Light Response of Retinal ON Bipolar Cells Requires a Specific Splice Variant of $G\alpha_o$

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Glutamate released onto retinal ON bipolar neurons binds to a metabotropic receptor to activate a heterotrimeric G-protein (G_o) that ultimately closes a nonspecific cation channel. Signaling requires the α subunit ($G\alpha_o$), but its effector is unknown. Because $G\alpha_o$ is transcribed into two splice variants (α_{o1} and α_{o2}) that differ in the key GTPase domain, the next step in elucidating this pathway was to determine which splice variant carries the signal. Here we show by reverse transcription-PCR and Western blots that retina expresses both splice variants. Furthermore, *in situ* hybridization and immunostaining on mouse retina deficient in one splice variant or the other show that both α_{o1} and α_{o2} are expressed by ON bipolar cells but that α_{o1} is much more abundant. Finally, electroretinography performed on mice deficient for one splice variant or the other shows that the positive b-wave (response of ON bipolar cells to rod and cone input) requires α_{o1} but not α_{o2}. Thus, the light response of the ON bipolar cell is probably carried by its strongly expressed splice variant, $G\alpha_{o1}$.

Key words: G-protein; G_o splice variants; splice variant knock-out mouse; mGluR6; metabotropic glutamate receptor; retina; ERG

At the first visual synapse, glutamate modulates three-quarters of the postsynaptic neurons (ON bipolar cells) via a metabotropic glutamate receptor (mGluR6) (Nomura et al., 1994; Vardi and Morigiwa, 1997; Vardi et al., 1998, 2000) that couples to a heterotrimeric G-protein (G_o) (Weng et al., 1997; Nawy, 1999; Dhingra et al., 2000). Signaling requires the α subunit ($G\alpha_o$), but, the next step in this pathway, which ultimately closes a nonspecific cation channel, is unknown (Nawy and Jahr, 1990; Shiells and Falk, 1990; de la Villa et al., 1995; Euler et al., 1996). To identify an effector for $G\alpha_o$ seems important even beyond the visual system because, although effectors are known for the β/γ subunits of this most abundant brain G-protein (Dolphin, 1998), no effectors have yet been identified for the α subunit.

$G\alpha_o$ is transcribed into two splice variants, α_{o1} and α_{o2}, which contain identical helical domains near the N terminus (NT) but different GTPase domains near the C terminus (CT) (Goldsmith et al., 1988; Hsu et al., 1990; Strathmann et al., 1990; Tsukamoto et al., 1991; Horn and Latchman, 1993). Because the GTPase domain binds both receptor and effector, it is crucial for coupling (for review, see Neer, 1994; Clapham, 1996; Gudermann et al., 1997). Therefore, the next step in elucidating this pathway was to determine which splice variant carries the signal. Previous studies that colocalized $G\alpha_o$ with mGluR6 in ON bipolar dendrites could not distinguish between different splice variants because the antibody (Ab) used recognizes both (Vardi, 1998), nor did the α_o null mouse, which lacks the ON response, resolve this question because it is missing both splice variants (Jiang et al., 1998; Dhingra et al., 2000). Here, using mice deficient in one splice variant or the other, we show that both are expressed by ON bipolar dendrites but that only $G\alpha_{o1}$ matters for the light response.

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MATERIALS AND METHODS

Animals were deeply anesthetized by intraperitoneal injection (for rat, 45 mg/kg pentobarbital; for mouse, a mixture of 85 μg/gm ketamine and 13 μg/gm xylazine), and the eyes were enucleated; animals were then killed by anesthetic overdose (three times the initial doses). Animals were treated in compliance with federal regulations and University of Pennsylvania policy. The eye was incised at the ora serata and fixed by immersion in buffered 3 or 4% paraformaldehyde with (for immunocytochemistry) or without (for *in situ* hybridization) 0.01% glutaraldehyde for 1 hr. It was then rinsed in buffer, soaked overnight in 30% buffered sucrose, and embedded in a mixture of two parts 20% sucrose in phosphate buffer and one part of tissue freezing medium. Radial cryosections were 10–15 μm thick.

Reverse transcription-PCR. Retina was homogenized in solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol, and total RNA was isolated by the acid-guanidium phenol–chloroform method (Chomczynski and Sacchi, 1987). The reverse transcription (RT) reaction was performed at 42°C for 50 min with 1–5 μg of total RNA in 20 μl of buffer containing 50 mM Tris-HCl, pH 7.4, 60 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 U/ml RNase inhibitor, 0.5 mM of each dNTP, 500 pmol of random hexamer (or 100 pmol of oligo-dT), and 200 U of SuperScript II Moloney murine leukemia virus reverse transcriptase (Invitrogen, Gaithersburg, MD). PCR reaction was performed in a buffer containing 10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 0.2 μM 5′ and 3′ primers, 2 μl of reverse-transcribed cDNA, and 2.5 U of AmpliTaq (PerkinElmer Life Sciences, Branchburg, NJ). Thirty cycles (94°C for 1 min, 52°C for 1 min, and 72°C for 2 min) were performed on a programmable thermocycler (PerkinElmer Life Sciences). The sequences of PCR primers (synthesized by Invitrogen) were the same as those used to generate the long probe for *in situ* hybridization (see below).

