Localization of Iodopsin in the Chick Retina During In Vivo and In Vitro Cone Differentiation

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Using highly specific antibodies against a chick red-sensitive cone pigment, iodopsin, we investigated the localization of iodopsin in the developing and mature chick retina. The chick retina contains several different photoreceptor types, including a rod, a double cone with a principal and accessory cone, and four different types of single cones. Immunocytochemical observations revealed that outer segments (OS) of one of the single cones (type I) and both cells of the double cone were strongly immunoreactive to anti-iodopsin antibodies. The Golgi regions and small vesicular structures in the inner segments (IS) of these cells also were intensely stained, indicating a continuous synthesis of iodopsin and its addition to the newly formed cone OS. In the differentiating cones of the developing but immature chick retina, iodopsin immunoreactivity was found at the plasma membranes of both the IS and the terminals (pedicles). This suggests that unidirectional transport of iodopsin to the outer segment may be established during cone differentiation. Immunostaining in the outer plexiform layer (OPL) produced two bands, suggesting that the pedicles of the double cones and type I single cones terminate at different positions in this layer. Application of the antibodies to a cell culture system of the chick retina revealed that cells immunoreactive to anti-iodopsin differ slightly in morphology from those reactive with anti-rhodopsin. Since antibodies to iodopsin and rhodopsin stained different types of photoreceptors in the intact chick retina, it will be possible to analyze cell lineage of rods and cones in vitro by use of these antibodies.


The visual pigments located in the outer segments (OS) of retinal photoreceptors are the photosensors for phototransduction. A variety of different pigments are known, each of which is specified by its wavelength of maximal absorption1,2 and is localized in a particular type of photoreceptor. Photoreceptors show a remarkable diversity of morphology and distribution in vertebrate retinas. In the chick retina, morphologic studies have revealed several forms of photoreceptors3,4—a rod, a double cone comprising a principal and an accessory cone, and single cones. Single cones can be classified further into four different types by the colors of their oil droplets.5

Rhodopsin is specifically localized in the rod outer segment. Cone visual pigments of the chick retina, on the other hand, have not yet been localized. Among them, iodopsin, the red-sensitive cone pigment, is found exclusively in the chick retina and has been widely investigated spectroscopically and biochemically.6-10 Recently, we have obtained four kinds of monoclonal antibodies against R-phptopsin, the protein moiety of chicken iodopsin.11 All of the antibodies bound directly to iodopsin, but neither to other chicken cone visual pigments nor to rhodopsin, as analyzed by immunoblots and immunoprecipitation methods. Preliminary immunocytochemical experiments revealed that these antibodies reacted with only some of the cone OS.11 Thus, it is essential to determine precisely the cell types of cones that contain iodopsin.

In the current study, using an electron microscopic (EM) immunocytochemical method, we examined the localization of iodopsin in the chick retina. We also studied developmental changes in iodopsin localization during cone differentiation to obtain information on how iodopsin molecules are integrated into the OS. Since cell cultures are useful for analysis of the regulatory mechanism involved in the divergent differentiation of photoreceptors,12,13 the antibodies were further applied to the in vitro system to identify differentiating cone cells. This will enable us to investigate the lineage analysis of chick photoreceptor cells in further studies.

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Materials and Methods

In Situ Preparation

Chick embryos at 13, 14, 15, 17, and 19 days of incubation and chicks at 1, 3, and 13 days after hatching were studied, and were used according to the ARVO Resolution on the Use of Animals in Research. (Fertilized eggs of the chick were purchased from Ishii Poultry Co. Ltd., Tochigi, Japan.) Chicks were maintained under cycled light (7 AM to 7 PM) and were killed at about 1 PM. Eyeballs were enucleated in room light immediately after decapitation and dissected into anterior and posterior halves with a razor blade. The posterior halves were briefly washed with Hank's saline and fixed for 30 min in an ice-chilled mixture of 2% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.3), followed by a postfixation for 10 hr in a second fixative consisting of 2% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.3) at 4°C. For light microscopy, the fixed tissues were washed with phosphate buffered saline (PBS: 50 mM sodium phosphate, 150 mM NaCl; pH 7.3) at 4°C and then sectioned at 20-μm thickness with a freezing microtome. For EM, the fixed tissues were washed with PBS, cut into 50-μm sections with a Microslicer (Dosaka EM, Japan) without freezing the tissue.

In some cases, retinas were processed for EM immunocytochemistry without sectioning. For this purpose fresh chick retinas were separated from the pigment epithelium to enhance the antibody binding to the exposed OS, and were treated with the same series of fixatives. After washing the tissues with PBS, they were cut into small pieces with a razor blade and processed for immunohistochemical staining by the same procedure.

