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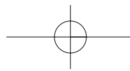

ORIGINAL PAPER

Eur. J. Histochem.
46: 333-340, 2002
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Comparative immunolocalization of the plasma membrane calcium pump and calbindin D_{28K} in chicken retina during embryonic development

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Accepted: 4/02/02

Key words: calbindin D_{28K}, calcium pump, chicken retina, development

SUMMARY

The immunolocalization of the plasma membrane calcium pump (PMCA) was studied in 4-week-old chick retina in comparison with calbindin D_{28K} (CaBP) immunostaining. We have demonstrated that the monoclonal anti-PMCA antibody 5F10 from human erythrocyte plasma membrane cross-reacts with a Ca^{2+} pump epitope of the cells from the neural retina. The immunolocalization of both proteins was also studied during the embryonic development of the chicken retina. At age 4.5 days, the cells of the retina were faintly immunoreactive to PMCA and CaBP antibodies, but the lack of cellular aggregation and differentiation did not allow discrimination between the two proteins. A clear difference in the localization was seen from the tenth day of development through post-hatching with slight variation. PMCA localized mainly in the outer and inner plexiform layers, in some cells in the ganglion layer, in the nerve fiber layer and slightly in the photoreceptor cells. CaBP was intensely stained in cones, cone pedicles and some amacrine cells. The number of CaBP positive amacrine cells declined after hatching. A few ganglion cells and several nerve fibers were CaBP immunoreactive. The role of these proteins in the early stages of retinal development is unknown, but the results suggest that Ca^{2+} homeostasis in the retina is well regulated, probably to avoid excessive accumulation of Ca^{2+}, which often leads to neurodegeneration.

INTRODUCTION

The calcium ion is a significant factor in the visual process, participating in several steps in the complex path from light absorbance by the photoreceptor cells to the formation of electrical signals that are neuronally transmitted to the visual cortex to be deciphered therein as a viewed image. In the absence of light, the photoreceptor cells maintain a dark current due to the influx of Na^+ and Ca^{2+} through a cyclic guanylate (cGMP)-gated cation channel followed by subsequent extrusion of both ions. Upon light exposure, a series of reactions involving rhodopsin and other proteins results in a decrease in cGMP concentration, which closes the cGMP-gated ion channel. The closure of this channel and continued operation of the $Na^+/(Ca^{2+} + K^+)$ exchanger decreases the calcium concentration of

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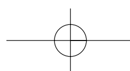

the photoreceptor cell. The lowered Ca^{2+} concentration is considered a requirement for the process of light adaptation. Other calcium-dependent processes have been identified, their action mediated by the binding of calcium to calcium-binding proteins that contain the characteristic E-F hand amino acid sequence originally described by Kretsinger *et al.* (1982). Other cell types of the retina, i.e., horizontal, bipolar, amacrine and ganglion cells, also have E-F hand-containing calcium-binding proteins, which further accentuates the role of calcium in the visual process. The exact cellular location of these proteins varies among species and among specific cell types. Identified within one or more of these cells are calmodulin (Pochet *et al.*, 1991), calbindin (Schreiner *et al.*, 1985; Pochet *et al.*, 1991; Pasteels *et al.*, 1987; Veccino *et al.*, 1993; Papzafiri *et al.*, 1995; Yan, 1997; Haley *et al.*, 1995; Dalil-Thiney *et al.*, 1994), calretinin (Rogers, 1989; Pasteels *et al.*, 1990; Papzafiri *et al.*, 1995; Völgyi *et al.*, 1997), parvalbumin (Uesugi *et al.*, 1992; Yan, 1997), hippocalcin (Bastianelli *et al.*, 1995) and caldendrin (Menger *et al.*, 1999). The function of many of these proteins, except the ubiquitous calmodulin, is the subject of speculation and, in certain cases, controversial.