In situ *hybridization.* α_{o1} and α_{o2} sequences were amplified by RT-PCR from whole retina. The sequences of PCR primers were as follows: α_{o1} upstream, 5′-catcctccgaaccagggtc-3′; α_{o1} downstream, 5′-caagccacagccccggag-3′; α_{o2} upstream, 5′-catcctccgaaccagggtc-3′; and α_{o2} downstream, 5′-ggcgatgatgacgtccgt-3′. We also designed a set of probes at the most diverse region of α_o (which gave a shorter reaction product): α_{o1} upstream, 5′-gctcttcgactccatctgt-3′; α_{o1} downstream, same as for the first set; α_{o2} upstream, 5′-gacagcatctgcaacaac-3′; and α_{o2} downstream, same as for the first set. PCR products were subcloned into PCRII vector (Invitrogen) or pBluescript (Stratagene, La Jolla, CA), and the authenticity of the products was verified by direct sequencing. ^{33}P-labeled riboprobe was made by *in vitro* transcription. Briefly, the reaction was performed by incubating the linearized plasmid DNA in 20 μl of solution containing 40 U of RNA polymerase, 10 mM DTT, 20 U of RNase inhibitor, 0.5 mM ATP, GTP, and CTP, 250 μM UTP, and 25 μCi of [α-^{33}P]UTP. After incubating at 37°C for 2 hr, the reaction was treated with RNase-free DNase I and precipitated by LiCl. RNA was further purified by phenol–chloroform extraction, precipitated by ethanol, and finally dissolved in DEPC water.

To test whether the antisense probes cross-hybridized, we applied the sense transcripts of α_{o1} and α_{o2} to a nitrocellulose membrane and then tried to hybridize them with the antisense probes. Each antisense hybridized to its own sense but not to the other; therefore, the *in situ* hybridization probably reflects genuine distribution of α_{o1} and α_{o2} mRNAs.

Retina sections were hybridized overnight (~18 hr) with ^{33}P-labeled probes in *in situ* hybridization buffer containing 10% dextran sulfate, 50% formamide, 4× SSC, 0.1% SDS, and 2× Denhardt's solution at 10^6 cpm/50 μl. After overnight hybridization at 58 or 65°C, slides were washed twice at 10°C above the hybridization temperature in 2× SSC–50% formamide, once at room temperature in 2× SSC, followed by one wash at 10°C above the hybridization temperature in 1× SSC–0.1% SDS. Slides were rinsed in 0.1× SSC, incubated with 300 mM ammonium acetate, dehydrated in ethanol, and finally dipped in photographic emulsion (Kodak NTB-2; Eastman Kodak, Rochester, NY) and exposed for 1–5 weeks.

Immunocytochemistry. Staining was performed according to a standard protocol: soak in diluent containing 10% normal goat serum, 5% sucrose, and 0.3% Triton X-100 in phosphate buffer; incubate in primary antibodies overnight at 4°C; wash and incubate (3 hr) in anti-rabbit $F(ab)_2$ fragment conjugated to a fluorescent marker; and rinse and mount in Vectashield (Vector Laboratories, Burlingame, CA). To stain a 4% paraformaldehyde-fixed retina for protein kinase C (PKC), we needed to retrieve the antigenicity with 0.5% sodium borohydrate (8 min) and use both Triton X-100 (0.75%) and Tween 20 (0.2%) as detergents. For double staining, incubation was done as for single labeling, with both primary antibodies and both secondary antibodies simultaneously. Some sections were incubated in horseradish peroxidase-conjugated secondary antibody and visualized with 3,3′-diaminobenzidine (DAB) reaction product. For electron microscopy, DAB reaction product was intensified by the gold-substituted silver-intensified peroxidase method. The tissue was then osmicated (1.5% osmium tetroxide, 60 min), stained with 1% uranyl acetate in 70% methanol (60 min), dehydrated in 80, 90, and 100% methanol, cleared in propylene oxide, and embedded in Epon 812. Ultrathin sections were mounted on Formvar-coated slot grids and stained with uranyl acetate.

Antibodies. The antibody against α_{o1} (Ab 1718) was raised in rabbit against the peptide EYPGSNTYED, and the antibody against α_{o2} (Ab 1715) was prepared against peptide EYTGPSAFTE (both are gifts from Dr. D. Manning, University of Pennsylvania, Philadelphia, PA). In addition, three antibodies against α_o that recognize both splice variants on Western blots were used. (1) A polyclonal antibody (Ab 9072) was raised in rabbit against the peptide ANNLRGCGLY located at the C terminus (gift from Dr. D. Manning). This sequence is identical to that of α_{o1} peptide but is different in one amino acid from the corresponding peptide of α_{o2} (in which the bolded asparagine is replaced by lysine). (2) A polyclonal antibody was raised in rabbit against a peptide DGISAAKDV located at the N terminus (gift from Kwen-Jen Chang, The Burroughs Wellcome Co., Research Triangle Park, NC) (Chang et al., 1988; Codina et al., 1991). This peptide is identical in both splice variants. (3) A monoclonal antibody (mAb 3073) raised in mouse against the purified bovine protein (Chemicon; Li et al., 1995). The antibody to PKCα was monoclonal raised in mouse (mAb 5; Amersham Biosciences, Little Chalfont, UK).

