Ontogeny of plasma membrane Ca\textsuperscript{2+} ATPase isoforms in the neural retina of the postnatal rat

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Abstract
Calcium ion (Ca\textsuperscript{2+}) signaling has been widely implicated in developmental events in the retina, but little is known about the specific mechanisms utilized by developing neurons to decrease intracellular Ca\textsuperscript{2+}. Using immunocytochemistry, we determined the expression profiles of all known isoforms of a key Ca\textsuperscript{2+} transporter, the plasma membrane Ca\textsuperscript{2+} ATPase (PMCA), in the rat retina. During the first postnatal week, the four PMCA isoforms were expressed in patterns that differed from their expression in the adult retina. At birth, PMCA1 was found in the ventricular zone and nascent cell processes in the distal retina as well as in ganglion and amacrine cells. After the first postnatal week, PMCA1 became restricted to photoreceptors and cone bipolar cells. By P10 (by postnatal day 10), most inner retinal PMCA consisted of PMCA2 and PMCA3. Prominent PMCA4 expression appeared after the first postnatal week and was confined primarily to the ON sublamina of the inner plexiform layer (IPL). The four PMCA isoforms could play distinct functional roles in the development of the mammalian retina even before synaptic circuits are established. Their expression patterns are consistent with the hypothesis that inner and outer retinal neurons have different Ca\textsuperscript{2+} handling needs.

Keywords: PMCA, Development, Differentiation, Ganglion cell, Photoreceptor

Introduction
During development, changes in [Ca\textsuperscript{2+}], accompany cell division and differentiation in many neuronal tissues, including the vertebrate retina (Gu et al., 1994; Sugioka et al., 1998; Wong et al., 1998; Zirpel et al., 2000). Developing retinal cells are subjected to significant [Ca\textsuperscript{2+}], loads, arising from ion entry into the cytoplasm via voltage-gated Ca\textsuperscript{2+} channels, ionotropic glutamate receptor channels, or release from internal stores (Sugioka et al., 1998; Schmid & Guenther, 1999). Ca\textsuperscript{2+} fluxes across the plasma membrane, through gap junctions, and between intracellular store compartments and cytoplasm are associated with early synchronized waves of transiently increased [Ca\textsuperscript{2+}] (Pozzan et al., 1994). Ca\textsuperscript{2+} waves may be important for proper development of neuronal circuits in many tissues of the central nervous system (CNS), including the retina (Penn et al., 1998).

Whereas the mechanisms of Ca\textsuperscript{2+} entry into the cytoplasm of developing retinal cells are relatively well understood (Rörg & Grantyn, 1994; Sugioka et al., 1998; Schmid & Guenther, 1999), less is known about clearance of cytoplasmic calcium ions. Retinal neurons actively maintain very low baseline levels of cytoplasmic [Ca\textsuperscript{2+}], in the range of 30–100 nM, and can quickly return to these levels following depolarization or agonist-induced [Ca\textsuperscript{2+}]i elevation (Bindokas et al., 1994; Kobayashi & Tachibana, 1995; Wong et al., 1995; Krizaj & Copenhagen, 1998; Hurtado et al., 2002). The plasma membrane Ca\textsuperscript{2+} ATPases (PMCA\textsubscript{s}) are an important Ca\textsuperscript{2+} clearance system found in all neurons that extrude Ca\textsuperscript{2+} across the plasma membrane (Hammes et al., 1994; Stauffer et al., 1995; Filoteo et al., 1997; Zacharias & Kappen, 1999; Burette et al., 2003; reviewed in Strehler & Zacharias, 2001; Strehler & Treiman, 2004). PMCA\textsubscript{s} play a key role in Ca\textsuperscript{2+} signaling of sensory tissues, such as the cochlea (Furuta et al., 1998; Boyer et al., 2001; Dumont et al., 2001) and the retina (Krizaj & Copenhagen, 1998; Morgans et al., 1998). The four PMCA isoforms exhibit differences not only in Ca\textsuperscript{2+} pumping rates but also in the regulatory mechanisms associated with them. These include differences in kinetics, affinities for Ca\textsuperscript{2+} and calmodulin, interactions with dics-large homology (PDZ) domain-containing proteins, and susceptibility to modulation by second messengers (Caride et al., 2001; DeMarco & Strehler, 2001; reviewed in Guerini, 1998; Strehler & Zacharias, 2001).

The different PMCA isoforms are expressed in a cell type-specific manner in the adult mouse retina, rat cochlea, and rat brain.
(Krizaj et al., 2002; Dumont et al., 2001; Burette et al., 2003). Only a few studies have documented the specific cellular pattern of expression of the different PMCA isoforms in developing brain tissues (Brandt & Neve, 1992; Furuta et al., 1998; Kozel et al., 1998; Zacharias & Kappen, 1999). None have described this for the developing mammalian retina. Using an antibody recognizing all PMCA isoforms, Tolosa de Talamoni et al. (2002) recently showed that chick retinal neurons express PMCAs before hatching, with immunoreactivity seen mainly in the plexiform layers. This suggests that PMCAs play a role in retinal development. Regulation of Ca\(^{2+}\) in the developmental program of the mammalian retina could be accomplished in part by utilizing the marked differences in biophysical and physiological parameters of the various PMCA isoforms (Strehler & Treiman, 2004).

We therefore examined the distribution of expression of all four PMCA isoforms during postnatal development of the rat retina using isoform-specific antibodies. We found that the different PMCA isoforms are expressed in specific developmental sequence in cell bodies and processes of all classes of retinal neuron. Moreover, changes in developmental isoform expression were observed for both inner and outer retinal cells. Because the different isoforms have known differences in Ca\(^{2+}\) handling characteristics, the data suggest that developing retinal neurons may have specific Ca\(^{2+}\) handling needs that differ from mature neurons and that the developmental expression of each isoform is separately regulated.