In this report, we have focused our attention on the immunohistochemical localization of the ATP-dependent plasma membrane calcium pump (PMCA) and calbindin D_{28k} (CaBP) in the chicken retina. CaBP, originally identified in chick intestinal mucosa as a vitamin D-dependent protein (Wasserman *et al.*, 1966), was shown to bind Ca^{2+} in the micromolar range (Bredderman *et al.*, 1974), and was found to be present in a wide variety of tissues of various species (Taylor, 1974; Thomasset *et al.*, 1982; Wasserman, 1992 a) - including a population of peripheral and central nerve cells of birds and mammals, and in the retina of several vertebrate species (Pochet *et al.*, 1991). The ATP-dependent calcium pump, which extrudes calcium against a steep concentration gradient, is ubiquitously expressed in calcium transporting epithelia (Glendenning *et al.*, 2000 a). Its presence has also been shown in placenta (Borke *et al.*, 1989 a), mammalian choroid plexus (Borke *et al.*, 1989 b), avian and mammalian cerebella (Tolosa de Talamoni *et al.*, 1993), sheep brain (McDonald *et al.*, 1995), rat hippocampus and cortex (Filoteo *et al.*, 1997) and several cell lines (Glendenning *et al.*, 2000 a, b). It has also been demonstrated biochemically to be present in the retina (Puckett *et al.*, 1985). The immunolocalization of this protein in the human eye was reported by Johnson *et al.* (1995).

The aim of this study was to know the immunolocalization of PMCA in comparison with that of CaBP in chicken retina during embryonic development. In addition, the comparative study was also extended to retina of 4-week -old chicks.

MATERIAL AND METHODS

Four-week-old White Leghorn chickens were anesthetized with Nembutal (50mg/kg) and perfused through the cardiac ventricle with 60 ml of phosphate-buffered saline (0.01 M phosphate/0.15 M NaCl, pH 7.4; PBS), followed by 300 ml of fixative solution containing 4% (w/v) paraformaldehyde and 1% glutaraldehyde in PBS. The eyeballs were dissected out and placed in the same fixative for 24 h at 4° C and then immersed in 0.1 M sodium phosphate/0.15 M NaCl pH 7.4 with 0.03% sodium azide. Paraffin sections (4µm) were used for immunostaining. Embryonic eyeballs were directly placed in Carnoy´s fixative for several hours and processed to obtain 2-3 µm thick paraffin sections. The criteria defined by Hamburger and Hamilton (1951) were taken into account to determine the exact staging of the embryonic eye.

Antisera

The polyclonal anti-calbindin D_{28k} produced in the rabbits from chick intestine was a generous gift from Dr. Curtis Fullmer (Cornell University, Ithaca, NY). The anti-plasma membrane calcium pump monoclonal antibody (5F10) was produced against the purified human erythrocyte PMCA and was a gift from Dr. John Penniston (Mayo Clinic, Rochester, MN).

Immunoblotting

Fresh retinas free of pigment epithelium were carefully removed from the eyecups and homogenized in saline solution. The proteins were separated by SDS-PAGE employing 7% acrylamide and 0.1% SDS (Laemmli, 1970); protein standards of known molecular weight were run in the same gel (Sigma Co., St. Louis, MO). The separated proteins were transferred to nitrocellulose sheets (Towbin *et al.*, 1979). The blots were incubated

for 2-3 hs in Tris-buffered saline solution containing 0.02 M Tris and 0.5 M NaCl, pH 7.4 and mouse anti-calcium pump antibody (1:500 dilution) at room temperature. Peroxidase-conjugated goat anti-mouse immunoglobulin G (1:500 dilution) (Sigma Co., St. Louis, MO) was used as a second antibody. The peroxidase substrate for color development was 4-chloro-1-napthol incubated in the presence of hydrogen peroxide.

Immunohistochemical staining

PMCA and CaBP localization was accomplished by the indirect immunoperoxidase technique. Dewaxed rehydrated paraffin sections of eye were preincubated with 10% (vol/vol) normal goat serum for 15 min at room temperature. The post-hatched tissue was incubated with anti-PMCA at 1:1000 dilution or anti-CaBP at 1:500 dilution. Embryonic tissue was incubated with 1:100 dilution of anti-PMCA or 1:150 dilution of anti-CaBP. In each case, primary antibody was incubated 2 hours at 37°C. After rinsing, the solution containing the biotinylated second antibody was added to the tissue and, after a 15 min incubation period, the section was rinsed. After a 15 min incubation period with the streptavidin-peroxidase conjugate, the tissue was rinsed and color was developed by incubation with 0.05% diaminobenzidine solution and 0.01% H_2O_2 for 5-10 min. All incubations were carried out in a humid chamber at room temperature. Counterstaining was done by using pure hematoxylin. For the negative controls, accomplished in each studied time point, the primary antibody was substituted for nonimmune serum.

RESULTS

The immunoblot of reactivity of the 5F10 anti-PMCA antibody with chicken retina free of pigment epithelium is shown in Fig. 1. The average molecular size of the doublet band was estimated to be 148 kDa, within the range given in previous reports (Wasserman *et al.*, 1992 a).