Selective disruption of $G\alpha_{o1}$ and $G\alpha_{o2}$ expression. Standard molecular biology techniques, to be reported in detail elsewhere, were used to construct targeting vectors of two types (Rudolph et al., 1993, 1994; Jiang et al., 2002). For disruption of $G\alpha_{o1}$, a replacement-type targeting vector was made. It contained a genomic segment of the $G\alpha_o$ gene with exons 6, 7.2, 8.2, 7.1, 8.1, and 9.1, of which exon 7.1 was disrupted by insertion of a neomycin selection cassette, followed by an internal ribosome entry site, and the open reading frame of β-galactosidase. $G\alpha_{o1}$-targeted embryonic stem (ES) cells were obtained by selection for G418 resistant clones. For disruption of $G\alpha_{o2}$, an insertion-type vector was made with the same genomic segment, but, instead of disrupting exon 7.1, the codon for Cys-255 of exon 7.2 was replaced by a stop codon. In addition, a double neomycin–thymidine kinase selection cassette was placed at the end of the genomic segment to allow for positive and negative selection strategies.

$G\alpha_{o2}$-targeted ES cells were generated by the "hit-and-run" procedure in which insertion of the mutated homology and selection cassettes is selected for in the presence of G418, and subsequent excision of the wild-type duplicate with attending loss of both selection cassettes was obtained by negative selection in the presence of FIAU (2-fluoro,2-deoxy-5-iodouracyl-β-D-arabinofuranoside). After germline transmission, the resulting F2 mice (50:50 129Sv/C57BJ/6) either lacked $G\alpha_{o2}$ but preserved expression of $G\alpha_{o1}$ or lacked $G\alpha_{o1}$ but preserved expression of $G\alpha_{o2}$ and, in addition, expressing β-galactosidase (M. Jiang and L. Birnbaumer, unpublished observations) (*vide infra* for differential G_o-protein expression data).

Electroretinographic recordings. The experimental apparatus, methods of light stimulation and quantification, electroretinographic (ERG) recording, and cone signal isolation have been described in detail previously (Lyubarsky et al., 1999, 2002). Briefly, a mouse was dark adapted for 2 hr, and then, under dim red light, it was deeply anesthetized by intraperitoneally injecting ketamine (20 μg/gm) plus xylazine (8 μg/gm). The animal was immobilized in a holder, the pupils were dilated with 1% tropicamide, and the eyes were protected with a drop of methylcellulose. A platinum recording electrode contacted both corneas, and a tungsten reference electrode was inserted subcutaneously on the forehead. The animal in its holder was then placed inside a light-proof Faraday cage, and light stimuli were delivered through several ports. Stimulus intensity and spectral composition were controlled with neutral density and bandpass interference filters. Light intensities were calibrated and converted to estimated numbers of photoisomerization per photoreceptor (R^*) as described previously (Lyubarsky et al., 1999, 2000).

RESULTS

Both splice variants of $G\alpha_o$ (α_{o1} and α_{o2}) are expressed in retina

To determine which transcripts are expressed, we first performed RT-PCR on rat whole retina. Both transcripts were amplified: the bands were at the expected sizes (530 for α_{o1} and 510 for α_{o2}), and the PCR products were cut at the expected positions by the appropriate restriction enzyme (*Cla*I applied to α_{o1} and *Hin*dIII applied to α_{o2}) (Fig. 1*A*,*B*). In several experiments in which the PCR products were sequenced, there was good agreement with published sequences for hamster α_{o1} or α_{o2} sequences.

To determine whether both proteins are expressed, we used antibodies specific for each splice variant. The specificity of the antibody was tested by dot blots: the antibody for α_{o1} reacted with the α_{o1} peptide but not with the α_{o2} peptide, and the antibody for α_{o2} reacted with the α_{o2} peptide but not with the α_{o1} peptide (Fig. 1*C*). By SDS-PAGE of retinal homogenates followed by Western blotting, the α_{o1} antibody detected a prominent band at ~43 kDa, and the antibody for α_{o2} detected a prominent band at ~40 kDa; both agree with their known molecular weights of 40 and 39 kDa (Fig. 1*D*). The antibody for α_{o2} detected an additional band at ~50 kDa. We conclude that the retina expresses RNA transcripts and proteins of both splice variants of α_o.

α_{o1} is expressed by rod bipolar cells

First, we tested the distribution of α_{o1} mRNA by *in situ* hybridization. Antisense riboprobe for α_{o1} transcript applied to sections of fixed rat retina showed strong labeling in the inner nuclear

