Expression of Soluble Phototransduction-Associated Proteins in Ground Squirrel Retina

Malcolm von Schantz,*% Agoston Szél,*† Theo van Veen,* and Debora B. Farber§

Purpose. This study describes the expression and distribution of arrestin, phosducin, and recoverin in the cone-dominant retina of the ground squirrel *Spermophilus tridecemlineatus*.

Methods. mRNA expression was studied by blot hybridization of ground squirrel retinal RNA, with human and murine RNA as controls. The distribution of the gene products in the ground squirrel retina was investigated by immunocytochemistry using radial and consecutive tangential sections.

Results. Northern blot hybridization showed messages for arrestin (1.9 kb), phosducin (1.4 kb), and recoverin (1.2 kb) in ground squirrel retinal RNA. Both controls showed transcripts of the same or similar sizes. Rod-like cells and blue cones were stained by antibodies against arrestin and phosducin. The arrestin antiserum stained the whole cell bodies, most intensely in the myoid region, whereas phosducin immunoreactivity was confined to the outer and inner segments, which were stained with approximately equal intensity. The strongest immunoreaction was found in the photoreceptor plasma membrane. Recoverin antibodies recognized the entire soma of all photoreceptor cells. The myoid region and the synaptic pedicles were most heavily stained. No light-dependent migration was observed with either antiserum in any photoreceptor type.

Conclusion. The presence of arrestin immunoreactivity in rod-like cells and blue cones is consistent with previous reports on other mammals. However, it has not been reported previously that phosducin immunoreactivity is distributed in the same way. The colocalization of arrestin and phosducin in rod-like cells and blue cones is yet another trait distinguishing blue cones from red and green cones.

The retina of the 13-lined ground squirrel *Spermophilus tridecemlineatus* contains approximately 90.6% green cones, 6.1% rod-like cells, and 3.1% blue cones. In contrast to the rod-like cells found in the European ground squirrel *Spermophilus citellus*, those in *S. tridecemlineatus* appear to be a homogeneous group with respect to structural traits and biochemical markers. The photopigment in the rod-like cells of *S. tridecemlineatus* consistently cross-reacts with an antibody against blue cone pigment. We have previously studied the expression and distribution of the membrane-associated components of the phototransduction cascade in the ground squirrel retina. Other investigators have taken advantage of the dominance of cones in the ground squirrel retina to prove that interphotoreceptor retinoid-binding protein (IRBP) and peripherin/rds are expressed in cones as well as in rods.

The traditional subdivision of retinal photoreceptors into rods and cones was originally based on differences in general structure and function. It was later found that rod outer segment disks are separate membrane enclosures within the cell membrane, whereas cone outer segment disks are invaginations of the cell membrane. Moreover, cones are equipped with their own phototransduction cascade, which consists of proteins similar to but distinct from those found in rods.
There are, however, several traits that distinguish cones sensitive to short wavelengths (i.e., blue-sensitive cones) from those sensitive to long or medium wavelengths (i.e., red-sensitive or green-sensitive cones). The most prominent of these is the greater diameter of blue cones. Biochemical distinctions include differences in fucose incorporation and in staining by procion yellow and by antibodies against carbonic anhydrase. In the ground squirrel retina, we have also reported a difference in peanut agglutinin binding to the interphotoreceptor matrices of blue and green cones.

Arrestin, phosducin, and recoverin are all soluble proteins that are expressed in relatively high amounts in the mammalian retina. Initially discovered and described in the retina, where they are thought to interact with the proteins of the phototransduction cascade, they were all subsequently found to be expressed in other tissues as well. The three proteins are also known to be members of larger families.

Arrestin (S-Antigen, 48-kd protein) is a strongly immunogenic, soluble retinal protein that takes part in the quenching of the light signal by binding to phosphorylated rhodopsin, thereby preventing its interaction with α-transducin. Arrestin immunoreactivity is almost ubiquitous in photoreceptors throughout the animal kingdom. The mammalian pineal organ contains a form of arrestin that is encoded by an mRNA identical to that of retinal arrestin. The arrestin promoter also directs expression into the lens and the brain. Arrestin is now known to be part of a family of at least four homologous proteins.