Fig. 2a shows the immunolocalization of CaBP in the retina of the 4-week-old chicken and, at the same time, this figure was used to identify the different regions of the retina of the 4-week-old chicken. The anti-CaBP antibody heavily stained the photoreceptor cells; the outer limiting membrane; regions within the outer nuclear layer including the synaptic pedicles of the receptor cells, and the outer plexiform layer. A few cells intensely stained in the inner component of the inner nuclear layer, presumably amacrine cells (Schreiner *et al.*, 1985) and in the inner plexiform layer. Some ganglion cells and processes in the nerve fiber layer were also CaBP positive.

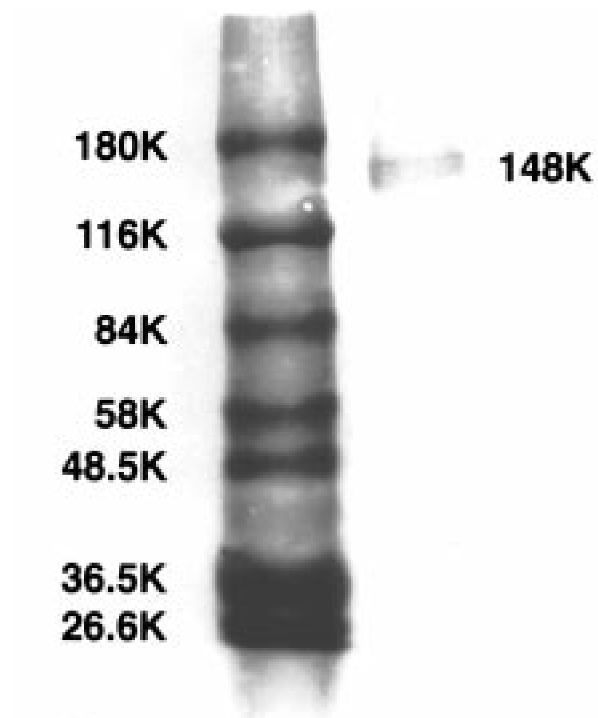

Fig. 1 - Western blot analysis of the reactivity of the monoclonal antibody 5F10 with the PMCA from chicken retina. Left lane: protein standards of known molecular weights. Right lane: neural retina homogenate proteins were separated by SDS/PAGE and transferred electrophoretically to a nitrocellulose membrane. The primary antibody was the 5F10 antibody against human erythrocyte PMCA and the second antibody was peroxidase-conjugated goat anti-mouse immunoglobulin G.

As can be seen in Fig. 2b, PMCA immunoreactivity distribution of the chicken retina of 4-week-old birds showed differences compared to that of CaBP. The primary localization was in the inner plexiform layer where several sublayers, two being more prominent, could be distinguished. The outer plexiform layer was also stained with 5F10 antibody. No staining was observed in the cell bodies located in the inner nuclear layer or the outer nuclear layer. The outer segment of the photoreceptor cells were minimally stained by the

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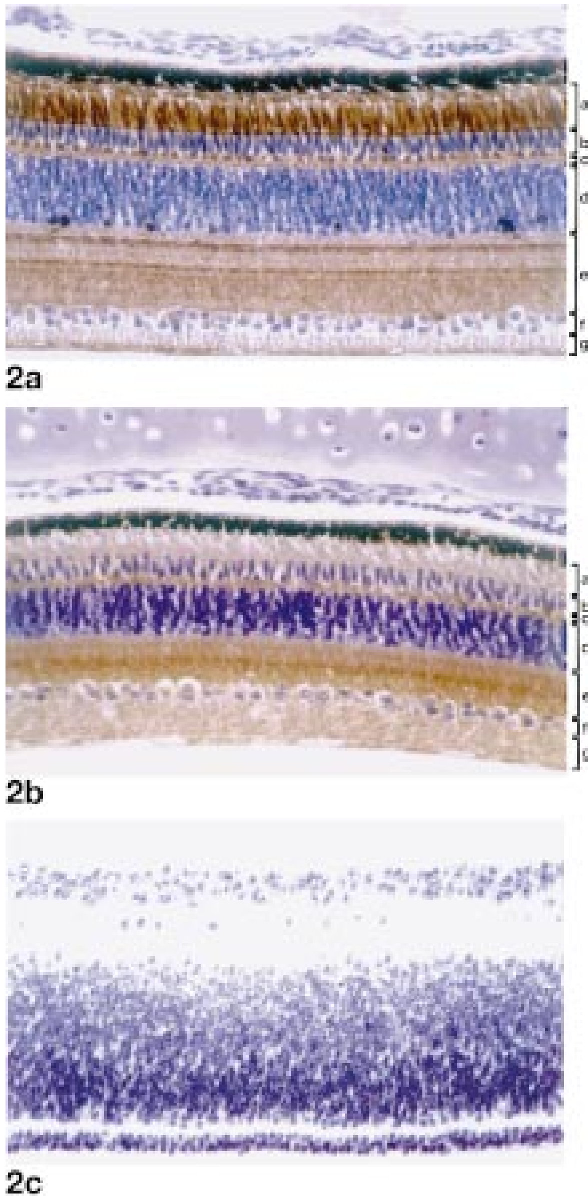