**Materials and methods**

**Animals and tissue preparation**

Retinas from Long-Evans rats (Simonsen; Gilroy, CA) at different postnatal ages were investigated with the day of birth designated as postnatal day (P)0. Rats older than P60 were considered to be adults. All procedures were in accordance with NIH guidelines and were approved by the Committee on Animal Research at UCSF. P0 and P3 rats were sacrificed by decapitation, and older animals were killed by CO\(_2\) asphyxiation followed by cervical dislocation.

**PMCA antibodies and immunocytochemistry**

Isoform-specific, anti-PMCA antibodies were gifts from J. T. Penniston (Mayo Clinic, Rochester, MN) and were purchased from Affinity BioReagents (ABR; Golden, CO) and Swant (Bellinzona, Switzerland). The anti-PMCA rabbit polyclonal antibodies (NR1, NR2, and NR3 against PMCA1, PMCA2, and PMCA3, respectively) were generated against 13–18 residue peptide sequences from the amino terminus of the corresponding rat PMCA (or human PMCA in the case of the Swant antibodies). Mouse monoclonal antibody JA9, specific for PMCA4, was generated against PMCA4 protein isolated from human red blood cells and has also been extensively characterized, including mapping of the epitope recognized by the antibody to a peptide present in both the human and rat proteins (Caride et al., 1996). All these antibodies were previously characterized against rat tissue using Western blotting and were shown to selectively immunostain rodent tissues, including brain, cochlea, and retina (Stauffer et al., 1997; Dumont et al., 2001; Krizaj et al., 2002). Furthermore, antibody specificities for each rat PMCA isoform have been fully characterized using microsomes prepared from transfected COS cells (transformed simian fibroblasts) overexpressing PMCAs of known identity (Filoteo et al., 1997). The staining we obtained with the commercial PMCA1, 2, and 3 antibodies was similar to that obtained with their counterparts from the Penniston laboratory.

To obtain retinal sections, eyes were enucleated, corneas were opened with a razor blade, and the eyes were fixed by immersion for 30 min to 1.5 h in 4% (w/v) paraformaldehyde in phosphate buffer (PB; 0.1 M; pH = 7.4). The tissue was rinsed two times in PB and cryoprotected in 30% sucrose overnight at 4°C. Fixed tissue was embedded in medium (Tissue Tek OCT; Ted Pella, Redding, CA), frozen, sectioned vertically at 14-μm thickness on a cryostat, and collected on Super Frost Plus slides (Fisher; Pittsburgh, PA). For each age, sections were prepared from retinas of at least two and up to four separate animals, and multiple slides from each preparation were probed using each of the four PMCA isoform antibodies.

Retinal sections on slides were washed in PB for 15 min before being permeabilized and blocked in a solution containing 0.5% Triton X-100 and 10% goat serum. The antibody dilutions were 1:300–1:500 for NR1, 1:200–1:300 for NR2, 1:200–1:300 for NR3, and 1:100 for JA9. Antibodies against several classes of protein markers for retinal cells were coapplied with those against individual PMCA isoforms in double-labeling experiments. These antibodies included those reactive for calbindin (made in mouse; clone CB955; obtained from Sigma, St. Louis, MO; used at 1:500 dilution), glutamic acid decarboxylase (GAD65) (mouse; gad-6; Developmental Studies Hybridoma Bank, University of Iowa; 1:500), SV2 (mouse; SV2; Developmental Studies Hybridoma Bank, University of Iowa; 1:100), the vesicular gamma aminobutyric acid (GABA) transporter (guinea pig; AB5855; Chemicon, El Segunda, CA; 1:2000), tyrosine hydroxylase (mouse; MAB3815; Chemicon; 1:100), and choline acetyltransferase (mouse; Chemicon MAB305; 1:50–1:300).

We used goat anti-mouse, goat anti-guinea pig, 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, as secondary antibodies. After incubation, sections on slides were washed in PB, mounted with Vectashield (Vector, Burlingame, CA), and sealed with nail polish. Negative controls for nonspecific staining of secondary antibodies were performed for every set of experiments by omitting the primary antibodies. Immunofluorescent images of these sections showed occasional nonspecific staining of photoreceptor outer segments in some slides, but none showed specific staining patterns. Representative examples are shown in the figures.

**Image acquisition and processing**

Images were taken from midperipheral regions of the cryostat sections. The ages we report in this study are the age of the animal on the day of sacrifice. 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, suitable band-pass or long-pass filters for emission detection, and either a 40×/0.8NA or 63×/1.4NA objective (Zeiss). The DIC images were acquired by detection of 488-nm transmission through the specimen. Single-label experiments showed that no signal from the inappropriate secondary antibody was detected in either channel in our setup (data not shown). For the PMCA2, 3, and 4 developmental series (but not including the adult sections) shown in Figures 3, 4, and 5, images were acquired at microscope settings that were the same for each image within a series from slides processed in parallel. Other images were acquired at higher gain.
settings, as indicated in the figure legends, to reveal details of lower staining intensity. This procedure was used to facilitate comparison of image brightness among ages within an individual experiment. Acquired images were resized, adjusted, and labeled using Adobe Photoshop (v7.0) software; no changes were made to the pixel values of the developmental series images (i.e. those acquired at similar gains and laser settings), while the contrast of other images was adjusted using the maximum and minimum settings (while keeping a slope of 1.00) in the “Levels” command in the software.

Results

Distributions of PMCA proteins involved in Ca\(^{2+}\) extrusion in both the adult and developing postnatal rat were examined in vertical retinal sections using antibodies that recognized individual PMCA isoforms.