Phosducin (MEKA, 33-kd phosphoprotein) is another abundant soluble retinal protein that can form a heterotrimeric complex with the βγ-transducin heterodimer. Similar to arrestin, it is present in the pineal organ. It appears to be widely expressed among vertebrates, including teleosts. A recent study has described the inhibition of the interaction between the β-adrenergic receptor and its G-protein by a 33-kd protein. This protein was found to be similar or identical to the retinal phosducin. There are no data available to indicate whether a similar mechanism involving phosducin is at work in the retina. However, phosducin seems to interact with the proteins of the phototransduction cascade competing with α-transducin for binding to the βγ-transducin subunits. In another study, a molecule that has sequence similarities with phosducin has been demonstrated in a wide variety of tissues.

Recoverin is a 26-kd calcium-binding protein initially observed in the bovine retina and presumed to promote a recovery of the photoreceptor cGMP concentration after illumination by acting on guanylate cyclase. Recent data, however, demonstrate that recoverin does not activate guanylate cyclase, as previously thought. Another recent report shows that in the frog retina, the regulator protein S-modulin prolongs the lifetime of activated phosphodiesterase at high calcium ion concentrations by inhibiting rhodopsin phosphorylation. Recoverin is thought to be a mammalian homologue of S-modulin, and it may therefore play the same role. Recoverin immunoreactivity has also been found in cone bipolar neurons and in the pineal organ. Again, several similar molecules have been reported to be present in the nervous system.

In this investigation, we have studied the expression of arrestin, phosducin, and recoverin in the ground squirrel retina using RNA blot hybridization and immunocytochemistry. The immunocytochemical experiments were performed using radial sections or consecutive tangential sections. In the latter, we used a battery of photopigment antibodies to discriminate among the three types of photoreceptors, as described previously. Briefly, green cones are identified by the antibody COS-1. Both blue cones and rod-like cells are recognized by the blue-cone pigment antibody OS-2 and are distinguished by their shapes and by the fact that only rod-like cells are labeled by the opsin antiserum AO. Part of this investigation has previously been published in abstract form.

MATERIALS AND METHODS

Animals and Tissue Preparation

All animals were maintained and killed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Wild ground squirrels (S. tridecemlineatus) were trapped by commercial vendors in Illinois and Wisconsin, shipped to our laboratory, and maintained on a grain–nut diet under a 12-hour light/12-hour dark cycle. Animals were killed and the retinas prepared for RNA analysis or immunocytochemistry as described previously. Light-adapted animals were killed at 9 AM; dark-adapted animals were killed and dissected under infrared light after 3 hours of darkness.

Human eyes were enucleated 2 to 12 hours after death and kept at −80°C until thawed and dissected for RNA extraction. The tenets of the Declaration of Helsinki were followed, with informed consent and approval by the institutional human experimentation committee obtained.

RNA Analysis

RNA was purified as described previously. Poly-A RNA was obtained using the Mini-Oligo(dT) Cellulose Spin Column kit (5 Prime → 3 Prime, Boulder, CO). Total RNA (10 μg) or poly-A RNA (1 to 2 μg) was separated in a 1.2% agarose–formaldehyde gel. RNA
was blotted overnight onto Hybond N+ membranes (Amersham, Little Chalfont, Bucks, UK) in 20 × SSC. Cross-linking of RNA to the membrane and stripping of the probe between hybridizations were performed according to the manufacturer's instructions. The following cDNA clones were used for hybridization: pHL1301, a 1.2-kb Eco RI fragment encoding human α-tubulin; BSC70, a 1.6-kb Eco RI fragment encoding bovine arrestin; zr.414, a 1.1-kb Eco RI fragment encoding murine phosducin (Bowes-Rickman C, Rapoport A, Farber DB, unpublished data, 1994); and RecV, a 0.5-kb Pst I-Eco RI fragment encoding bovine recoverin. DNA (50 ng) was labeled using the Klenow fragment of DNA polymerase (Promega, Madison, WI) and α-32P-dCTP (NEN Research Products, Boston, MA). Hybridization was carried out overnight in a hybridization oven (Bellco Biotechnology, Vineland, NJ) at 65°C in a phosphate-buffered 1% BSA solution containing 7% SDS. Washing and visualization were performed as described previously. Each blot was initially hybridized with the tubulin probe to ensure that equal amounts of intact RNA were present in all lanes.

