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SERCA ISOFORM EXPRESSION IN THE MAMMALIAN RETINA

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Abstract

The sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) is a key intracellular calcium transporter, which regulates cellular calcium concentration $[Ca^{2+}]$ by transporting Ca^{2+} ions from the cytosol into the endoplasmic reticulum. SERCA-mediated Ca^{2+} sequestration controls proper folding of newly synthesized proteins within the ER as well as the timing and spatial patterning of depolarization-evoked Ca^{2+} responses in the cytoplasm. We studied expression and distribution of all three SERCA isoforms in the mouse retina using isoform-specific antibodies. No immunostaining was observed with the SERCA1 antibody. SERCA2 was expressed in photoreceptor inner segments, amacrine and ganglion cells of the mouse retina. Similar SERCA2 localization was observed in adult rat, macaque and ground squirrel retinas. Analysis of distribution of SERCA2 immunofluorescence in the developing mouse retina revealed prominent SERCA2 signals throughout postnatal development. The antibody raised against the SERCA3 isoform labeled inner segments of photoreceptors and cell bodies in the inner nuclear layer of the mouse retina. The SERCA3 signal was detected in the inner plexiform layer of the early postnatal retina, but moved by P10 to the outer retina where it was concentrated in outer segments of cones. These results indicate that SERCA2 represents the dominant SERCA isoform in the mammalian retina. SERCA3 may contribute to calcium regulation in photoreceptors and bipolar cells.

Keywords

calcium store; photoreceptor; endoplasmic reticulum; mouse; squirrel; rat

Introduction

Endoplasmic reticulum (ER) is the largest intracellular compartment (accounting for more than 50% of all endomembranes) in eukaryotic cells. In neurons, the ER consists of a network of cisternae extending throughout the cell, from the synaptic terminal to dendritic branches (Mercurio and Holtzman, 1982; Verkhratsky, 2005). Ca^{2+} release from and sequestration into neuronal ER can exert both local and global control over Ca^{2+} -dependent cytoplasmic events, allowing it to regulate many cellular functions, including gene expression, protein synthesis, synaptic transmission and apoptosis (reviewed in Meldolesi and Pozzan, 2002; Berridge et al., 2003; Breckenridge et al., 2003; Verkhratsky, 2005).

The high intraluminal concentration of Ca^{2+} (in the range of 0.1–1 mM) is maintained by the activity of SERCA (sarcoplasmic-endoplasmic reticulum Ca ATPase) pumps, *E1–E2* type ATPases with an affinity for Ca^{2+} of $\sim 0.2 - 1.5 \mu M$ (Dode et al., 2002; Strehler and Treiman, 2004). By maintaining high luminal $[Ca^{2+}]_{ER}$ SERCAs support post-translational modification and transit of newly synthesized proteins across the ER (Cooper et al., 1997), maintain the ability of the ER to control intracellular signaling by ryanodine- and inositol

triphosphate receptors (Berridge et al., 2003) and regulate Ca^{2+} influx via store-operated Ca^{2+} channels (Hofer et al., 1998). Molecular cloning has identified three separate SERCA genes termed SERCA1 (ATP2A1), SERCA2 (ATP2A2), and SERCA3 (ATP2A3). The protein products of ATP2A1–3 (SERCA1–3) are characterized by tissue-specific expression and differences in catalytic properties (Wu et al., 1985; Wuytack et al., 2002; Strehler and Treiman, 2004).