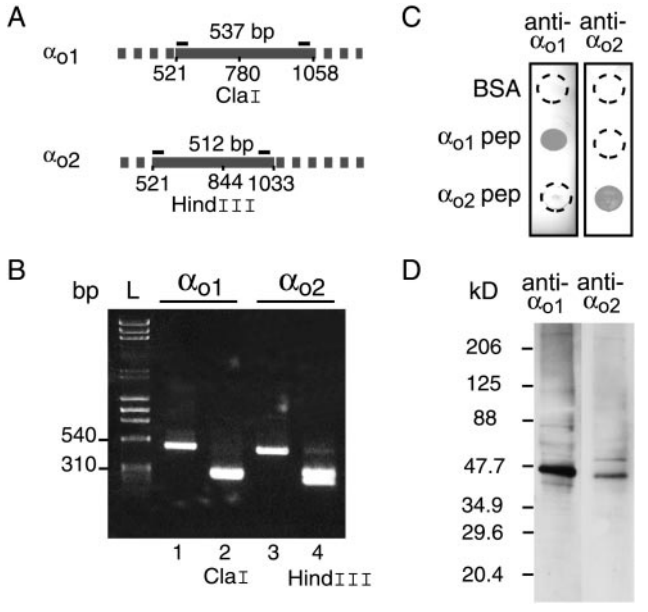

Figure 1. Retina expresses both splice variants of α_o. *A*, Primers for amplifying α_{o1} and α_{o2} mRNA; restriction enzymes cutting sites are also indicated. *B*, mRNA from rat retina was reverse transcribed and amplified using the specific primers shown in *A*. In *lanes 2* and *4*, the PCR products were cut with restriction enzymes. *C*, Dot blots show that α_{o1} antibody does not recognize the α_{o2} peptide (*α_{o2} pep*) and that α_{o2} antibody does not recognize the α_{o1} peptide (*α_{o1} pep*). Neither antibody reacted with bovine serum albumin (*BSA*). *D*, Western blots of whole rat retina show, for α_{o1}, a single prominent band at ~43 kDa and, for α_{o2}, a prominent band at ~40 kDa plus a weak band at ~50 kDa.

layer (INL) and ganglion cell layer. Labeling was strongest in the upper tiers of the INL, the location of rod bipolar cell somas. Labeling in the outer nuclear layer (ONL) and in photoreceptor inner segments was weak and was not significantly different from background obtained with sense probes for α_{o1} (Fig. 2*A*,*B*). A shorter, more specific probe gave less signal but the same labeling pattern. Also, the same probes applied to mouse retina gave weaker but similar labeling.

Next, we tested the distribution of α_{o1} protein. An antibody specific for this splice variant, applied to both rat and mouse retinas, showed staining similar to that obtained previously for α_o (Vardi et al., 1993; Vardi, 1998). Stained somas were numerous in the upper tiers of the INL, with dendrites reaching high in the outer plexiform layer (OPL) (Fig. 2*C*,*F*). Electron microscopy showed the stain in dendrites that invaginate the rod terminal (data not shown). Thus, rod bipolar somas and dendrites stain for α_{o1}. The bipolar axons crossing the INL were stained for α_{o1}, but, as they cross the inner plexiform layer (IPL), they were unstained. The IPL was also stained but more weakly than OPL. By confocal microscopy, the IPL showed two intense strata at ~25 and 55% of IPL depth, a lack of stain between ~25 and 35%, and weak stain elsewhere (0% is the interface between INL and IPL). Photoreceptors were unstained. Staining pattern for α_{o1} was specific because (1) retinal sections from rat incubated with preimmune serum or with antibody preabsorbed with the α_{o1} peptide were negative (Fig. 2*D*,*E*), and (2) retinal sections from an α_{o1} null mouse and an α_o null mouse were negative (Fig. 2*G*,*H*).

α_{o1} is expressed by all types of ON cone bipolar cell

Because rod bipolar cells and ON cone bipolar cells differ in response kinetics, components of their transduction cascades