The staining procedure for iodopsin was carried out with the same method as described previously. Briefly, the sections or the tissues were incubated in turn with 2% fetal calf serum for 30 min at room temperature, mouse monoclonal anti-iodopsin IgG (0.2 μg/ml diluted with PBS containing 0.2% Triton X-100) for 48 hr at 4°C. They were further processed with avidin-biotin-peroxidase system (Vectorstain ABC kit; Vector Laboratories, Inc., Burlingame, CA). After each incubation, the sections were thoroughly washed with PBS containing 0.2% Triton X-100. The immunoreactive sites were finally visualized by use of hydrogen peroxide and diaminobenzidine as substrates of the peroxidase. Characterization of the two monoclonal antibodies (designated as R2 and R3) were fully described in a previous paper.

For a control staining, nonimmununized mouse IgG was substituted for anti-iodopsin.

In Vitro Preparation

Chick embryos at 6–6.5 days of incubation were used for cell culture. Methods for the culture have been described in a previous paper. Briefly, isolated neural retinas were dissociated with 0.1 M EDTA in Ca2+ - and Mg2+-free Hanks' saline for 20 min at room temperature, followed by incubation in 0.2% trypsin (1:250; Difco, Detroit, MI) for 20 min at 37°C. About 5 × 10^6 cells were inoculated into a culture dish of 3 cm diameter precoated with collagen (Nitta Gelatin, Osaka, Japan). The medium was Eagle's minimum Essential medium supplemented with 5% fetal calf serum (Flow, Irvine, Scotland), 1% heat-inactivated chicken serum (Flow), 0.6% glucose, and 0.5 mM sodium pyruvate.

For immunohistochemistry, cultured cells were fixed with the same fixatives used for the chick retina. The fixation time was 10 min for the first fixative and 10 hr for the second. After washing, cultured cells were processed by the procedure described above.

Results

Exactly the same results were obtained in the separate immunohistochemical stainings with the two antibodies. No significant staining was observed in the control experiments.

Localization of Iodopsin in the Mature Chick Retina

In a fully differentiated chick retina, the antibodies stained cone OS of several cell types (Fig. 1A, C; see also Fig. 2E). These included the two members of double cones and some single cones. The lateral plasma membranes of the inner segments (IS) and other parts of the cell membranes were devoid of immunoreactivity.

The immunoreactive single cones were found to be of one particular type (Fig. 1A). They always possessed the same type of oil droplet, which contained homogeneous, pale material when observed with the ordinary EM staining procedure of lead citrate and uranyl acetate. These oil droplets were larger in size and often located at a more scleral position than those of neighboring nonimmunoreactive single cones. These observations indicate that the type 1 single cone positively reacted to anti-iodopsin.

In the cytoplasm, intensely immunoreactive material was accumulated in the Golgi region (Fig. 1B). Small dotlike structures near the Golgi region and within the paraboloid also were stained. Some of them were associated with small vesicles.

Localization of Iodopsin in the Developing Retina

During the cone differentiation, iodopsin did not necessarily show typical polarized distribution in
Fig. 1. Electron micrographs of iodopsin localization in the 13-day chick retina. (A, C) Some of cone cells have immunocytochemically stained outer segments. These include type 1 single cone (SI) and both principal (Pr) and accessory (Ac) cones. Other unidentified types of single cone (type 2, 3, or 4) are negative for iodopsin. Immunostaining was carried out with a whole retina preparation after removing pigment epithelium and without cutting sections. (B) In the inner segments of double cones the Golgi region is intensely stained (arrowheads). Tiny dotlike stainings (arrows) are scattered in the cytoplasm and the paraboloid (P) of the accessory cone. Müller cells (M) extend microvilli at the outer limiting membrane. (A X9000, B X8000, C X9200; bars = 1 μm.)
cones, as is found in the mature retina. No immunoreactivity was found in the cones until the day 13 of incubation. On day 14 (Fig. 2A), a short, thin process located at the scleral side of the growing IS was positively stained. At this stage, the plasma membrane of the IS also was immunoreactive, and very faint immunoreactivity was seen in the OPL. Other parts of the retina were completely free of immunoreactivity. On day 15 (Fig. 2B) immunoreactivity was very prominent in the OS, IS, and OPL. In the outer nuclear layer (ONL), the plasma membranes were only weakly stained. On day 17 (Fig. 2C) the localization of immunoreactive materials was similar to that on day 15, except that the OPL and IS were much less intensely stained than at the previous stage. A dot-like structure located at the supranuclear region was stained. On day 19 (Fig. 2D) the staining pattern was very similar to that of day 17. Occasionally, type 1 single cones displayed immunoreactivity throughout, producing a Golgi-like image (Fig. 3). Their pedicles extended laterally in the vitreal part of the OPL. Pedicles of the double cones seemed to be located in the scleral part of the OPL (Fig. 2D), terminating in a different layer of the OPL from that of type 1 single cones.

