spassova et al., 2004). Functionally, TRPC6-mediated Ca²⁺ entry in rod perikarya could mediate Ca²⁺ entry in cell bodies independently of membrane voltage (e.g. Li et al., 1999; Tesfai et al., 2001), thus providing adequate access of Ca²⁺ to the cell nucleus even under light-adapted conditions, when most VOCCs are closed. It remains to be determined whether TRPC6, ryanodine receptor and SERCA2 pump are also expressed in mouse cones.

In summary, I provide here immunocytochemical evidence suggesting that TRPC6 is expressed in the cell bodies of rod photoreceptors. By contrast, VOCCs are mostly localized to the ellipsoid/subellipsoid and the synaptic terminal. These data, together with the fact that every photoreceptor region contains a unique set of intracellular organelles, strongly suggest that both plasma membrane Ca²⁺ entry and release from stores are local and region-specific, providing a framework for our understanding of compartmentalization of intracellular signaling pathways in vertebrate photoreceptors.

Abbreviations

GCL, ganglion cell layer; INL, inner nuclear layer; InsP₃, 1,4,5 inositol triphosphate; IPL, inner plexiform layer; IS, inner segment; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; RyR2, ryanodine receptor isoform 2; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase; TRPC, transient receptor potential channel; VGLUT1, vesicular glutamate transporter isoform 1; VOCC, voltage-operated calcium channel.

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