PMCA1 expression during development is transient in the inner retina and sustained in photoreceptors and bipolar cells

At birth, most of the retina consists of a broad neuroblast layer (NBL) containing precursors for future photoreceptors, bipolar cells, horizontal cells, and Müller glia. Although the future cones as well as a few of the rods express opsins (Hicks & Barnstable, 1987; Jasoni & Reh, 1996), they are largely undifferentiated, appearing as neuroblasts which occupy the outer third of the NBL. In the inner retina, newly formed dendrites of amacrine cells mingle with retinal ganglion cell (RGC) dendrites to form the nascent inner plexiform layer (IPL) (Weidman & Kuwabara, 1968; Braekevelt & Hollenberg, 1970).

PMCA1 immunoreactivity was detected in the outer third of the neuroblast layer (NBL) at birth (Figs. 1 & 2), suggesting that PMCA1 is expressed in at least some photoreceptors early in differentiation of the outer retina. As the photoreceptors develop over the course of the first postnatal week and the primitive inner segments begin to mature, they send an axon toward the developing outer plexiform layer (OPL). During this time, PMCA1 immunoreactivity began to concentrate in processes that extended towards the OPL (Figs. 1B–1D & 2A–2C). The PMCA1 signal was especially prominent in growth cone-like processes which also expressed the synaptic marker SV2 (Fig. 6A), suggesting that they represent the developing photoreceptor synaptic terminals. Indeed, by P12, most of the PMCA1 in the OPL became localized to photoreceptor terminals (Fig. 1E) and exhibited an expression pattern similar to the adult (Fig. 1G). In the adult, PMCA1 signal in photoreceptor terminals predominated over all other PMCA1-immunopositive structures, including bipolar cells. These results suggest that PMCA1 may play a key role in Ca\(^{2+}\) extrusion from both developing and mature photoreceptor terminals.

Bipolar cells are among the last retinal neurons to differentiate fully (Jasonti & Reh, 1996). PMCA1 has been considered a marker for type 3 (OFF) and type 5/6 (ON) cone bipolar cells in the adult mouse retina (Krizaj et al., 2002; Haverkamp et al., 2003). At P5 in the rat, however, most of the PMCA1-positive cells in the

Fig. 1. Expression of PMCA isoform 1 in the postnatal and adult rat retina. This and the following figures show vertical, transverse retinal sections oriented with the ganglion cell layer at the bottom that were probed with PMCA isoform-specific antibodies using fluorescent immunocytochemistry and examined with a confocal microscope. A–G show retinas from P0, P3, P5, P9, P12, P15, and adult rats, respectively. Gain settings on the confocal microscope were identical for A–C and were lower but also identical for D–G; the slides of each group were processed together. To show more clearly the structure of the developing early retina, A–C also show differential interference contrast (DIC) images of the same section. Representative negative controls, which contained secondary but lacked primary antibodies, are shown for the P0 and adult sections in A and G. (A) At birth, PMCA1 immunostaining is concentrated in the distal NBL and in the IPL. (B, C) During the first postnatal week, the outer retinal PMCA1 signal is observed in cells and their processes extending toward the proximal retina. At P5, these processes contain high-intensity puncta, likely corresponding to developing photoreceptor terminals. Many ganglion cell bodies are labeled. (D) At P9, PMCA1 is expressed in both plexiform layers. Immunostaining is also observed in perikarya from presumed bipolar neurons in the distal half of the INL. (E–G) PMCA1 is expressed in photoreceptor terminals in the OPL and in presumed bipolar cell bodies and processes in the INL and IPL, respectively. The IPL signal is seen in both sublaminae of the IPL. NBL: neuroblast layer; IS: photoreceptor inner segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; and GCL: ganglion cell layer. Scale bar = 25 \(\mu\)m in panels A–F and 17 \(\mu\)m in panel G.
developing INL appeared to be amacrine cells (Figs. 1C & 2D), adjacent to the IPL, with only a few cells labeled in the middle of this layer where the bipolar cells are developing. PMCA1 appeared in presumed bipolar cell bodies (in the distal INL) and processes by P9 (Figs. 1C & 1D). The IPL from P9 onward though lightly stained for PMCA1 throughout its extent, was seen to develop two distinct bands of stain. One band was expressed in sublamina a (the OFF sublayer) and the other in sublamina b (the ON sublayer), similar to the expression pattern in cone bipolar types observed in the adult mouse retina (Krizaj et al., 2002; Haverkamp et al., 2003). In the adult, many bipolar cell perikarya were labeled by the antibody, and their axons could be seen running into the IPL (Fig. 1G). These cell bodies were not in the upper row of cells generally occupied by the rod bipolars but somewhat lower in the INL, consistent with PMCA1 expression in some, but not all, cone bipolars. Additional IPL staining was seen, but it was fainter than the two major bands. This stain may be due to a third ON bipolar cell type, possibly including the rod bipolar cell; similar fainter labeling in the other strata of the IPL has been seen in the mouse retina (see Krizaj et al., 2002; Haverkamp et al., 2003). Alternatively, PMCA1 may be weakly expressed within processes of amacrine or ganglion cells within the IPL.

More prominent PMCA1 signal at birth was detected in cell processes in the inner retina. PMCA1 was transiently detected in the proximal NBL, where amacrine cells are developing (Figs. 1A & 2D). This putative amacrine PMCA1 immunoreactivity appeared to be maintained through about P9 (Fig. 1D). In addition to the signal within the IPL, most of the cells in the ganglion cell layer (GCL) had relatively low levels of PMCA1 immunoreactivity at early postnatal ages (Figs. 1A–1C). This immunostaining pattern is much different than that seen in the adult, where neither amacrine nor ganglion neurons were labeled with the PMCA1 antibody (Fig. 1G).