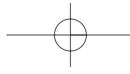
Fig. 2 - a. Localization of calbindin D_{28k} in a retinal section of 4 week old chicken detected by immunoperoxidase reaction. a: photoreceptor layer, b: outer nuclear layer, c: outer plexiform layer, d: inner nuclear layer, e: inner plexiform layer, f: ganglion cell layer, g: nerve fiber layer. **b.** Localization of PMCA in a retinal section of 4-week-old chicken detected by immunoperoxidase reaction. **c.** Negative control of a 4-week-old chicken retinal section treated with non-specific rabbit serum.

5F10 antibody. Some cells from the ganglion layer were labeled with the antibody against PMCA and the nerve fibers were clearly positive. The optic nerve formed from the axons of the ganglion cells had also positive staining for PMCA (not shown). Fig. 2c shows a negative control of a retinal section after reacting with non-specific rabbit serum. Similar negative controls were obtained at each stage of eye development.

The comparative distribution of PMCA and CaBP in the retina of the chicken during embryonic development can be seen in Fig. 3. At age 4.5 days, cells in the retina were densely packed but were not yet segregated into distinct layers. The retina was faintly immunoreactive to PMCA and CaBP. As described by Barnstable (1987), cell differentiation is initiated from the central retina towards the periphery. At the stage of 10 days, there was a clear difference between PMCA and CaBP staining. In the central retina, the inner plexiform layer showed PMCA and CaBP staining. Heavy CaBP staining was also present in some amacrine cells of the inner nuclear layer. The growth of the processes increased in the following days giving more prominence to the inner and outer plexiform layers. PMCA immunoreactivity was also increasing in those layers during development of the chicken retina. Some cells from the ganglion layer also showed PMCA staining. At 18.5 days of development, the PMCA distribution was quite similar to those at one-day post-hatching and in 4 week-old chicks. During the last days of embryonic development, CaBP immunoreactivity of the photoreceptor cells was increasing. The number of CaBP-stained amacrine cells declined after hatching (Fig. 2a), and the CaBP immunoreactivity of the cell bodies from the outer component of the inner nuclear layer and the inner segments of photoreceptor, which was clearly visible at the hatching day, was not discernable at 4 weeks post-hatching.

DISCUSSION

The primary localization of PMCA in four-week-old chicken retina is the inner plexiform layer, where a large number of neuronal connections exist, and in the outer plexiform layer formed by synapses of the photoreceptor cells and processes of the horizontal cells. PMCA appears to be absent in the inner nuclear layer and only stains faintly positive in the photoreceptor cells. This distribution seems to be different from that of CaBP. Although CaBP immunoreactivity is also present



Embryonic Age (days) | Calbindin | Calcium Pump

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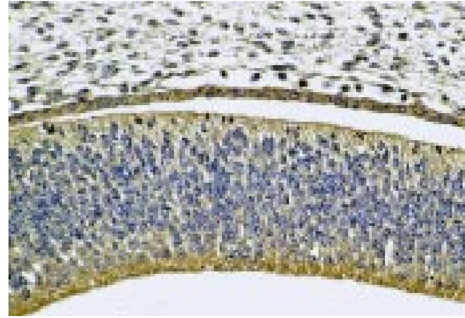