EM observations of Day 15 retinas revealed that the plasma membranes of the IS were stained intensely (Fig. 4A). Immunoreactivity on the surface of

![Fig. 2. Iodopsin immunohistochemistry of the developing chick retina. (A) Embryonic day 14, (B) day 15, (C) day 17, (D) day 19, and (E) 2 days after hatching. Arrows indicate stained outer segments and arrowheads Golgi regions. Bars and arrowheads below the bars indicate outer limiting membrane and outer plexiform layer, respectively. (A) The outer segment starts differentiation on day 14 at the distal tip of the inner segment. The pigment epithelium has separated from the neural retina because of their poor interaction at this stage, and is not seen in this figure. (B, C) The inner segments and outer plexiform layer are intensely stained on day 15, but are only faintly stained on day 17, when the outer segments grow rapidly. (D) When the retina matures, only outer segments and the Golgi region remain intensely stained. In the outer plexiform layer faint staining is found in two separate bands (small arrows). (E) In the mature retina the outer segments of particular cones show strong immunoreactivity. The Golgi regions are still immunoreactive. PE, pigment epithelium; OS, outer segment; IS, inner segment. (×1100; bar = 10 μm).](http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933156/)
Fig. 3. Two examples of iodopsin immunoreactive type 1 single cones in embryonic day-19 retina. Each shows a whole image of the cell, including the pedicle. The pedicles lie in the vitreous side of the outer plexiform layer. Bars indicate the approximate position of the outer limiting membrane (×1100; bar = 10 μm).

In Vitro Differentiation of Cone Cells

In the monolayer culture of dissociated retinal cells, numerous cells grown on the glial cell sheet were positively stained (Fig. 5). Morphologically, two types of cells immunoreactive to anti-iodopsin were found, one of which was round in shape and the other elongated. The round cells (Fig. 5A) showed a typical neuronal morphology. The elongated cells (Fig. 5B) were polarized and monopolar with a long process at one end. Both of these cells had one or two long neuritic processes which arborized as many short processes. Immunoreactivity was found on the plasma membranes of the cells and processes. Immunoreactive deposits were often found within the cell bodies. Thick and short stout processes which were often found in cells immunostained with anti-rhodopsin were rarely observed in cells immunoreactive to anti-iodopsin.

Discussion

The present paper is the first description of iodopsin localization in photoreceptors during maturation of the chick retina. Monoclonal antibodies to iodopsin used here have been well characterized in a previous report. The antibodies recognize the protein moiety of only the chicken red-sensitive cone visual pigment (iodopsin), R-photopsin, which has an apparent molecular weight of 34,000. Immunoprecipitation-spectrophotometric analysis indicates that the antibodies are highly specific for R-photopsin.

Localization of Iodopsin in the Chick Retina

Morphological studies have shown that chick retinas contain a rod and several different types of cones with one double cone and at least three types of single cones. Since we identified four kinds of chicken cone visual pigments by chromatographic procedures and spectrophotometric measurements of CHAPS-phosphatidylcholine extract, it is important to know the localization of these cone pigments in the chick retina for understanding the primary process of color vision. The present immunocytochemistry clearly demonstrates that iodopsin is localized in the OS of both the double cone and one type of single cone. This cone is identified as type I single cone by the size, position and morphology of its oil droplet. The oil droplet is considered to have red color in light microscopic preparations. Unlike frog and tadpole, both members of the double cone, one principal and one accessory cone, have the same visual pigment. These two cones may be electrically coupled by gap junctions in the chick retina. In the turtle retina the two members of the double cone have the similar electrophysiologic properties. Since the colors of oil droplets of the principal and accessory cones are slightly different in the chick retina, the two elements may serve as a supplementary component to control chromatic sensitivity.
Fig. 4. Electron micrographs of iodopsin localization in the embryonic day-15 retina. (A) The plasma membrane of the inner segment is heavily stained (arrowheads). The Golgi region (G) of the double cone is also immunoreactive. (B) Immunoreactive materials are found on the surface of short processes extending from the cone pedicles (arrows). Synaptic ribbons are indicated by arrowheads. (A \( \times 11,000 \), B \( \times 16,200 \); bars = 1 \( \mu m \)).