In cold-blooded vertebrates, Ca^{2+} release from and sequestration into the ER regulate Ca^{2+} homeostasis in many classes of retinal cell, including photoreceptors (Krizaj et al., 1999; 2003), horizontal cells (Linn and Christensen, 1991; Micci and Christensen 1998, Solessio and Lasater, Hayashida and Yagi, 2002), amacrine cells (Hurtado et al., 2002), ganglion cells (Akopian and Witkovsky, 1998; 2001; Han et al., 2001) and Müller glia (Keirstead et al., 1995). The functional roles of Ca^{2+} stores in retinal neurons include control of development, membrane excitability, AMPA and GABA receptor function and synaptic transmission (Krizaj et al., 1999; Hurtado et al., 2002; Mitra and Slaughter, 2002; Akopian and Witkovsky, 1998, 2001). It is likely that Ca^{2+} release/sequestration play an essential role in the development, signaling and apoptosis of retinal neurons in mammals and birds (Lohmann et al., 2002). Apoptosis of mammalian photoreceptors tends to be associated with a Ca^{2+} overload and SERCA dysfunction (Linden et al., 1999; Chiarini et al., 2003; Donovan and Cotter, 2002; Fox et al., 2003; Sharma and Rohrer, 2004). Both SERCA2 and SERCA3 isoforms are expressed in salamander retina (Krizaj et al., 2004). The data on intracellular Ca^{2+} stores in warm-blooded animals is scarce and there is no information on SERCA localization and function in the mammalian retina. Given the large differences in Ca^{2+} affinity, catalytic turnover rate, Ca^{2+} -dependence of transcription and modulation by intraluminal proteins between different SERCA isoforms (Wu et al., 2001; Gelebart et al., 2002; Strehler and Treiman, 2004) it is of particular interest to identify and localize SERCA isoforms in the mammalian retina. I report here that SERCA2 is expressed in an ubiquitous manner in most classes of retinal cell. In contrast, SERCA3 staining appears to be confined to photoreceptors and bipolar cells.

Methods

Animals and tissue preparation

Retinae from mice (CBA, BALBc and C57B strains), rats (Long Evans), macaque monkeys and ground squirrels were studied. All procedures were in accordance with NIH guidelines and were approved by the Committee on Animal Research at UCSF. P0 and P3 mice were sacrificed by decapitation, and older mice and rats were killed by CO_2 asphyxiation followed by cervical dislocation. The eyes were enucleated and corneas were cut with a razor blade and the eyecups with the retinas were immersion-fixed for one half to one hour in 4% (w/v) paraformaldehyde in phosphate buffer (PB; 0.1M; pH=7.4). Isolated squirrel and macaque retinas were generously provided by Drs. Julie Schnapf and Jan Verweij (UCSF) and were fixed by the same protocol used for mice and rats. The retinas were rinsed two times in PB and cryoprotected in 30% sucrose overnight at 4° C. Pieces of retinas were mounted in OCT, sectioned vertically at 14 μm thickness on a cryostat, collected on Super-Frost Plus slides (Fisher, Pittsburgh, PA) and stored at -20°C until use.

Retinal sections on slides were washed in PB for 15 min before permeabilization and blocking in a solution containing 0.5% Triton X-100 and 10% goat serum. SERCA1 and SV2 antibodies (developed by Judith Airey and Kathleen Buckley, respectively) were obtained from the Developmental Studies Hybridoma Bank. The mouse monoclonal calbindin 28K antibody was obtained from Chemicon (Temecula, CA), the monoclonal SERCA2 antibody was from Affinity Bioreagents (ABR; Golden, CO); the rabbit polyclonal SERCA2 antibody from Bethyl Laboratories (Montgomery, TX). The rabbit polyclonal

SERCA3 N89 antibody, originally developed in Dr. Frank Wuytack's laboratory (Wuytack et al., 1994), was purchased from ABR and Sigma (St. Louis, MO). This antibody has been previously characterized in the mouse tissue by Western blot (Krizaj et al., 2004). Antibody dilutions were 1:100–1:200 for SERCA2 and 1:100–1:500 for SERCA3. As secondary antibodies, goat anti-mouse or goat anti-rabbit IgG (H+L) conjugated to fluorophores (Alexa 488 and Alexa 594 conjugates (Molecular Probes, Eugene, OR), diluted 1:500 or 1:1000 were used. The PNA lectin (Molecular Probes) was diluted 1:20. After incubation, sections on slides were washed in PB, mounted with Vectashield (Vector, Burlingame, CA), and sealed with nail polish. Negative controls for non-specific staining of secondary antibodies were performed for every set of experiments by omitting the primary antibodies; immunofluorescent images of these sections occasionally showed some non-specific staining of photoreceptor outer segments in but were otherwise blank. Control experiments for SERCA3 were also performed with a synthetic neutralizing peptide whose sequence VTDARERYGPN was taken from the conserved amino end of mouse, rat and human SERCA3 (ABR; PA1–910).