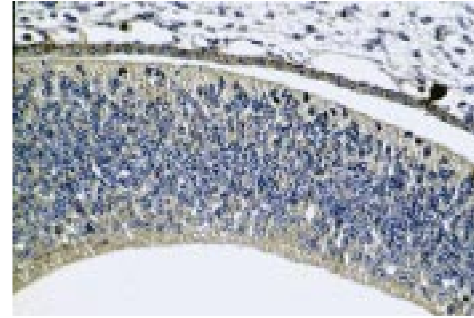

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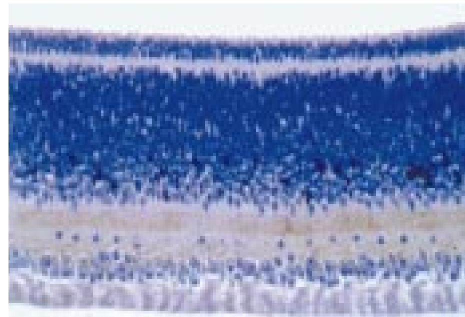

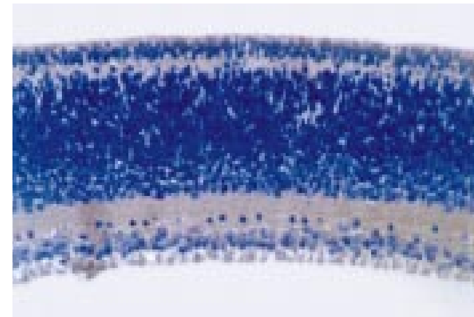

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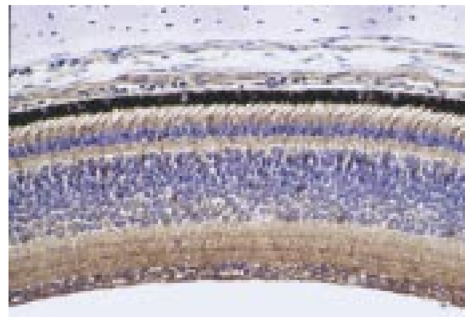

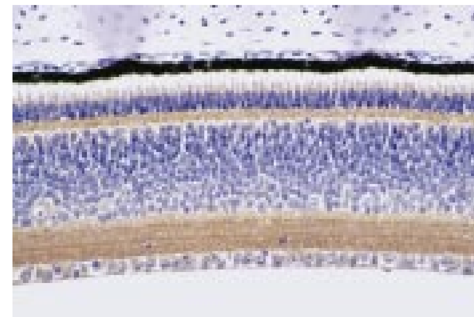

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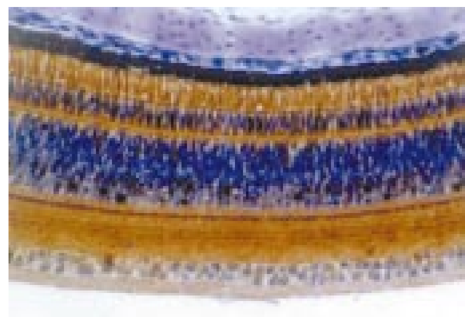


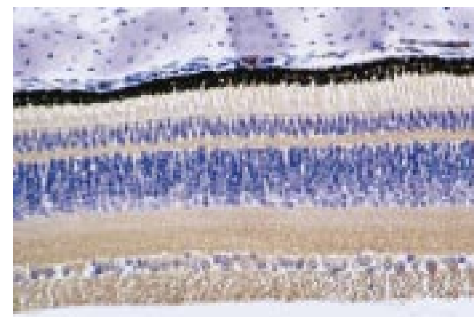
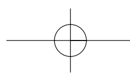


Fig. 3 - Localization of calbindin and calcium pump in chicken retina during the embryonic development.



in the inner plexiform layer, the highest CaBP concentration is located on the outer segment of the cones and the amacrine cells of the inner nuclear layer. As pointed out (Hamano *et al.* 1990), the elements directly related to phototransmission are labeled with CaBP: the cones, cone pedicles and ganglion cells. Our data on CaBP localization in chicken retina is quite similar to that found by Hamano *et al.* (1990) and Ellis *et al.* (1991). In contrast, Pasteels *et al.* (1987) did not observe the presence of CaBP in the nerve fiber layer of the pigeon retina, nor did Schreiner *et al.* (1985) in chicken retina.

The prominent appearance of PMCA seems to coincide with the establishment of synapses, mainly in the inner plexiform layer. The intensity of labeling with 5F10 antibody also increases with the growth of the processes in the inner plexiform layer and in the outer plexiform layer. No further modifications in the PMCA expressions were observed in the retina of birds beyond hatching.

CaBP immunoreactivity is clearly different from that of PMCA at the tenth day of embryonic development. Within this period of development, more amacrine cells are reactive with the CaBP antibody than at the 4 week-old post- hatching age. This might occur because of some special function of CaBP in the development of amacrine cells that is not required in mature cells (Ellis *et al.* 1991).