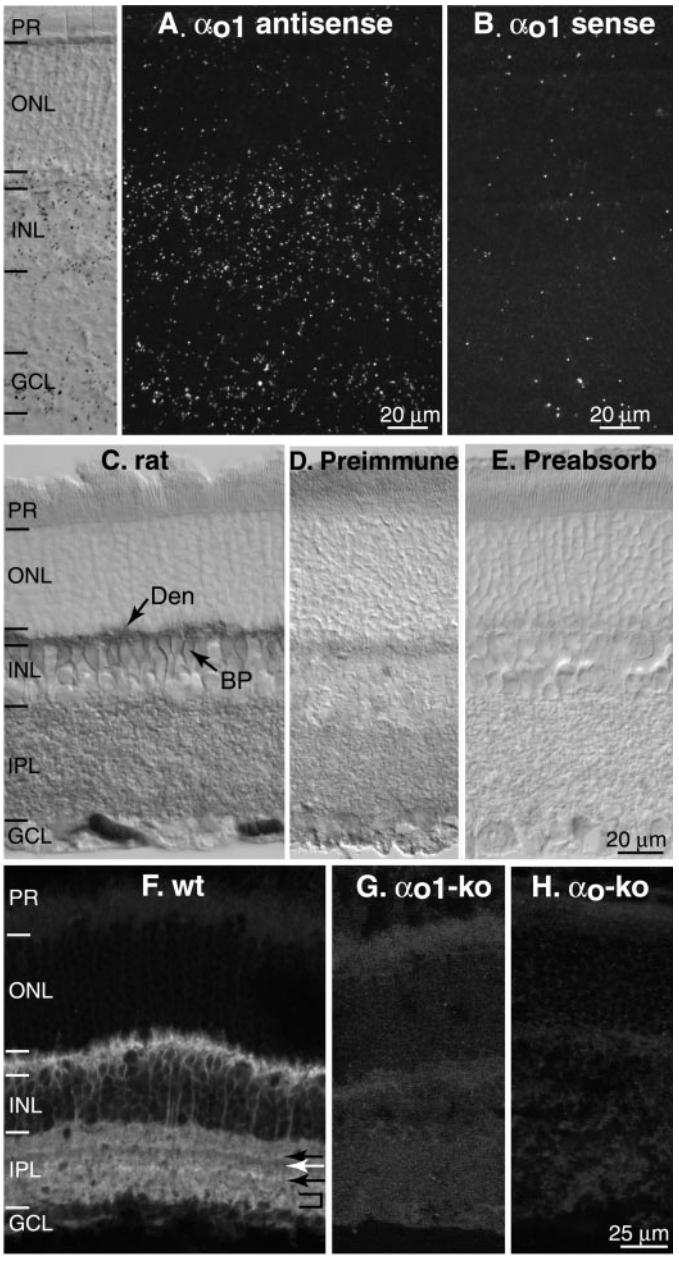

Figure 2. Bipolar cells strongly stain for the α_{o1} splice variant. *A*, *B*, *In situ* hybridization (rat). Antisense probe gave labeling in the INL and in the ganglion cell layer (*GCL*). Strongest labeling is in the upper tier of the INL, the location of rod bipolar somas. Sense probe gave low background. *Left panels*, Differential interference contrast shows the retinal layers of the section in *A*. *PR*, Photoreceptors. *C–E*, Immunostaining with the α_{o1}-specific antibody visualized with 3,3′-diaminobenzidine (rat). *C*, Staining is strong in bipolar dendrites (*Den*) in OPL and weaker in bipolar somas (*BP*) in the INL and in the IPL. *D*, Staining with the preimmune serum was negative. *E*, Staining with the antibody preabsorbed with the α_{o1} peptide was also negative. *F–H*, Immunostaining with the α_{o1}-specific antibody visualized with FITC (mouse). *F*, In the OPL and INL, staining in wild-type mouse resembles that in rat. In the IPL, note the thin bands of higher intensity (*black arrows*), a thicker band of lower intensity (*brackets*), and a band devoid of staining (*white arrow*). *G*, *H*, No staining above general background was observed when the same antibody was applied, respectively, to an α_{o1} or α_{o1+2} null mouse. *ko*, Knock-out; *wt*, wild type.

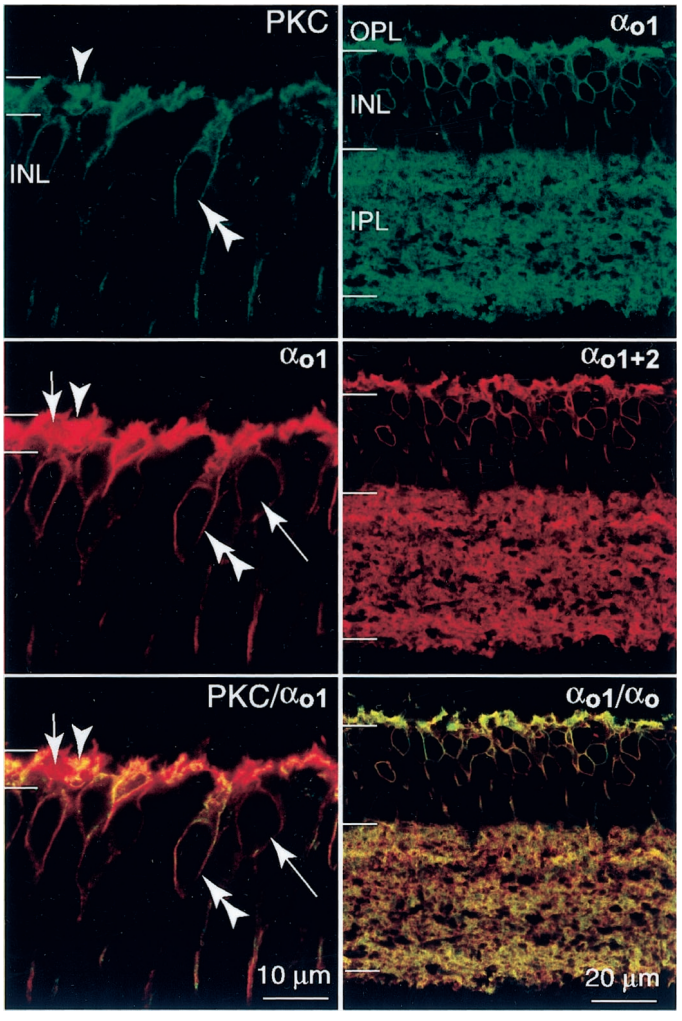

Figure 3. All ON cone bipolar cells stain for α_{o1} (rat). *Left*, All rod bipolar somas (stained for PKC) also stained for α_{o1} (*double arrowhead*), but some somas stained only for α_{o1} (*long arrow*). In the OPL (*bracketed*), rod bipolar dendrites stained for both PKC and α_{o1} (*arrowhead*), but some dendrites stained only for α_{o1} (*short arrow*). These probably belong to ON cone bipolar cells. *Right*, All ON bipolar somas were identified by staining with the monoclonal antibody for α_o. All of these somas also stained for α_{o1}. Also, in the IPL, the two staining patterns were the same, indicating that the retina expresses primarily the α_{o1} splice variant.

might also differ (Berntson and Taylor, 2000; Wu et al., 2000). Therefore, we asked whether rod and cone bipolar cells express the same splice variant of G_o. Double immunostaining showed that all ON bipolar cells express α_{o1}. First, rod bipolar cells identified with antibody for PKC stained for α_{o1}, but additional somas positive for α_{o1} were negative for PKC; these are cone bipolar cells (Fig. 3, *left*). Similarly, rod bipolar dendritic terminals projecting high in the OPL stained for both PKC and α_{o1}, but processes at midlevel in the OPL, in which cone bipolar dendrites terminate, stained only for α_{o1} (Fig. 3, *left*). Second, we labeled all ON bipolar cells using a monoclonal antibody for G_o. This antibody is suitable because it stains exactly the same set of cells as the antibody against the C terminus, which was shown to label all ON bipolar cells (Vardi, 1998). In this double staining, all somas stained by the monoclonal antibody also stained for α_{o1} (Fig. 3, *right*). Interestingly, stain for α_o and α_{o1} completely colocalized, even in the inner plexiform layer, suggesting that in retina all the α_o-positive cells express at least the α_{o1} splice variant.