**Immunocytochemistry**

Enucleated eyecups were immediately immersed in 4% paraformaldehyde and 0.1% glutaraldehyde dissolved in 0.1 M Sorenson phosphate buffer (pH 7.2) for 4 hours. The material was embedded either in araldite (Durcupan ACM; Fluka, Buchs, Switzerland), or in diethylene glycol distearate wax (DGD; Polysciences, Warrington, PA). The araldite and the DGD blocks were sectioned on an ultramicrotome (1.5 μm) with glass knives. Serial tangential and radial sections were obtained. Immunoreaction, visualization with the avidin–biotin–peroxidase system (Vectastain; Vector Laboratories, Burlingame, CA), and mounting of slides were performed as described previously. The antibodies used are summarized in Table 1. The monospecificity of all antibodies had been checked by Western blotting and by blocking with competing antigen (see references in Table 1). All antibodies have been found to perform consistently in a wide variety of mammalian species. The results of the immunocytochemistry were visualized with an Axiophot photomicroscope (Zeiss, Oberkochen, Germany) using Nomarski optics. For demonstration of colocalization of photoreceptor-specific proteins, corresponding areas of adjacent tangential sections were photographed.

The embedding techniques were selected according to the specific properties of the primary antibodies. Although the antibodies exhibited the same staining pattern regardless of the method chosen, the intensity of the immunoreaction was subject to considerable variation depending on the embedding medium used.

**RESULTS**

Single 1.9-kb arrestin transcripts were detected in all three species (Fig. 1A). The mouse phosducin probe also hybridized to single transcripts, sized 1.2 kb in murine, 1.4 kb in ground squirrel, and 1.1 kb in human retina (Fig. 1B). The signal was considerably stronger in the lane containing mouse retinal mRNA. The recoverin probe visualized one abundant transcript in all three species (Fig. 1C). The transcript size was 1.0 kb in murine and ground squirrel RNA and 1.2 kb in human RNA.

A strong labeling with the arrestin antibody was observed in the whole soma of a minor subpopulation of photoreceptors (Fig. 2A). The most intense labeling was localized to the vitreal half of the inner segment (myoid). The blue pigment antibody OS-2 recognized blue cones, which were less abundant and had a larger outer segment diameter than did green cones, and rod-like cells, identified by their smaller diameter (Fig. 3A). Only the latter cell type was recognized by the rhodopsin antibody AO (Fig. 3B). Immunolabeling of consecutive sections showed that all blue opsin-positive elements were also positive for arrestin, whereas no green cones were recognized by the arrestin antibody (Fig. 3E).

The phosducin antiserum labeled rod-like cells, as well as a subpopulation of cone cells (Fig. 2B). As with arrestin, the labeling was present in the entire cell soma; unlike arrestin, however, the labeling was

---

**TABLE 1. Antibodies Used for Immunocytochemistry**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Antigen</th>
<th>Origin</th>
<th>Host</th>
<th>Type</th>
<th>Dilation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO</td>
<td>Opsin</td>
<td>Bovine</td>
<td>Rat</td>
<td>P</td>
<td>1:10,000</td>
<td>Szél and Röhlich³²</td>
</tr>
<tr>
<td>OS-2</td>
<td>Blue opsin</td>
<td>Chicken</td>
<td>Mouse</td>
<td>M</td>
<td>1:5,000</td>
<td>Szél and Röhlich³²</td>
</tr>
<tr>
<td>COS-1</td>
<td>Green opsin</td>
<td>Chicken</td>
<td>Mouse</td>
<td>M</td>
<td>1:50</td>
<td>Szél and Röhlich³²</td>
</tr>
<tr>
<td>4/17/85</td>
<td>Arrestin</td>
<td>Bovine</td>
<td>Rabbit</td>
<td>P</td>
<td>1:1,000</td>
<td>T. Shinohara³³</td>
</tr>
<tr>
<td>Anti-32</td>
<td>Phosducin</td>
<td>Bovine</td>
<td>Rabbit</td>
<td>P</td>
<td>1:1,000</td>
<td>T. Shinohara³³</td>
</tr>
<tr>
<td>S6K22</td>
<td>Recoverin</td>
<td>Bovine</td>
<td>Rabbit</td>
<td>P</td>
<td>1:300</td>
<td>A. S. Polans³⁵</td>
</tr>
</tbody>
</table>

P = Polyclonal; M = monoclonal.
DISCUSSION

The intensity of arrestin cDNA hybridization was roughly equal in all three species studied—a surprising result, because arrestin immunoreactivity is found in fewer cells in the ground squirrel retina than in the murine and human retinas. The phosducin cDNA hybridized more strongly with murine retinal mRNA than with mRNA from other species. This is to be expected, because the probe is of murine origin. The signal was, however, weaker in the ground squirrel lane than in the lane containing human mRNA, consistent with the fact that ground squirrel rod-like cells are much less abundant than human rods.