Immunoreactivity to CaBP antibody is not only present in some neurons from retina but also in the visual pathway from retina to the thalamus and then to the visual cortex. Recently, we have shown that either monocular enucleation or retinal lesions in 4-week-old chicks produce reduction of CaBP immunoreactivity in the contralateral tectum opticum and in the nucleus rotundus (Díaz de Barboza *et al.* 2000).

The functions of the calbindins in biological systems have yet to be conclusively defined. In transcellular transport, as occurs in renal tubules and intestinal mucosa, calbindin has been proposed to facilitate the movement of calcium from the apical to basal region of the transporting cell (Wasserman *et al.* 1992 a). Calbindin could certainly serve as a calcium buffer since its binding affinity (about 2×10^6 M^{-1}) is suitable for this role. Some data obtained either in neurons from the hippocampus (Chard *et al.* 1995) or in pig duodenal enterocytes (Schröeder *et al.* 1996) supports Ca^{2+} buffering as one of the significant functions of CaBP. These functions of CaBP are particularly suitable for cells, in which there is a rapid intracellular movement and turnover of Ca^{2+}, such as the cone photoreceptor in the “dark state”. The epithelial hypothesis of CaBP function would theorize that this high affinity calcium-binding protein facilitates the transfer of Ca^{2+} from the cGMP-gated cation channel to the vicinity of the $Na^+/(Ca^{2+} + K^+)$ exchanger and, at the same time, maintains intra-conal Ca^{2+} at non-toxic concentrations. In calbindin-null mutant mouse, calbindin immunoreactivity was completely absent but those cells that express the protein in wildtype retinas, such as horizontal cells, were still present and appeared normal, an indication that CaBP is not required for the maintenance of retinal structure (Wäsle *et al.*, 1998). The possibility that CaBP has a more specific function in developmental processes has already been suggested.

The ATP-dependent membrane calcium pump, in one or more of its several forms, is a common feature of cells in general, extruding Ca^{2+} from cell to extracellular space to offset the possibility of calcium-mediated toxicity and to reset the basal calcium level for agonist action dependent on rises in intracellular calcium. In epithelia, as it occurs in kidney and intestine, the calcium pump is heavily concentrated on the basolateral membrane, giving parenteral directionality to calcium movement. The role of PMCA in the retina is most likely similar, to maintain intracellular Ca^{2+} at preexcitation basal concentrations.

The absence or minimal level of PMCA in cones of the post-hatched chicks, as noted herein and by Johnson *et al.* (1995), is particularly intriguing and perhaps understandable in terms of the origin of the dark current. This current is dependent on calcium cycling from extracellular fluid through the cGMP-gated channel into the cone outer segment proper and extruded by way of the $Na^+/(Ca^{2+} + K^+)$ exchanger. The presence of another Ca^{2+} extruding mechanism, such as PMCA, would add another feature that needs to be controlled and would further complicate the origin and function of the dark current.

Vitamin D, in its hormonal form as 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), is an important factor controlling the synthesis of calbindin and the plasma membrane calcium pump in intestinal cells (Wasserman 1992 a, b, Pannabecker *et al.* 1995, 1996) and distal kidney tubular cells

(Glendenning *et al.* 2000 a). Schreiner *et al.* (1985) had observed that the presence of CaBP on horizontal cells of chicken retina is dependent on nutritional vitamin D status. Overall, immunostaining of CaBP in chicks fed a rachitogenic diet was lower than that of chicks of the same age fed a normal diet. The dependency of retinal PMCA synthesis on nutritional vitamin D has not been shown. It should be noted that the vitamin D receptor has been shown to be present in a number of cells in the human retina, including the inner and outer segments of photoreceptor cells and some cells in other regions of the retina (Johnson *et al.*, 1995).

In conclusion, our data show that the distribution of PMCA is different from that of CaBP in chicken retina, not only in mature chicks but also along the different stages of development. The early appearance of both proteins in the embryonic period is unknown, but it could occur to protect cells from calcium overloading, which can cause neurotoxicity.

ACKNOWLEDGMENTS

This work was supported by NIH Grant DK-04652 (Dr. Wasserman), FONCYT (Dr. Tolosa de Talamoni) and SECYT, U.N.C. (Dr. Tolosa de Talamoni). Dr. Nori Tolosa de Talamoni is a Career Investigator of the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina.

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