Confocal microscopy

Immunofluorescent and differential interference contrast (DIC) images were acquired at depths of 8 bits on a confocal microscope (LSM 5 Pascal; Zeiss, Tarrytown NY) using 488 nm and 594 nm lines for fluorophore excitation and suitable band-pass or long-pass filters for emission detection. 40× water or 63× oil objectives were used.

Pre-embedding electron microscopy

Salamander eyecups were fixed in 2% PFA and 1% glutaraldehyde in PB for 10 min. The retina was dissected and fixed 3 hours at room temperature, rinsed and placed in PB, cut into thin longitudinal strips, and stored in PB at 4° C. Retinas were placed in 30% sucrose in PB overnight at 4° C, frozen in 2-methylbutane/LN2 and slowly thawed in a cold block. Subsequently, retinas were washed with 0.1% bovine serum albumin/Tris Saline and 0.05% Tween-20, pH = 7.4, quenched with 3% H2O2 in BTS and fixed in Karnovsky's medium for 60 min. Retinas were then reduced in 2% aqueous OsO4 and 1.5% ferrocyanide for 60 min, rinsed, and added to Kellenbuger (2% buffered with UA) at 37° C for 30 min. Finally, retinas were dehydrated, embedded in Eponate 812 (Ted Pella Inc.) and processed for electron microscopy.

Results

Light microscopic localization of SERCAs in the mammalian retina

SERCA-mediated Ca²⁺ sequestration occurs via transporter proteins encoded by three separate genes (ATP2A1–3; reviewed in Wuytack et al., 2002, Strehler and Treiman, 2004). The SERCA1 gene is translated into a 115 KDa Ca²⁺ ATPase protein, which is expressed mainly in fast twitch skeletal fibers (Brandl et al., 1987). Consistent with this, immunostaining with SERCA1 antibodies produced no staining in the mammalian retina (data not shown).

While Western blots indicated that both SERCA2 and SERCA3 gene products are expressed in the mouse retina (Krizaj et al., 2004), the spatial localization of these two isoforms in the mouse is not known. To determine the distribution of both isoforms, we used SERCA2-specific antibodies. Following immunostaining, a pronounced SERCA2 signal was detected in both plexiform layers (Fig. 1). No staining was observed in the absence of the primary antibody (Fig. 1C). At higher confocal gains, SERCA2 immunofluorescence was observed in mouse photoreceptor inner segments (ISs; arrowheads in Fig. 1B), consistent with SERCA2 expression in the IS region that contains large amounts of ER cisternae (Mercurio

and Holtzman, 1982). The most pronounced SERCA2 signal in mouse was detected in cell bodies of presumed amacrine cells lining the proximal end of the INL and in cells localized to the ganglion cell layer (arrows in Fig. 1B), suggesting this isoform plays an important role in Ca^{2+} signaling in the inner retina (e.g., Lohmann et al., 2002). To identify the SERCA2-immunopositive cell types in the OPL, retinas were double-labeled with the horizontal cell marker calbindin and presynaptic markers SV2 and PSD-95 (Krizaj et al., 2002). Although a weak SERCA2 signal was detected in horizontal cells, most of the OPL signal was seen at the level of photoreceptor terminals (Fig. 7F–H). This finding was further confirmed in double-labeling studies with SV2 and PSD-95 (data not shown). This data suggests that SERCA is expressed in most classes of retinal neuron, including photoreceptors, horizontal cells, amacrine cells and ganglion cells.

We also studied the pattern of SERCA2 expression in other mammalian retinas. As seen in Fig. 2, rat, ground squirrel and macaque monkey retinas exhibit a similar pattern of SERCA2 immunoreactivity to that seen in mouse (Fig. 1) and tiger salamander (Krizaj et al., 2004) retinas. These results indicate that SERCA2 isoform is ubiquitous in the vertebrate retina, similar to its expression in the CNS (Baba-Aissa et al., 1996; Wu et al., 1995).