Over the course of the first two postnatal weeks, synaptic function matures, RGCs develop their intrinsic excitability (Röhrig & Gränty, 1993; Schmid & Guenther, 1996), and populations of ganglion cells refine their dendrites to specific sublaminar locations. Comparing the P5 GCL (Fig. 1C) to the P9 GCL (Fig. 1D), it can be seen that PMCA1 signal was mostly gone from the GCL after the first postnatal week, suggesting that PMCA1 may be involved in early developmental events, such as Ca\(^{2+}\)-dependent remodeling of amacrine and ganglion cell processes (e.g., Lohmann et al., 2002), but not in RGC synaptic signaling or spike generation.

**PMCA2 expression during development is concentrated in the inner retina**

PMCA2 and PMCA3 are generally considered to represent neuronal PMCA isoforms (Hammes et al., 1994; Stauffer et al., 1995; Guerini et al., 1999). Due to their high sensitivity for Ca\(^{2+}\)/calmodulin (Kd = 2 to 5 nM), both isoforms can be more effective at Ca\(^{2+}\) extrusion than PMCA1 and PMCA4 (Hilfiker et al., 1994; Caride et al., 2001; Brini et al., 2003; Strehler & Treiman, 2004).

At birth, PMCA2 immunoreactivity was found in the rat retina using relatively high gain settings, suggesting a low level of expression. PMCA2 was found throughout the NBL in early postnatal retina (Figs. 3A–3C & 3G–3H). In contrast to PMCA1, the PMCA2 signal within the NBL was much more uniform and was expressed around the perikaryon, rather than in the growth cones extending towards the developing OPL, as seen by double immunostaining at P3 with the synaptic marker SV2 (Fig. 6B). The PMCA2 signal in the NBL decreased to very low levels by P6. PMCA2 was detected in the OPL at P10 (Fig. 3H), and this signal increased markedly by P14, where cells with horizontal cell morphology were labeled (Fig. 3D). In the adult, the inner segments of photoreceptors and the OPL were lightly labeled with the PMCA2 antibody, with most of the staining occupying the proximal side of the OPL. This OPL signal colocalized with calbindin (Fig. 6C) and at higher magnification showed dendritic knobs characteristic of horizontal cells (data not shown). Thus, much of the OPL staining for PMCA2 can be attributed to horizontal cells.

Immunostaining with the PMCA2 antibody poorly labeled presumed bipolar cell bodies in the distal inner nuclear layer (INL) of the rat retina (Figs. 3D & 3E). Because of this poor label and because PMCA2 immunoreactivity was detected in amacrine cells, which likely account for much of the PMCA2 label in the IPL, it

**Fig. 2.** PMCA1 immunostaining in the developing outer and inner retina. (A) In the P0 outer retina, PMCA1 staining is concentrated in cells of the ventricular zone (VZ). (B–C) In P3 and P5 outer retina, PMCA1-immunopositive processes are seen extending toward the future OPL; prominent puncta are seen in presumed growth cones of photoreceptors in the developing OPL. (D) In the P5 inner retina, the PMCA1 antibody labels cell bodies of presumed amacrine cells in the proximal INL in addition to processes within the IPL. Scale bar = 10 \(\mu\)m in all panels.
was difficult to determine a possible developmental expression pattern of PMCA2 in bipolar cells. Some PMCA2-immunopositive signal was seen in the IPL by P6 (Fig. 3B). At P10, the antibody labeled cell bodies in the INL that resembled amacrines, suggesting that many of the PMCA2-positive processes in the IPL at this time arise from these amacrine cells (Fig. 3C and at higher gain in Fig. 3H). By P14, the signal was much stronger with amacrines still clearly labeled, and two IPL bands that partially colocalize with choline acetyltransferase (ChAT) in the adult (described below) became more obvious (Fig. 3D). The staining intensity continued to increase in the IPL through P28 (Figs. 3E & 3F). At early ages, PMCA2 was also detected at a low level in the GCL (Figs. 3A & 3G); the immunostaining was observed in cell bodies in the developing GCL until P14 (Fig. 3D). Later time points showed more intense signal in the IPL and less expression in the GCL cell bodies. After P14, the majority of the PMCA2 signal was confined to the dendritic trees of inner retinal cells (i.e. IPL staining), but cell bodies in the GCL and INL had a PMCA2 signal on their surfaces that decreased from P14 to adulthood.

In the adult, PMCA2 staining was concentrated within the inner retina. Immunopositive signal was detected in the plasma membranes of cell bodies in the proximal layer of the INL, where amacrine cells reside, suggesting that a significant fraction of the labeled IPL processes belongs to these amacrines. Some but not all of these cells were GABAergic because many also stained with an antibody that recognizes GAD65, an enzyme used for GABA synthesis (Fig. 6D). In the IPL, PMCA2 signal was particularly prominent in two brightly stained bands. These two bands partially colocalized with ChAT, which is contained in the cholinergic starburst amacrine cells, and many ChAT-immunopositive cell bodies in both the GCL and INL contained PMCA2 immunoreactivity (Fig. 6E). The PMCA2 bands in the IPL were typically broader than the ChAT bands, consistent with the large number of presumed amacrines that were ChAT negative but positively labeled for PMCA2 (Fig. 6E). These results are similar to what was observed in adult mouse (Krizaj et al., 2002) and suggest that PMCA2 may play a prominent role in Ca\(^{2+}\) extrusion in the inner retina of the rat.

