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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\textsuperscript{2+} in photoreceptors are poorly understood. I have investigated the compartmentalization of voltage-dependent and independent Ca\textsuperscript{2+} channels in mouse photoreceptors. Transient receptor potential channels isoform 6 (TRPC6), a putative store-operated Ca\textsuperscript{2+} channel, was selectively localized to the cell body of rods. By contrast, voltage-operated Ca\textsuperscript{2+} channels were expressed in the synaptic terminal and in the ellipsoid/subellipsoid regions. Likewise, Ca\textsuperscript{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\textsuperscript{2+} entry mechanisms in mammalian photoreceptors and bipolar cells are highly compartmentalized, consistent with local, region-specific activation of Ca\textsuperscript{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\textsuperscript{2+} influx and extrusion into the non-OS region occur mainly via L-type voltage-operated Ca\textsuperscript{2+} channels (VOCCs) and plasma membrane Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} signaling within different non-OS regions. In this paper I present evidence that VOCCs, Ca\textsuperscript{2+} stores and potential store-operated Ca\textsuperscript{2+} channels are distributed to different regions in a highly compartmentalized fashion.

The transient receptor potential (trp) genes encode a set of Ca\textsuperscript{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\textsubscript{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\textsuperscript{2+} stores, cytoskeleton and mechanical activation via InsP\textsubscript{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 α1 antibodies indicates that voltage-gated Ca\textsuperscript{2+} channels and Ca\textsuperscript{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\textsuperscript{2+} release and sequestration from the endoplasmic reticulum can be highly localized, depending on distribution of Ca\textsuperscript{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
animals were anesthetized and killed via CO₂ 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™ 488- and Alexa™ 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, 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, NH₂-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 Ca²⁺ ATPase; 1:100), calbindin (1:500), the pan α₁ 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™ 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, AAAGATATCTTCAAATTCAAGT (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).

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**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.
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, NH2-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 Ca2+ influx pathways in photoreceptors, retinal sections were double-immunostained for TRPC6 and a monoclonal pan α1 antibody, which recognizes all α1 subunits of L-type Ca2+ channels. As seen in Fig. 3B, puncta immunoreactive for the pan α1 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 Ca2+ channels containing the α1F subunit near the active zones (Morgans, 2001). Unlike α1F, which appears to be expressed in synaptic terminals of rodent rods (Morgans, 2001), the pan α1 subunit antibody also immunolabeled the subellipsoid/ellipsoid space localized between the outer segment and the cell body (arrows in Fig. 3B). Little pan α1 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 Ca2+ influx mechanisms in mammalian photoreceptors are compartmentalized: whereas Ca2+ entry into the synaptic terminal and subellipsoid/ellipsoid occurs via different VOCC subtypes, Ca2+ entry into the cell body may occur via store-operated and/or receptor-operated cation channels.

In a number of tissues Ca2+ entry through TRPC6 is activated by depletion of intracellular Ca2+ stores (Trebak et al., 2003). Immunolabeling for SERCA2 shows that this ubiquitous Ca2+ 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 Ca2+ 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 Ca2+ regulation in mammalian rods. (1) TRPC6, a putative store-operated and/or receptor-operated Ca2+ 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 α1F (Morgans, 2001; Berntson et al., 2003), the identity of the α subunit located distally within the IS is not known. (3) The key Ca2+ 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 &
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Holtzman, 1982). Taken together, these results suggest that Ca\textsuperscript{2+} influx into mammalian photoreceptors is compartmentalized between different regions. It remains to be determined how this localized expression of Ca\textsuperscript{2+} channels and transporters regulates metabolism (ellipsoid), translation (subellipsoid), transcription (cell body) and synaptic release (synaptic terminal).

The precedent for TRP-mediated Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} influx into vertebrate photoreceptors 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.

Several elements potentially involved in TRPC signaling have been identified in rod ISs. These include phospholipase Cβ, ryanodine and InsP\textsubscript{3} 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 Ca\textsuperscript{2+} stores and in a store-independent fashion via phospholipase C and diacylglycerol (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 InsP\textsubscript{3} receptors, which are known to be potential modulators of TRPC-mediated Ca\textsuperscript{2+} entry (Spassova et al., 2004). Functionally, TRPC6-mediated Ca\textsuperscript{2+} entry in rod perikarya could mediate Ca\textsuperscript{2+} entry in cell bodies independently of membrane voltage (e.g. Li et al., 1999; Tesfai et al., 2001), thus providing adequate access of Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} 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.

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Abbreviations

GCL, ganglion cell layer; INL, inner nuclear layer; InsP$_3$, 1,4,5 inositol triphosphate; OPL, outer plexiform layer; OS, outer segment; RyR2, ryanodine receptor isofom 2; SERCA, sarcoplasmic-endoplasmic reticulum Ca$^{2+}$ ATPase; TRPC, transient receptor potential channel; VGLUT1, vesicular glutamate transporter isofom 1; VOCC, voltage-operated calcium channel.

References