Ca^{2+} release from intracellular stores has been suggested to play an important role in stabilization of contacts between inner retinal cells in dendritic stratification that occurs during retinal development (Lohmann et al., 2002). Given the prominent localization of SERCA2 within the IPL we also investigated developmental expression of this isoform in the postnatal mouse retina. Fig. 3 shows that, at P1, the inner plexiform layer (IPL) was strongly SERCA2-immunopositive, as were cell bodies of presumed amacrine neurons in the proximal INL. At high confocal gains, SERCA2 signal was observed throughout the neuroblast layer (NBL) and was particularly marked in the ventricular zone (VZ) in which the neuroblasts hosting future rods and cones are localized (Jasoni and Reh, 1996) (Fig. 3C). SERCA2 immunofluorescence in the outer retina was relatively weak throughout development and could be seen only at high confocal gain, which saturated the signal from the inner retina (Fig. 3C, 7J). Unlike the developmental expression of the SERCA3 immunosignal (see below, Fig. 5), the SERCA2 signal in the inner retina remained strong throughout development of the postnatal mouse retina.

SERCA3 is localized to photoreceptors

The SERCA3 isoform is expressed at significant levels only in a selected number of tissues (Wu et al., 1995, Wuytack et al., 2002), usually in cells that co-express SERCA2 (Shull, 2000; Strehler and Treiman, 2004). When the mouse retina was immunostained for SERCA3 using the commonly used N89 antibody (Wuytack et al., 1994), a marked signal was detected in cellular regions that were largely devoid of SERCA2 expression (Fig. 4). The SERCA3 antibody immunostained both outer retina and in the inner nuclear layer (INL); a diffuse cytoplasmic signal was occasionally observed in the GCL (Fig. 4B). The INL signal consisted of diffuse cytoplasmic label in rod bipolar cells as well as sharply delimited ellipsoid-shaped puncta throughout the INL (Figs. 4B, 7I). Clearly demarcated structures were labeled in the IS of rods and cones at the level of the connecting cilium (Fig. 7E; arrowheads in Fig. 4B); these structures were significantly shorter compared to those labeled by SERCA2 (Fig. 7E). Somewhat unexpectedly, prominent SERCA3 expression was also seen in the outer segment (OS) layer (Fig. 4B, D, 7A–E). The labeling in the outer retina can be seen more clearly at lower confocal gain in Figs. 4D and 7A–D.

To further localize the SERCA3-immunopositive signal within the OS layer, retinas were incubated with the lectin peanut agglutinin (PNA). PNA has been shown to selectively label the plasma membrane of OSs of mammalian cones while it is absent from rod OSs (Ogilvie et al. 1997). In sections double-labeled for SERCA3 and fluorescein-conjugated PNA, a

substantial degree of colocalization was observed (Fig. 7B–D). In parallel series of experiments we immunolabeled retinæ with the antibody raised against the cone OS heteromeric G protein (transducin; G_t), with similar results (data not shown). This data suggests that the N89 antibody immunostains outer segments of mouse cone photoreceptors. To determine whether the SERCA3 signal was an anomaly confined to one particular strain of mice (the C57BL) we compared the immunostaining with CBA and BALBc strains. No difference in SERCA3-immunostaining was detected between these three mouse strains (data not shown).

Given the localization of SERCA3 immunosignal in the outer retina, a series of control experiments was performed to determine the specificity of the SERCA3 antibody. No staining was observed when the retina was incubated with the secondary antibody alone (Fig. 4F). To test whether the primary antibody was specific for SERCA3, we co-incubated it with a synthetic neutralizing peptide whose sequence VTDARERYGPN was taken from the conserved amino end of mouse, rat and human SERCA3. Incubation with the peptide completely eliminated the SERCA3-mediated signal (Fig. 4H). Western blots showed a signal at the appropriate molecular weight (Krizaj et al., 2004). This data suggests that the antibody we used recognizes a specific epitope within the mouse retina. To determine whether the staining with the N89 antibody was limited to the mouse, we immunolabeled rat and ground squirrel retinæ with the SERCA3 antibody. As illustrated in Fig. 5B, D, SERCA3 signal was confined to the outer retina in both mammalian species; similar results were seen in the monkey retina (D.K. and T.Szikra, to be published elsewhere, but see Figs. 7M, N).