ON bipolar cells and certain amacrine cells express low levels of α_{o2}

A specific antisense probe for α_{o2} mRNA distributed similarly to that of α_{o1}: strong labeling in the INL, somewhat weaker in the ganglion cell layer, and none in the outer nuclear layer and photoreceptor inner segments (Fig. 4*A*). A shorter, more specific probe distributed similarly, but less intensely, whereas the sense probe distributed randomly and rather weakly (Fig. 4*B*).

To test the distribution pattern of the α_{o2} protein was difficult. The antibody for α_{o2} capriciously stained cone outer segments in rat (but not in mouse), and it also stained Muller cells. The staining of the Muller cell was clearly unspecific because this cell also stained with the preimmune serum applied to wild type and with the antibody applied to α_o or α_{o2} null mice. Thus, the antibody for α_{o2}, under our staining conditions, was unsuitable for immunocytochemistry. Instead, we used three antibodies shown by Western blots to recognize both α_{o1} and α_{o2} and applied them to retina of the α_{o1} null mouse. The monoclonal antibody gave no staining. However, the other two antibodies that recognize both splice variants, one against the CT and one against the NT, both gave faint staining in the OPL, bipolar somas, and stratum 1 of the IPL (Fig. 4*C*,*D*). To distinguish this stain from background, we first imaged staining from the α_o null retina. This general background served as a reference at the confocal microscope (Fig. 4*E*). Then, using the same laser intensity and gain parameters, we imaged staining from the α_{o1} null retina. For both CT and NT antibodies, staining was stronger than in α_o null retina, and the pattern was repeatable (Fig. 4, compare *E* with *C*, *D*). Both antibodies applied to the α_{o2} null retina gave identical staining as in the wild type (Fig. 4*F*). Thus, both by *in situ* hybridization and by immunocytochemistry, bipolar cells weakly express α_{o2}.

Next, to determine whether the stained bipolar cells represent only a subset of bipolar cells, we stained the α_{o1} null retina for PKC and anti-α_o (C terminus). All cells that stained for PKC also stained for α_o, but ~8% of the cells (13 of 75 in one animal; 1 of 100 in the other) that stained for α_o were unstained for PKC (Fig. 5). The difference between the two animals could be attributed to weaker expression in cone bipolar cells or to regional differences. The cells that are α_{o2} positive (but PKC negative) were not identified. We can rule out the possibility that they are OFF bipolar cells because it has been established (using the same antibody as in this experiment) that α_o is absent from OFF cone bipolar cells (Vardi, 1998). Thus, α_{o2} is expressed in rod bipolar cells and in at least some ON cone bipolar cells.

The light response requires α_{o1} but not α_{o2}

To test which splice variant is crucial for the ON bipolar cell light response, we examined the electroretinogram in mice lacking either α_{o1} or α_{o2}. Three different lighting conditions were used: scotopic (dark adapted plus dim stimulus), saturated (dark adapted plus saturating stimulus), and photopic (light adapted plus strong stimulus). Under all conditions, mice lacking α_{o1} also lacked the b-wave, which is generated by the ON bipolar cells (Robson and Frishman, 1995) (Fig. 6, *middle column*; two animals, four eyes). This lack of b-wave does not reflect lack of photoreceptor activity because the a-wave, which originates in suppression of the photoreceptor dark current (Hagins et al., 1970; Hood and Birch, 1993; Breton et al., 1994; Lyubarsky and Pugh, 1996; Pugh et al., 1998), was present. Under the "saturated" condition, α_{o1} null mice gave a reduced, but still profound, a-wave (78 ± 34 μV). Under photopic conditions, in which the negative wave is likely a combination of a-wave and OFF bipolar response,