Szél et al. (1986)\(^{21} \) have reported that a monoclonal antibody reacts with the same types of cones as shown in the present study. Their antibody recognizes a single molecule with a molecular weight of 33,000, and cross-reacts with a protein in frog, turtle, gecko and mammalian retinas. Thus the epitopes of their antibody and ours appear to be different from each other, because ours do not react with gecko\(^{11} \) and monkey.

Fig. 5. Immunohistochemistry of chick retinal cells cultured for 10 days. Positively stained cells are found as round neuronal cells with varicose neuritic processes (arrow) (A) or as elongated monopolar cells (B). (A, B \( \times 820 \); bar = 10 \( \mu m \)).
retinas (data not shown). Our antibodies are highly specific for chicken iodopsin.

The Golgi regions in the 13-day retina after hatching were intensely stained with anti-iodopsin, suggesting active synthesis of iodopsin in the mature retina. This implies that the OS of the cones are actively renewed as is observed in rods.22 Our observation that small vesicular structures with immunoreactivity were seen in the vicinity of the Golgi regions suggest that iodopsin may be transported in the vesicular structures to the OS through the cytoplasm, as postulated for rods.23

Our previous study revealed that a polyclonal antirhodopsin reacts with the type 2 and type 3 single cones in addition to the rods.14 Similar results were also obtained with polyclonal anti-rhodopsin antibodies raised in different laboratories.24,25 It seems that some of cone visual pigments other than iodopsin have several epitopes in common with rhodopsin.

Localization of Iodopsin During Cone Differentiation

In the developing chick retina, several structures other than the OS were immunoreactive, such as the IS and nerve terminals both of which lost immunoreactivity when the differentiation terminated. In the early stage of cone differentiation iodopsin did not seem to be transported only to the OS region but rather appeared to be randomly incorporated into the growing membranes along both sides of the connecting cilium and pedicles. The formation of the OS begin at around Day 15 and they then grow actively.12 Some mechanism of incorporation of iodopsin into the membrane must be necessary at the base of the OS. Since the IS and OPL were much less reactive on embryonic Day 17, a unidirectional transport mechanism of iodopsin may have been established by that time.

On Day 19 (Fig. 3) few of the type 1 single cones displayed the whole cell contour, and it was shown that their pedicles (nerve endings) terminated in the vitreal side of the OPL, different from that of the double cones. Such staining can give precise information about the terminal configuration and the synaptic formation with the secondary neurons (bipolar and horizontal cells), when analyzed by EM.

Localization of Iodopsin in the Culture

Previous studies have shown that photoreceptors of the developing chick retina undergo cell differentiation in culture, including development of several characteristic structures and opsinline immunoreactivity.12,26 In those studies, cells immunoreactive to anti-rhodopsin have a morphology similar to that of the immunostained cells differentiating in vitro in the current study, but still differ in several morphologic respects. Cells immunoreactive to anti-rhodopsin usually are elongated or ovoid in shape and often display a thick, stout process. They extend quite short neuritic processes.12 On the other hand, cells immunoreactive with anti-iodopsin seldom have a thick process, but extend prominent neuritic processes. These differences may indicate a morphologic difference between rods and cones in vitro. Anti-rhodopsin and anti-iodopsin are good markers for in vitro photoreceptor differentiation, because they stain different types of photoreceptors in the intact chick retina. This will make it possible to investigate the control mechanism of the wide diversity of photoreceptor differentiation and the receptor mosaic formation.

Immunoreactive cells to anti-iodopsin could be classified into two types: one had a round soma with a neuronal appearance, and the other a polarized shape. Since each type did not necessarily tend to be juxtaposed with a cell of the same type, it was difficult to decide which type of cell corresponded to the double cone of the chick retina. Lack of juxtaposition with a cell of the same type may also indicate that each member of the double cone was postmitotic when the retina was transferred to the culture (at 6–6.5 days of incubation) provided that they derived from the same precursor cell. In our future studies it will be possible to identify the cell type in cultures by using retinas of earlier stages as culture materials.

In the culture conditions used in the current study, polarized distribution of iodopsin, as is seen in the mature retina, was not observed. Rods of the newborn rat also do not acquire cellular polarity under similar culture conditions.27 Since lack of polarity in iodopsin distribution was seen in vivo in the early stage of cone differentiation (before day 17), our in vivo and in vitro observations may indicate that the current culture conditions promote photoreceptor differentiation but are not sufficient for its maturation (polarized distribution of a protein). It is essential to investigate which components of the microenvironment (including cellular interactions) are maturation factors in the developing retina, and the current culture system will afford a good opportunity for this purpose.

Key words: chick retina, cone cell, iodopsin, development, cell culture

References