**PMCA3 in developing rat retina**

PMCA3 staining in the outer retina was weak at all ages compared to inner retinal staining. A few cells in the outer edge of the NBL exhibited low PMCA3 signal at P5 and had morphologies that
resembled horizontal cells (Fig. 4C, arrows). By P8, these cells were more apparent (Figs. 4D & 4I). By P21, the labeling seemed decreased to lower levels, similar to the adult (Fig. 4H), where the low PMCA3 signal in the proximal half of the OPL costratified with calbindin-positive horizontal cells (Fig. 6F).

PMCA3 was expressed at birth in the inner retina, particularly in the GCL (Fig. 4A). Some GCL cells, immunoreactive for PMCA3, were GAD65-negative, suggesting that they were RGCs (data not shown). Quite early in postnatal development, at P5, PMCA3 was detected in presumed amacrine of the proximal NBL (i.e. in those cells closest to the IPL) (Fig. 4C). The inner retinal PMCA3 signal encompassed the entire extent of the IPL after the first postnatal week (Fig. 4). In the adult, some PMCA3-positive amacrine perikarya appeared to colocalize with GAD65 (Fig. 6G). Many others did not, and a large proportion of the PMCA3-immunopositive processes in the IPL were GAD65-negative as well (Fig. 6G). Unlike in the adult mouse retina, in which the IPL was uniformly labeled by the PMCA3 antibody (Krizaj et al., 2002), the rat IPL exhibited two prominent, PMCA3-immunopositive bands in strata 2 and 4 (e.g. Fig. 4H) with fainter signal in the other strata. These bands colocalized with the cholinergic amacrine cell marker ChAT (Fig. 6H) and did not overlap with the GABAergic marker GAD65 (Fig. 6G). These bands were thus similar to those seen with the PMCA2 antibody. However, the band in sublamina a was narrower in PMCA3- than in PMCA2-stained material.

Tissue sections from animals P14 and older generally showed rings of stain, indicative of plasmalemmal labeling of cell bodies in the cellular layers (Figs. 4F–4H). In many cells of the early postnatal retina from P0 through about P10, however, cells had both plasma membrane and cytoplasmic staining with the PMCA3 antibody (Figs. 4A–4E & 4I). Our impression was that cells with cytoplasmic PMCA3 signal were more numerous in the first postnatal week and decreased after that, with more of the immunopositive cells in retinas from older animals having plasmalemmal labeling (e.g., compare Fig. 4C to Fig. 4F). From P8 through P14, PMCA3 immunoreactivity was seen increasingly to label the plasma membranes of inner retinal neurons, leading to the adult pattern by P21 (Fig. 4G).

**PMCA4 in developing rat retina**

Strong PMCA4 immunoreactivity appeared relatively late in the postnatal rat retina. The pattern of PMCA4 immunoreactivity

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**Fig. 4.** Expression of PMCA isoform 3 in the postnatal and adult rat retina. A–I show retinas from P0, P3, P5, P8, P10, P14, P21, adult, and P8 rats, respectively. Gain settings on the confocal microscope were identical for A–G and were higher for I. The slides in A–G were processed together. The insets in A, D, and G show DIC images of the same section; the inset in F shows a negative control which contained secondary but no primary antibodies. Arrows point to presumed horizontal neurons. Abbreviations are as in Fig. 1. Scale bar = 25 µm in panels A–G and 17 µm in panels H and I.
observed in the adult retina, in amacrine and ganglion neurons and in the IPL, was formed by P21 (Fig. 5D). Using this same detection gain on the confocal microscope for P14 samples, much lower levels of staining were observed, but PMCA4 immunoreactivity was still present in a similar pattern (Fig. 5C). At higher gain settings, however, PMCA4 could be detected in inner retina at P8 (Fig. 5F), again in a similar pattern as in older retina, and in a few GCL cells and IPL processes of P3 retina (data not shown). Scattered PMCA4-positive cells were seen in the GCL and in the INL, typically adjacent to the IPL (Fig. 5E), but the most prominent immunoreactivity was observed in sublamina b of the IPL. PMCA4 antibody appeared to stain the OPL faintly in some sections. This stain was unreliable yet seemed positive in many instances. For this reason, we cannot exclude the possibility that small amounts of PMCA4 are present in horizontal cells or photoreceptors.

The PMCA4-immunopositive cells in the INL were also labeled by an antibody against vesicular GABA and glycine transporter (VGAT) (Fig. 6I), as were a few of the cells in the GCL (data not shown). Other PMCA4-positive cells in the GCL appeared to be VGAT negative, suggesting that these were RGCs (data not shown). Thus, the labeled processes in the IPL could arise from both amacines and RGCs. Only a small proportion of the cell bodies of the total amacrine and RGC populations were definitively labeled (many fewer than appeared in PMCA2- or PMCA3-labeled retinas).

Within the late postnatal and adult IPL, PMCA4 immunoreactivity was seen in several bands, with the most intense staining observed in sublamina b (the ON sublamina), where cone ON bipolar and rod bipolar terminals are located (Figs. 5D & 5E).

Brightly stained, PMCA4-immunopositive fibers were also seen in sublamina a (the OFF sublamina), with fibers labeled in stratum 1, directly apposed to the INL; in stratum 2, at an apparently lower density than in stratum 1; and occasionally in the middle of the IPL, where many fibers appeared to be crossing the IPL (Fig. 5E). Stratum 1, the most distal layer of the IPL, is also the location of tyrosine hydroxylase (TH)-positive neurites that arise from dopaminergic amacrine within the retina, but the PMCA4 label did not completely colocalize with TH, either in the fibers or in the cell bodies of double-labeled material (Fig. 6J). Because the PMCA4-immunopositive cell bodies were distinct from the TH-positive ones, PMCA4 does not appear to be expressed by dopaminergic amacines. These results suggest that PMCA4 may have an important role in modulating transmission of the ON signaling pathway in the inner retina.