At birth, SERCA3 immunoreactivity was detected in the IPL and in the ventricular zone (VZ) at the vitread-most region of the neuroblast layer (NBL) (Fig. 6). The intensity of this signal increased through development simultaneously with a gradual decrease in the SERCA3 signal intensity in the inner retina (Fig. 6). At P3–P5, SERCA3 immunoreactive signals were seen in the developing INL as well as in cell bodies at the distal edge of the IPL and GCL. At ~P9–P10, a predominating SERCA3 signal is seen in the photoreceptor layer at the level of emerging inner and outer segments. Similar results were observed in the developing rat retina (D.K., unpublished data). Taken together, these data suggests that N89-immunoreactivity shifts during retinal development from the inner retina to the outer retina, concentrating within IS and OS of photoreceptors.

Electron microscopy

An electron microscopic analysis was performed to further investigate the SERCA3 localization to photoreceptors. The electron micrographs show uniform labeling of a subset of photoreceptor OSs with the SERCA3 antibody (Fig. 8). The gold particles were confined to the plasma membrane and the surfaces of disks in the proximal section of the OS layer, consistent with immunofluorescent data (Fig. 4). To test whether the SERCA3 signal was altered by the EM fixative (0.1% glutaraldehyde), glutaraldehyde-fixed retinal sections were checked by confocal immunofluorescence. The SERCA signal in these retinæ was identical to that in controls in which paraformaldehyde fixative (4%) was used (data not shown), suggesting that fixation does not interfere with the antigen recognized by the antibody.

Discussion

SERCA2 is prominently expressed in the mammalian retina

SERCA2 is the most widespread of all SERCA pumps and is phylogenetically the oldest (Baba-Aissa et al., 1996; Wuytack et al., 2002). Immunoblotting and immunofluorescence studies have shown that SERCA2 represents the main SERCA isoform in most neuronal

tissues (Wu et al., 1995; Wuytack et al., 2002). Consistent with the prominent SERCA2 expression in the brain (Baba-Aissa et al., 1996; Wu et al., 1995), intense staining was observed in mouse, rat, squirrel and macaque retinas. These results suggest that, SERCA2 is expressed in the mammalian retina across many species. The SERCA2 antibodies immunolabeled most classes of retinal neuron, including photoreceptors, amacrine cells and ganglion cells, indicating that SERCA2 represents the predominant SERCA isoform in the mammalian retina. Preliminary experiments suggest that the majority of the SERCA2 signal corresponds to the SERCA2b splice variant (D.K. and T. Szikra, in preparation). Our results also suggest that, in developing mammalian retina and possibly in the adult, SERCA2-mediated Ca^{2+} sequestration may occur in tandem with SERCA3 (Figs. 3, 5 & 7E).