Antibodies against arrestin and phosducin labeled equally intense in outer (Fig. 3C) and inner segments (Fig. 3D) and was most intense in the photoreceptor membrane. The strength of the immunoreaction was considerably lower than that found with the arrestin antibody. Immunolabeling of consecutive sections showed all identifiable blue opsin-positive elements (Fig. 3A) to be positive for phosducin (Figs. 3C, 3D). No immunoreaction was detected in green cones.

The recoverin antiserum labeled all photoreceptor cells (Fig. 4). The label intensity was found to be the strongest in the myoids and in the pedicles of the photoreceptors. The outer segments were stained weakly, and a diffuse label was observed in the entire outer nuclear layer. A few cells in the inner nuclear layer were also recognized (Fig. 4A). Tangential sections (Fig. 4B) were used to show that no photoreceptor cell was left unstained. All elements encountered at either the outer segment level or the myoid level were recognized by the recoverin antibody.

For all the antibodies used, the immunocytochem-

ical staining pattern within the photoreceptor mosaic was the same with light-adapted and dark-adapted animals.

FIGURE 1. Northern blots hybridized with cDNA probes for arrestin (A), phosducin (B), and recoverin (C). On all blots, murine RNA is seen in the left lane, human RNA in the middle lane, and ground squirrel RNA in the right lane. (A) and (C) contain 1.0 μg of poly-A RNA per lane, and (B) contains 2.0 μg per lane. (A) was exposed for 4 days. (B) is a composite, showing a 4-day exposure of the ground squirrel and human lanes, whereas the mouse lane has only been exposed for 6 hours. (C) was exposed for 16 hours.

FIGURE 2. Consecutive radial araldite sections of the ground squirrel retina reacted with arrestin (A) and phosducin (B) antibodies. Both antibodies stain only a few photoreceptors. In these cells, anti-arrestin stained the complete cell body, showing the most intensive reaction in the myoid. The phosducin antibody recognized an equally small subpopulation of photoreceptors. Note that the reaction is most intensive in the cell membrane. Some of the stained cells are marked with black arrows. The outer limiting membrane (OLM) and the retinal pigment epithelium (RPE) are marked with open arrow and asterisk (*), respectively. Magnification, ×825.
FIGURE 3. Parallel tangential araldite sections showing the same retinal area reacted with antibodies against blue opsin (A), rhodopsin (B), phosducin (C, D), and arrestin (E). (F) is a diagram made on figure (A) to show the individual photoreceptor types. Sections (A), (B), and (C) represent the outer segment level; at the lower edge of the sections, however, inner segments also appear. Sections (D) and (E) were taken from a more vitreal level; therefore, mainly inner segments are encountered. The honeycomb structure produced by the apical processes of the RPE cells is present only at the outer segment level. The antibody COS-1 labeled green cones (not shown). The wider blue cone outer segments are stained by OS-2 (A, thick arrow). The rod-like cells, which have a considerably smaller outer segment diameter, are also stained weakly by this antibody (A, thin arrow). Anti-rhodopsin exclusively stains rod-like cells (B, thin arrow). The phosducin antibody stains both blue cones (thick arrows) and rod-like cells (thin arrows) at both the outer (C) and inner (D) segment levels. Similarly, anti-arrestin reaction is found in both photoreceptor types (E). Note that the latter antibody gives the highest staining intensity in the vitreal level of the inner segments. Green cones are not stained by any of the antibodies used. The scheme in (F) shows the three photoreceptor types that can be identified in (A) through (E). Blue cones are black, green cones are left unmarked, and rod-like cells are labeled by small black dots. Magnification, ×840.

rod-like photoreceptors and a subpopulation of the cone photoreceptors that were identified as blue cones by immunocytochemistry. Previous reports are not conclusive regarding arrestin localization. Earlier studies demonstrated arrestin immunoreactivity in rods as well as in cones in several vertebrates, but other investigators could not detect arrestin in rhesus monkey or squirrel cones, human cone outer segments, or porcine cones. Positive immunoreaction was observed in the tree shrew in a subpopulation of cones that was probably identical to blue cones, similar to what has been found in the ground squirrel.
Phototransduction Proteins in the Ground Squirrel

To our knowledge, it has not hitherto been reported that phosducin immunoreactivity also displays a differential distribution among different cone types. Although no reports are available that show immunoreactivity with other phosducin antisera to red/green cones, it is possible that these photoreceptors are equipped with a homologue with a different primary structure. At least two phosducin genes with some differences in the 5'-flanking region are present in the mouse genome, and two are expressed in the retina. There is also evidence for expression of phosducin or a similar polypeptide in several different tissues of the body.