Discussion

We studied the expression of the four known plasma membrane Ca\(^{2+}\) ATPase isoforms (i.e. PMCA isoforms 1, 2, 3, and 4) in the rat retina. These proteins transport calcium ions out of the intracellular space and thus help return \([\text{Ca}^{2+}]\) in the cytoplasm to normal resting levels. The distribution of specific PMCA isoform immunoreactivity in postnatal and adult rat retina suggests that the expression of these four isoforms follows distinct spatial and temporal patterns. Immature amacrine and ganglion cells expressed three PMCA isoforms (PMCA1, PMCA2, and PMCA3) at early postnatal ages, but PMCA1 was almost absent from these cells after P9, suggesting that PMCA1 is required in these cells during their early development but not during their period of
Fig. 6. Double immunolabeling of the postnatal and adult rat retina reveals cell subtype-specific expression of PMCA isoforms. A1-3 and B1-3 show retinas from a P5 and P3 rat, respectively; all other panels show retinas from adult rats. Each row of three panels consists of images from the identical area of a retinal section. Panels labeled “1” (the left panels, which show retinas immunostained with PMCA antibodies) are the green channel and panels labeled “2” (the middle panels, which show retinas immunostained with retinal cell type markers) are the red channel of the merged images, which are labeled “3” (the right panels). Layers within the area of the retinal sections shown are indicated in the left panels. For each row of images, the antibodies used are indicated on the left and middle panels, and arrowheads in these panels point to identical locations in each section. SV2: synaptic vesicle-associated protein 2; GAD65: glutamic acid decarboxylase of 65 kD; ChAT: choline acetyl transferase; VGAT: vesicular GABA and glycine transporter; and TH: tyrosine hydroxylase; other abbreviations are as in Fig. 1. Scale bar = 10 µm for all panels except A1-3 and J1-3, where it is equal to 25 µm and 5 µm, respectively.
synaptogenesis. During the maturation process, the intensity of immunofluorescence increased for all four PMCA isoforms, consistent with developmental upregulation of PMCA expression. The results are summarized in Table 1. The elimination from some cell types, the subsequent segregation of PMCA isoforms into specific plexiform laminae, and the late expression of PMCA4 occurred in parallel with neurite extension and synapse formation across the retina. This suggests that different PMCA isoforms may be used to regulate Ca\(^{2+}\) dynamics in defined cell populations at specific time periods and that some PMCA isoforms are required to maintain Ca\(^{2+}\) ion homeostasis before synaptic circuits are functional.

**PMCA1**

This study demonstrates a developmental shift in the expression of PMCA1 in the developing rat retina. Its expression in the retina appears selective. In the adult retina, PMCA1 is expressed at the synaptic terminals of rod and cone photoreceptors as well as in at least two classes of bipolar cells (Krizaj et al., 2002; Haverkamp et al., 2003). These cell types possess ribbon synapses and are characterized by graded release of the synaptic transmitter glutamate. However, because only a few bipolar cell classes were labeled, not all terminals possessing ribbon synapses have prominent PMCA1 expression.

The distribution of PMCA1 immunoreactivity in adult rat was similar to that in the adult mouse retina (Krizaj et al., 2002; Haverkamp et al., 2003), as well as to that in the retina of other mammalian species, including macaque and ground squirrel (D.K., unpublished observations). In the rat, PMCA1 immunoreactivity was found in photoreceptors early in their development in the NBL. This occurred before both the period of morphological photoreceptor synapse formation and initial synaptic transmission between photoreceptors and horizontal cells, which begin around P5 in the rat retina (Weidman & Kuwabara, 1968; Blanks et al., 1974; Bachman & Balkema, 1993; Rich et al., 1997). At the end of the first postnatal week, PMCA1 signal was seen in neurites of photoreceptors extending toward the future OPL and the retinal pigment epithelium, suggesting PMCA1-mediated extrusion throughout the extent of the cell is needed for photoreceptor developmental progression. This is consistent with findings that neurite extension and elaboration of mammalian CNS dendrites and axons depend on signaling by transient increases in Ca\(^{2+}\). (Redmond et al., 2002; Tang et al., 2003). By about P10, most of the PMCA1 signal in photoreceptors was found in their synaptic terminals, presumably to regulate the level of glutamate release, which is highly dependent on [Ca\(^{2+}\)], (Witkovsky et al., 1997; Krizaj & Copenhagen, 2002).

The temporal expression pattern of PMCA1 in the retina is similar to that observed in the developing rat cochlea (Furuta et al., 1998), another sensory tissue with an abundance of synapses with ribbon-like structures and graded transmitter release. Cochlear expression of PMCA1 diminishes from birth to P14, becoming restricted to the synaptic region of hair cells in the adult (Dumont et al., 2001). Our results support the idea that PMCA1 plays an important role during development prior to synapse formation and possibly in development of synaptic connections by regulating Ca\(^{2+}\) homeostasis in the growth cone seeking its postsynaptic partners within the OPL. In addition, developing cells in the ventricular zone of the mammalian retina exhibit slow Ca\(^{2+}\) waves which appear to be coordinated with Ca\(^{2+}\) signaling in the inner retina (Syed et al., 2004). It thus seems possible that PMCA1 in photoreceptors early in their development may help restore [Ca\(^{2+}\)], to baseline levels following these influx events during growth.