SERCA3 signal in cone photoreceptors

In the mouse retina, the SERCA3 antibody immunostained inner segments of rods and cone photoreceptors as well as outer segments of cones. A moderate SERCA3 signal was also detected in the INL, possibly corresponding to bipolar cell bodies. The expression of both SERCA2 and SERCA3 isoforms in the inner segment region is consistent with physiological findings suggesting that intracellular Ca^{2+} stores regulate IS $[\text{Ca}^{2+}]_i$ (Krizaj et al., 2003). In contrast to SERCA2, the SERCA3 antibody labeled just a fraction of the IS (Fig. 6E) at the level of the connecting cilium and the anatomical substrate for this IS staining remains to be determined. Given the canonical view of Ca^{2+} regulation in photoreceptor outer segments (Fain et al., 2001; Korenbrot and Rebrink, 2002), the staining of outer segments of cones by the SERCA3 antibody was unexpected. Several lines of evidence suggest that the antigen recognition by N89, the SERCA3 antibody, was specific: (1) Western blots from whole mouse retinas probed with the SERCA3 antibody showed a distinct band at 97 kDa, corresponding to the molecular weight of SERCA3 (Krizaj et al., 2004). (2) No staining was observed in the absence of the primary antibody or when the N89 antibody was co-incubated with a selective neutralizing peptide; (3) Similar staining was seen in several mammalian species and (4) Electron microscopy also showed SERCA3 signal in OS membranes. The immunostaining experiments presented here suggest, but do not prove, that a SERCA isoform is expressed in the ISs and OSs of cones. This issue might be resolved in Ca^{2+} imaging experiments from isolated mouse cones (e.g.; Sampath et al 1999). However, Wang et al. (1999) recently provided data that support indirectly localization of an integral endoplasmic reticulum protein to plasma membrane of cone OSs. The authors showed that OS from rat, cat, rabbit and monkey cones abundantly express the isoform 1 of the IP_3 receptor family. Given that SERCAs and IP_3 Rs typically colocalize in ER membranes (Berridge et al., 2003), it is possible that SERCA3 plays a complementary role in cone OS membrane. Photoreceptor OSs contain many signaling elements typically associated with intracellular Ca^{2+} stores. $\text{PLC}\beta_4$, a key enzyme in the signaling cascade that typically initiates Ca^{2+} release from intracellular stores via production of IP_3 , has been found in both cones (Ferreira and Pak, 1994) and in rods (Jiang et al., 1996). Preliminary reports suggest light regulates IP_3 -mediated Ca^{2+} release and increase in OS $[\text{Ca}^{2+}]_i$ (Ghalayini and Anderson, 1984; Schnetkamp and Szerencsei, 1993; Schnetkamp and Kaupp, 1995) possibly through Ca^{2+} release from intracellular stores and/or buffering proteins (Matthews and Fain, 2001; Brockerhoff et al., 2003).

Functional implications

Given the role for ER in the modulation of the spatiotemporal pattern of $[\text{Ca}^{2+}]_i$ changes (Verkhatsky, 2005), SERCA2s in retinal neurons are likely to modulate the amplitude and kinetics of activity-dependent $[\text{Ca}^{2+}]_i$ elevations (Meldolesi and Pozzan, 2002) and decays (Majewska et al., 2000) in retinal cells under both light- and dark-adapted conditions. SERCA2 is likely to represent the isoform responsible for refilling the ER through the periods of sustained low baseline $[\text{Ca}^{2+}]_i$ in light-adapted neurons. Ca^{2+} release from

intracellular stores and SERCA-mediated Ca^{2+} sequestration are essential for sustaining intracellular Ca signaling during dendritic stratification (Lohmann et al., 2002), in control of membrane excitability (Mitra and Slaughter 2002; Hurtado et al., 2002), Ca^{2+} homeostasis (Solessio and Lasater, 2002) and synaptic signaling (Akopian and Witkovsky, 1996) of many classes of retinal neuron. Moreover, SERCA2 is likely to regulate Ca^{2+} signaling that underlies the developmental and functional plasticity of retinal networks, as documented in many types of central neurons (Harvey and Collingridge, 1992; Emptage et al., 2001; Cong et al., 2004). Similar to tiger salamander, SERCA2 in the mammalian retina is expressed throughout cell bodies and processes of retinal neurons that signal with graded potentials and/or via spiking.