As mentioned, phosducin is associated with the β/γ-transducin complex. It is now known from studies on cow and monkey retinas that rods and cones are equipped with different forms of β- and γ-transducin. Rods contain the subunits β1 and γ1, whereas cones contain the homologous β3 and γ2. We have previously found a different distribution of the β-transducin subunits in the ground squirrel retina. We found β1-transducin immunoreactivity in rod-like cells and blue cones, whereas a β3-transducin antibody labeled all photoreceptor types (γ-transducin was not studied). The apparent colocalization of phosducin and β1-transducin in the same photoreceptor types in the ground squirrel raises the question of whether this corresponds to a specific biochemical association as well. This is not supported by published data from the bovine retina, where the phosducin - transducing/γ complex reacted with antibodies against both β1- and β3-transducin. However, preliminary evidence indicates that bovine cones may have a specific phosducin with a slightly higher molecular weight. It should also be noted that the phosducin localization reported here differs from that found in the mouse retina, where a phosducin antibody labeled the entire cell but was more intense in the inner segments.

The localization of arrestin in blue cones and in rods reported here indicates that a mechanism of deactivation of the visual pigment by phosphorylation may be present in both blue cones and rods. It remains to be determined whether the interaction between arrestin- and phosducin-like proteins reported in brain preparations is also present in the retina. It is, however, established that long-wavelength cones also have a photopigment phosphorylation mechanism, at least in nonmammalian vertebrates. Thus, in the retina of the lizard Anolis carolinensis, which contains exclusively red and green cones, the opsins undergo a very strong light-induced phosphorylation. In chicken, the phosphorylation of the red opsin molecule has been specifically demonstrated. This phosphorylation is presumably part of an alternative mechanism for quenching of the visual pigment, possibly
involving a red and green cone-specific arrestin. The gene for an arrestin that is a likely candidate for this role has recently been identified. The blue cone photopigment sequence is one of several criteria that place blue cones as an intermediate between rods and red/green cones, sometimes with more similarities to the former than to the latter. The findings described here also indicate a closer evolutionary relationship between rods and blue cones.

We found an arrestin immunolabeling that was distributed throughout the entire cell soma but was strongest in the myoid region of the inner segment in rod-like cells and blue cones. In the light-adapted baboon retina, arrestin immunolabeling has been reported to be localized to the cone outer segment, whereas in rat and mouse cones, arrestin immunoreactivity remains in the inner segments for an hour after illumination. These discrepancies may emanate from interspecific differences.

Previous reports have described a rapid light-dependent migration of phototransduction proteins between inner and outer segments. Thus, arrestin immunoreactivity is found in light-adapted outer segments of rat, toad, and mouse retina, and it migrates into inner segments on dark adaptation. Moreover, the levels of arrestin mRNA are at their peak just before the onset of the dark period, as opposed to the mRNAs encoding the proteins of the phototransduction cascade. In contrast, phosducin and transducin are found in light-adapted inner segments and migrate to the outer segments in the dark. However, none of these reports describes the situation in cone photoreceptors. The results presented here indicate that migration of arrestin or phosducin does not take place in rod-like cells or in cones. We were also unable to detect any difference in α- or β-transducin or in γ-phosphodiesterase immunolabeling between light- and dark-adapted animals. These findings are consistent with those reported in other studies on rat and mouse retina. Apparently, cone photoreceptors do not have the same mechanism for rapid movement of phototransduction proteins that rods do. Interestingly, no light-dependent migration in rod-like cells was observed; the lack of such migration is yet another trait of these cells more reminiscent of cones than of rods. These issues would merit a special study with material collected at different time points after illumination.

The recoverin message hybridized strongly with the corresponding cDNA in all three species, and we found recoverin immunoreactivity in all photoreceptor types of the ground squirrel retina. These findings are in accordance with those in the rod-dominant bovine retina. A similar immunocytochemical staining has been found in human retina using the same antibody as in the present investigation.

The authors thank Artur S. Polans, Winston A. Salser, and Toshimishi Shinhara for the gift of DNA probes and antibodies. They also thank Clyde K. Yamashita for his invaluable help with the ground squirrel colony and with the handling and dissection of retinas, Katharina Rydén for technical assistance, and Andrea Viczian for sharing human retinal RNA samples.

References

8. Szél Á, von Schantz M, Farber DB, van Veen T. Differ-


42. Brookhuyse RM, Winkens HJ. Photoreceptor cell-spe...