Unlike in the adult, PMCA1 immunoreactivity was also detected at low levels in the ganglion cell layer and in amacrine cells in the first days after birth (a time when the bipolar cells are only beginning to be generated). We observed that PMCA1 immunoreactivity was mostly gone from the GCL after the first postnatal week, suggesting that PMCA1 is involved in early developmental events but not in RGC synaptic signaling or spike generation. During the second postnatal week in the inner retina, PMCA1 signal in the IPL gradually decreased, and strong PMCA1 immunoreactivity in the inner retina became restricted to two or three bands of terminals in the IPL and a subset of cell bodies in the INL. As discussed by Haverkamp et al. (2003), these cells resemble the type 3 (OFF) cone bipolar and the type 5 or 6 (ON) cone bipolar using the classification scheme of Euler and Wässle (1995) for rat bipolar cells. The downregulation in PMCA1 expression in the inner retina coincided with a prominent upregulation of PMCA2, PMCA3, and, subsequently, PMCA4 isoforms within the IPL, particularly within amacrine cells as judged by cell body labeling in the INL. PMCA1 immunoreactivity reached the adult expression pattern at the beginning of the third postnatal week.

**PMCA4**

PMCA4 was the latest isoform to be detected during postnatal development, appearing almost a week later than any of the others, with prominent immunoreactivity appearing around the time of eye opening. The small amounts of label in only a few scattered cells seen at the earliest postnatal ages suggest that PMCA4 is not necessary for the differentiation of retinal neurons. Although this isoform is expressed in many cells of most body tissues in the adult (Strehler & Zacharias, 2001), expression in the rat brain has been shown to be limited to specific cellular subpopulations (Buret et al., 2003). We found this to be true in retinas from both adult and developing rats, where PMCA4 was quite restricted in its expression pattern, being present in a subset of cell bodies in the INL and GCL and in processes of the IPL, primarily in sublamina b, where terminals from both ON cone bipolar and rod bipolar make contacts with many amacrine and ganglion cell types.

Our earlier results from adult mouse retina suggested that PMCA4 is uniformly expressed across the IPL (Krizaj et al., 2002). This finding is different from the present results in rat that

| Table 1. Summary by age and cell type of immunoreactivity for the four PMCA isoforms during development of the neural rat retina |
| PMCA isoforms | P0–P6 | P6–P11 | P11–P21 | Adult |
| Neuroblasts | 1 | 2 | n/a | n/a |
| Photoreceptors | n/a | 1 | 1, 2 | 1, 2 |
| Horizontal cells | 3 | 3 | 2, 3 | 2, 3 |
| Bipolar cells | 2 | 1 | 1, 2 | 1, 2 |
| Amacrine cells | 1, 2, 3, 4 | 1, 2, 3, 4 | 1, 2, 3, 4 | 1, 2, 3, 4 |
| Ganglion cells | 1, 2, 3, 4 | 1, 2, 3, 4 | 1, 2, 3, 4 | 1, 2, 3, 4 |

Perceived immunostain intensities: 1 < 1 < 1 < 1 as equivalent to +/− < + < ++ < ++++ for each isoform.
indicate a more prominent PMCA4 signal in sublamina b than in sublamina a. Unlike in the rat, where OPL labeling was seen in approximately half of our experiments, the PMCA4 antibody consistently labeled the mouse OPL (Krizaj et al., 2002). To address these discrepancies in the labeling of the IPL and OPL between mouse and rat, we performed a series of additional experiments in the mouse using new batches of the PMCA4 antibody obtained from two separate sources. We found that the mouse PMCA4 signal in the IPL was typically more pronounced in sublamina b, as in the rat retina, with these new antibody batches. However, the PMCA4 antibodies still consistently labeled the mouse OPL (data not shown). We hypothesize that the antibody aliquots originally used were partially degraded, resulting in a false immunopositive signal in sublamina a.

The time of PMCA4 appearance in the rat retina coincides with the beginning of ribbon synapse development in the IPL (Weidman & Kuwabara, 1968). The striking layer-specific expression in the rat retina of PMCA4 suggests a difference may exist in Ca\(^{2+}\) regulation between the ON and OFF pathways in the IPL, perhaps reflecting differences in Ca\(^{2+}\) elevation in ON and OFF inner retinal neurons in response to light stimuli (Protti & Llano, 1998). Other PMCA4-immunopositive processes were seen in two strata of the OFF sublamina, but these were much sparser. The processes in stratum 1 costratified with neurites from dopaminergic amacrine cells, but the PMCA4-positive cells were not dopaminergic. Nonetheless, the close apposition of their processes suggests that these PMCA4-positive neurons may directly communicate with dopaminergic amacrine cells. The position of the PMCA4-immunoreactive cell bodies in the inner part of the INL, near the IPL, and their expression of VGAT suggests that these cells were amacrine cells.

**PMCA2 and PMCA3**

Tissue distribution of PMCA2 and PMCA3 isoforms is generally restricted to neuronal tissue, suggesting that Ca\(^{2+}\) clearance from neurons differs from that of other cell types (Stauffer et al., 1995, 1997; Strehler & Zacharias, 2001). These isoforms were prominently expressed in the mammalian inner retina, with both PMCA2 and PMCA3 being upregulated during postnatal rat retinal development, mirroring similar findings obtained in the cerebellum (Guerrini et al., 1999) and the cochlea (Furuta et al., 1998). A key role of PMCA2 for development of the mammalian cochlea was revealed in PMCA2 mutant and knockout models, all of which exhibited severe phenotypes associated with abnormal development of the cochlea and the vestibular apparatus (Kozel et al., 1998; Dodson & Charalabapoulou, 2001). Both PMCA2 and PMCA3 possess a high affinity for Ca\(^{2+}\)/calmodulin and are therefore likely to support Ca\(^{2+}\)-dependent processes that require precision in [Ca\(^{2+}\)]\(_e\), regulation. The PMCA2 and PMCA3 signals in the inner retina are thus consistent with a prominent role for high-gain neurotransmission in the inner retina (Copenhagen et al., 1990).