Many studies have shown that prolonged exposure to elevated $[\text{Ca}^{2+}]_i$ or low $[\text{Ca}^{2+}]_i$ can be damaging to neurons. Depletion of ER Ca stores with the SERCA blocker thapsigargin or the ionophore A23187 often results in cell death via the “unfolded protein response” (Cooper et al., 1997; Chiarini et al., 2003; Breckenridge et al., 2003). Both SERCA2 and SERCA3 could play a protective role in the developing mammalian retina with the amount of releasable Ca^{2+} in developing inner retinal neurons determined by the SERCA2/SERCA3 ratio which could determine the ratio between baseline cytosolic $[\text{Ca}^{2+}]_i$ and lumenal $[\text{Ca}^{2+}]_i$ (Gelebart et al., 2002). SERCA3s are the least understood and appear to deviate the most from the other members of the family (Liu et al., 1997; Wuytack et al., 2002; Shull 2000). Biochemically, SERCA3 has a lower apparent affinity for Ca^{2+} than SERCA2 (Lytton et al., 1992), a faster catalytic turnover (Dode et al., 2002) and, unlike SERCA2, it is not regulated by the ER buffering protein phospholamban (Toyofuku et al., 1994). Although the expression pattern for SERCA3 is much more restricted compared to SERCA2, SERCA3 expression can be changed by stress or pathological conditions, suggesting SERCA3 could play a protective role (Wuytack et al., 2002; Strehler and Treiman, 2004).

Topologically, SERCA3 in the plasma membrane of cone OSs would be oriented outwards to support clearance of Ca^{2+} from the cytoplasm, possibly acting to speed the decrease in cone $[\text{Ca}^{2+}]_{OS}$. Ca^{2+} extrusion in cone OS is about six-fold faster than in rods (integration time in cones is ~ 50 msec; Nakatani and Yau, 1989; reviewed in Korenbrot and Rebrik, 2002). Ca^{2+} decay in cones may be faster than in rods due to greater surface-to-volume ratio of cone OSs or some difference in the properties of NCKX exchangers and/or buffering proteins. Although the density of NCKX exchange sites in cones appears to be the same as in rods (Yau, 1994), and the interactions between NCKXs and cGMP-gated channels similar in rods and cones (Kang et al., 2003), $[\text{Ca}^{2+}]_{OS}$ decline in cones, but not in rods, is biphasic, consistent with activation of two separate extrusion/buffering mechanisms. Furthermore, following exposure to light, $[\text{Ca}^{2+}]_{OS}$ falls to lower baseline levels than in rods (Fain et al., 2001). Although some early biochemical studies suggested that ATP-dependent Ca^{2+} pumps are responsible for Ca^{2+} sequestration in rod OSs (Mason et al., 1974; Kaupp et al., 1981; Schnetkamp and Kaupp, 1985; Puckett et al., 1985; Pepe et al., 2000) and that this effect is proton-dependent (Kaupp, 1981; Schnetkamp and Kaupp, 1983; 1985) as has been shown for SERCA-mediated Ca^{2+} transport (Naderali et al., 1997), there is at present no physiological evidence that ATP-driven Ca^{2+} pumps contribute to Ca^{2+} extrusion within the cone outer segment; the Na^+ - K^+ , Ca^{2+} exchangers are thought to act as the sole Ca^{2+} extruders at the cone OS plasma membrane (Nakatani and Yau, 1985; Prinsen et al., 2000; reviewed in Fain et al. 2001).

In conclusion, our experiments suggest that SERCA2 represents the dominant SERCA isoform in the mammalian retina. A marked SERCA3-immunopositive signal was detected in the outer segments of cones. Further physiological studies are needed to determine whether this signal corresponds to functionally active SERCA3 transporters within cone OSs.

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Fig.1.

Confocal fluorescence micrograph of a vertical frozen section through mouse retina labeled for sarcoplasmic-endoplasmic reticulum calcium ATPases isoform 2 (SERCA2). A. Differential contrast interference (DIC) view of the same retinal section as in B. B. SERCA2 immunofluorescence is prominent in the inner retina, including the IPL and GCL. Cell bodies of neurons at the proximal border of the IPL are labeled by the SERCA antibody (arrows). Moderate SERCA2 signal is seen in the OPL and at the level of photoreceptor inner segments (arrowheads). C. Same immunostaining protocol as in B., with no primary antibody. Scale bar = 20 μ m. Abbreviations: OS, outer segment; IS, inner segment; ONL outer nuclear layer; OPL, outer plexiform layer; INL inner nuclear layer; IPL inner plexiform layer; GCL, ganglion cell layer.