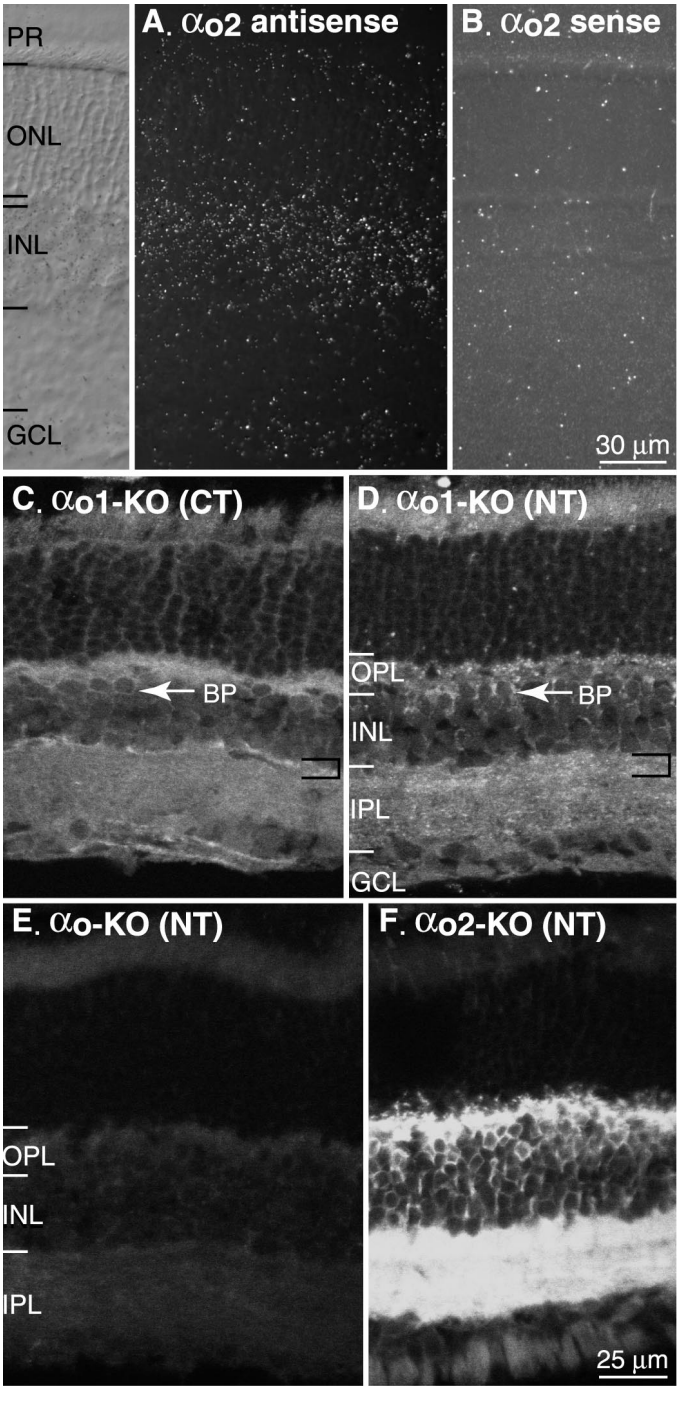

Figure 4. Bipolar cells and stratum 1 of the IPL weakly express the α_{o2} splice variant. *A*, *B*, *In situ* hybridization (rat). Antisense probes specific for α_{o2} message hybridized strongly in the INL and weakly in the ganglion cell layer (*GCL*). Sense probes gave weak, diffuse background. *Left*, Differential interference contrast for retinal section in *A*. *C–F*, Immunostaining of mouse retina, visualized with FITC. *D–F* were captured using the same confocal laser intensity and gain parameters. *C*, Antibody against the CT peptide of α_{o1} applied to the α_{o1} null retina faintly stained the OPL, bipolar somas (*BP*), and stratum 1 of the IPL (*brackets*). *D*, Antibody against the NT peptide applied to the α_{o1} null retina gave similar staining: faint in the OPL, bipolar somas, and puncta throughout the IPL, plus more intense staining in stratum 1. *E*, NT antibody applied to α_o knock-out (*KO*) retina gave weak diffuse background staining. *F*, NT antibody applied to α_{o2} knock-out retina gave strong labeling, resembling that obtained with this and other anti-α_o antibodies in wild-type retina.

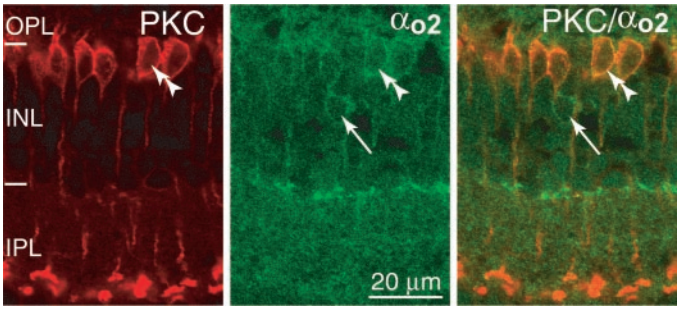

Figure 5. Rod bipolar and some cone bipolar cells stain for $G\alpha_{o2}$. Rod bipolar cells were identified by staining for PKC; staining for α_{o2} was achieved by staining an α_{o1} null mouse with anti-$G\alpha_o$-CT. All bipolar somas stained for PKC also stained for α_{o2} (*double arrowhead*), but some somas were stained only for α_{o2} (*arrow*).

the negative wave was as large or greater than in the wild type. This response pattern resembled that obtained when both splice variants were eliminated (α_o null) (Dhingra et al., 2000). Light responses in mice lacking α_{o2} were indistinguishable from wild type (Fig. 6, compare *left* with *right columns*; three animals, six eyes). The average peaks for $G\alpha_{o2}$ null mouse versus wild type were as follows (respectively, in microvolts): rod-generated b-wave, 249 ± 89 versus 220 ± 72; saturated a-wave, 283 ± 51 versus 319 ± 97; and cone-generated b-wave, 146 ± 49 versus 109 ± 32.

DISCUSSION

We showed by several techniques (RT-PCR, Western blotting, *in situ* hybridization, and immunostaining with antibodies directed against different domains) that both splice variants of $G\alpha_o$, $G\alpha_{o1}$ and $G\alpha_{o2}$, are expressed in retinal ON bipolar cells. However, $G\alpha_{o1}$ is much more abundant and is essential for the bipolar cell light response. Mice lacking the $G\alpha_{o1}$ splice variant are devoid of the scotopic and photopic b-waves, whereas mice lacking the $G\alpha_{o2}$ splice variant show a normal ERG. We noticed that the rod a-wave in the α_{o1} null mouse was reduced relative to the wild type. The difference is not clear and seems insignificant because in the complete $G\alpha_o$ null, both a-waves were within normal range. Nevertheless, the lower amplitude does not compromise the conclusion that the response of the bipolar cell is absent because this range of photoresponses should have elicited a b-wave ON response. Thus, the ON responses in rod and all ON cone bipolar cells depend only on G_{o1}.