Perikarya of many of the PMCA2- and PMCA3-immunoreactive cells in the IPL were GABAergic, suggesting that PMCA2 plays a role in inhibitory sculpting of the visual signal. In addition, the two prominent PMCA2-immunoreactive bands, observed in strata 2 and 4 of the IPL from about P10 onward, colocalized with ChAT-immunopositive bands. ChAT-positive amacrine cells in both the GCL and INL contained PMCA2 immunoreactivity, consistent with PMCA2 expression in cholinergic amacrine cells. Early cholinergic activity is thought to play a key role in the generation of retinal “Ca\(^{2+}\) waves” (Feller, 2002), providing laminar clues for stratification of a subset of ganglion cells (Stacy & Wong, 2003). Our results suggest that both PMCA2 and PMCA3 could play a role in the cholinergic activity of the developing retina. Furthermore, successful propagation of Ca\(^{2+}\) waves requires efficient extrusion of Ca\(^{2+}\) from the cytoplasm so as to maintain steady-state [Ca\(^{2+}\)]\(_e\), at low baseline levels (30 to 100 nM). PMCA isoforms 1, 2, and 3 could all participate in Ca\(^{2+}\) extrusion during early development, thereby regulating signaling during these waves (Wong et al., 1995; Syed et al., 2004).

**Are neuronal PMCAs involved in developmental cell death?**

From P0 to P10, PMCA2 and PMCA3 appeared to be localized to the cytoplasm of many neurons in immunostained sections. By P14, however, the localization of these PMCAs had moved to the plasma membrane and was plasmalemmal in adulthood. Recent results have demonstrated that at least two of the PMCA isoforms, PMCA2 and PMCA4, can be cleaved after caspase activation during apoptosis; furthermore, the cleaved products moved to a cytoplasmic localization in immunostained, apoptotic cells (Schwab et al., 2002), much like the localization we saw in early postnatal retina. A well known feature of retinal development is a period of apoptotic cell death for different neuronal types. In the rodent, up to 90% of the RGCs die by apoptosis, with the peak period from P3 to P5 (Galli-Resta & Ensini, 1996). The peak period of amacrine cell death is from P3 to P8 in the mouse (Young, 1984). Cytoplasmic localization of PMCA protein is typically observed in cells undergoing apoptosis (E. Strehler, unpublished observation; Schwab et al., 2002). Therefore, it seems possible that the cells in our material having a cytoplasmic localization of PMCA immunoreactivity were undergoing cell death at the time of fixation. The apoptotic cascade is initiated by Ca\(^{2+}\) entry and leads to activation of caspase enzymes, and an influx of Ca\(^{2+}\) has been shown to be necessary for apoptosis in rat RGCs (Cellerino et al., 2000). This would suggest that the presence of PMCAs early in development is not sufficient to keep cells alive and out of the pathway of programmed cell death. Further experiments, perhaps combining immunolocalization of these PMCA isoforms with TUNEL staining, would be necessary to examine this issue.

**Do PMCAs regulate neurotransmitter release?**

PMCAs are the primary Ca\(^{2+}\) extrusion mechanism in photoreceptor inner segments and in bipolar cell terminals (Morgans et al., 1998, 1999; Krizaj & Copenhagen, 1998; Krizaj et al., 2002; Zenisek & Matthews, 2000). Many synaptic terminal regions were intensely stained by PMCA antibodies. Given that [Ca\(^{2+}\)]\(_e\), at the terminal determines the level of neurotransmitter release (Heidelberger et al., 1994), it is plausible that PMCAs can regulate release. The most striking and intense staining for PMCA isoforms that we observed was the PMCA1 antibody labeling of mature photoreceptor terminals, suggesting PMCA1 could be a molecular regulator of release there. Functional experiments, perhaps using transgenic mice lacking PMCA1 expression in the retina, will be necessary to search for a link between regulation of specific PMCA isoforms and the control of transmitter release.

The specificity of PMCA isoform expression in certain cell types in the retina indicates a complexity of function, suggesting that neuronal subclasses possess distinct PMCAs as part of their “signaling toolkits” for Ca\(^{2+}\) handling (Berridge et al., 2003; Krizaj et al., 2004). For example, the early expression of multiple
PMCA isoforms in presumed retinal ganglion cells coincides with the active Ca\textsuperscript{2+} dynamics occurring in these cells during development. Moreover, the strong upregulation of all four PMCA isoforms in the retina is consistent with an increase in total Ca\textsuperscript{2+} content density, an increase in release of Ca\textsuperscript{2+} from intracellular stores, an upregulation of Ca\textsuperscript{2+} signaling molecules, and an increase in Ca\textsuperscript{2+}-dependent synaptic signaling seen during the differentiation and stratification of RGC and amacrine processes (Schmid & Guenther, 1999; Xue & Cooper, 2001).

The early expression of multiple PMCA isoforms coincides with the active Ca\textsuperscript{2+} dynamics occurring in the retina during development. In order for Ca\textsuperscript{2+} to be effective as an intracellular signal, Ca\textsuperscript{2+} influx must be counterbalanced by efflux and sequestration. We provide evidence for both short- and long-term expression of Ca\textsuperscript{2+} extrusion proteins during development of different neuronal classes in the mammalian retina. The developmental changes of PMCA isoform expression and their selective localization in specific subsets of retinal neurons are consistent with a key role of plasma membrane Ca\textsuperscript{2+} extrusion in both development and maintenance of retinal neurons and their circuits.

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