Fig. 2. Morphology and distribution of cells expressing SERCA2 in rat, ground squirrel and macaque monkey retina. Photomicrographs of vertical sections of (A, B) rat, (C, D) ground squirrel and (E, F) macaque monkey retina. The antibody labeled the cell bodies and neuropil in inner retina of all three species. Moderate staining occurs in inner segments (B, D, F) and cell bodies (B, D) of photoreceptors. Scale bar = 20 μ m.

Fig. 3. Developmental expression of SERCA2 in the postnatal mouse retina. A and F: DIC images of P0 and P11 retinas. Abbreviations are in Figure 1. Scale bar = 20 μ m.

Fig. 4.

Morphology and distribution of cells expressing SERCA3 immunoreactivity. A. DIC view of the fluorescent image in B. B. SERCA3 immunofluorescence at high gain. The antibody labels the OS layer and the IS layer (arrowheads). Diffuse staining of cell perikarya is visible in the INL and GCL with puncta of high fluorescence marking cells in the INL. C. DIC view of D. D. Low gain SERCA3 immunofluorescence shows a prominent signal in the outer segment layer. E. DIC view of F. F. Staining of the mouse retina in the absence of the primary antibody. G. DIC view of image H. H. 1:1 co-incubation of the primary antibody with a specific neutralizing peptide. Scale bars = 20 μ m. Abbreviations as in Fig. 1.

Fig. 5. SERCA3 expression in rat (A, B) and ground squirrel (C, D) retinas. The antibody labeled the inner segments of photoreceptors. A saturating signal is seen in outer segments of cones; only a subset of cones are labeled in the squirrel. Scale bars = 20 μ m.

Fig. 6. Developmental expression of SERCA3 in the postnatal mouse retina. A prominent signal in the outer retina is seen throughout the development, first in the VZ (P1 – P5) followed by the developing IS/OS region (P10 – P15). Abbreviations as in Figure 1. Scale bar = 20 μ m.

Fig. 7.

Colocalization experiments with SERCA2 and SERCA3. A. Combined DIC and fluorescent image of SERCA3 immunostaining in the mouse retina. B. SERCA3 signal in mouse outer retina. C. PNA lectin. D. Co-localization experiment: SERCA and PNA lectin both label the same population of cone OSs. Scale bar = 10 μ m. E. Double immunostaining for SERCA2 and SERCA3. Outer retina detail. SERCA3 immunofluorescence is confined to a subregion of the IS and to cone OSs. SERCA2 immunofluorescence is found throughout the IS. Scale bar = 10 μ m. F. – H. Colocalization of SERCA2 and calbindin. F. SERCA2 in the mouse retina labels the ISs and the OPL. G. Calbindin is selectively expressed in horizontal cells. H. SERCA2 is moderately expressed both in horizontal cells and presynaptically. Scale bar = 10 μ m. I. High resolution view of the mouse inner retina double-immunostained for SERCA3 and PKC. SERCA3 diffusely labels a population of rod bipolar cells (arrows). In addition, prominent punctate staining is seen throughout the INL (arrowheads). Scale bar = 10 μ m. J. Developing outer retina in P0 mouse immunostained for SERCA2 (Tx Red) and SERCA3 (FITC). Scale bar = 10 μ m. K. Developing outer retina in P10 mouse immunostained for SERCA3. Scale bar = 10 μ m. L. Developing inner retina in P1 mouse immunostained for SERCA2 (Tx Red) and SERCA3 (FITC). Scale bar = 10 μ m. M. Outer retina from macaque monkey immunostained for SERCA2 (Tx Red) and SERCA3 (FITC). Scale bar = 5 μ m. N. The ground squirrel retina, immunostained for SERCA3. Scale bar = 10 μ m.

Fig. 8. Pre-embedding immunoelectron microscopy of adult mouse retina. SERCA3 immunoreactivity was localized to outer segments in the proximal region of the OS layer. The mitochondria-rich ellipsoid region of the ISs is shown for orientation. Scale bars = 1.0 μm in A, 0.5 μm in B and 1.0 μm in C.