Importance of identifying the crucial splice variant in the mGluR6 cascade

The identification of α_{o1} as the critical splice variant permitted its use (in constitutively active form) as bait in a yeast two-hybrid assay. One strong interactor encodes the γ subunit of the photoreceptor phosphodiesterase (PDE6). However, available probes for PDE-γ did not localize it to ON bipolar cells, and therefore it is unlikely to be the effector in the ON response (Nawy, 1999). Another interactor encodes Ret-RGS1 (regulator of G-protein signaling), and this protein is expressed in dendritic tips of at least one type of ON cone bipolar cell (Dhingra et al., 2001). Ret-RGS is unlikely to be the actual effector for $G\alpha_{o1}$, although it may assist. Nevertheless, these preliminary observations suggest the value of having narrowed down the search for effectors for the specific splice variant of α_o.

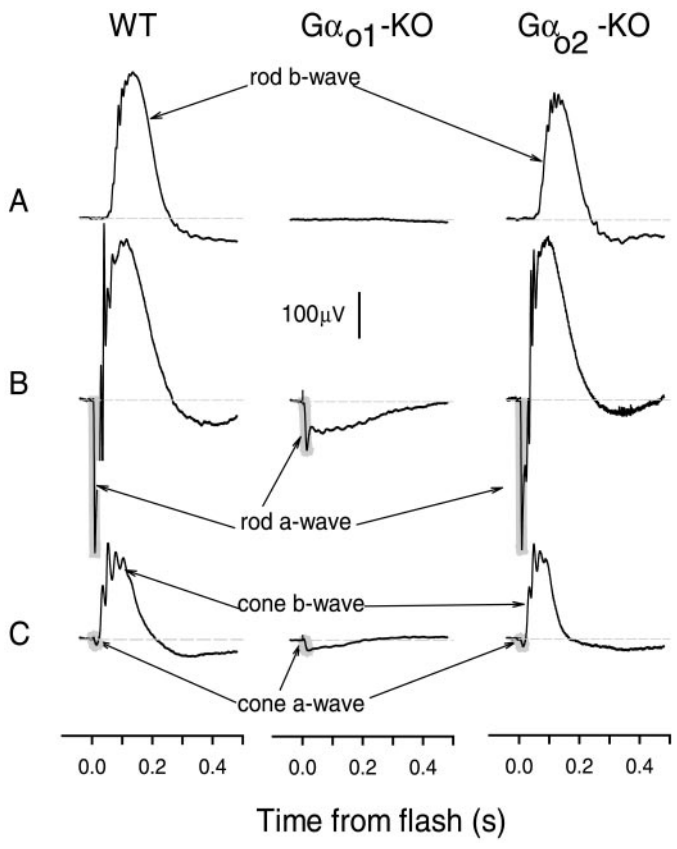

Figure 6. Rod- and cone-driven b-waves are present in the electroretinogram of $G\alpha_{o2}$ null mouse but absent from the $G\alpha_{o1}$ null mouse. *A*, Animals dark adapted for 2 hr were stimulated with dim flashes that elicited a rod-driven, corneal-positive b-wave in the wild-type mouse and in $G\alpha_{o2}$ null but not in the $G\alpha_{o1}$ null. Flash intensities estimated as photoisomerizations (R^*) per rod (Φ), and number of responses (n) averaged for each trace were as follows: $\Phi = 9$; $n = 18$. *B*, Dark-adapted animals were stimulated with an intense flash isomerizing ~0.1% of the rhodopsin. This elicited in wild-type and $G\alpha_{o2}$ null mice a negative a-wave (*shading*), followed by a positive b-wave. The $G\alpha_{o1}$ null mice showed an a-wave (although somewhat reduced) but no b-wave. $\Phi = 10^5$; $n = 4$. *C*, Mice were adapted to a background (white light, 9100 R^* rod^{-1} sec^{-1}) that completely suppressed the rod cGMP-activated current. Rods were then stimulated with an intense white flash that isomerized ~0.4% of the M-cone pigment and 0.03% of the UV-cone pigment. All of the animals showed a cone-driven a-wave. A typical cone-driven b-wave (positive-going response with superimposed oscillations, peaking ~70–90 msec after the flash) was present in wild-type and α_{o2} null mice but was absent in the $G\alpha_{o1}$ null animals. For all of the records, $n = 16$. *KO*, Knock-out; *WT*, wild type.

Possible function of G_{o2} in ON bipolar cells

If only G_{o1} is crucial for the ON response, what is the function of G_{o2}? We propose several possibilities. (1) $G\alpha_{o2}$ may contribute to fine-tuning of the mGluR6 cascade in certain ON bipolar cells. The ON bipolar cells are classified into rod bipolar cells and approximately five different types of cone bipolar cells that differ by their morphology (Cohen and Sterling, 1990; Boycott and Wässle, 1991; Euler and Wässle, 1995), and they are thought to divide the range of temporal frequencies by responding with different kinetics (Sterling, 1998; Freed, 2000a,b; Masland, 2001; Roska and Werblin, 2001). All ON bipolar cells express the same receptor (mGluR6) (Masu et al., 1995; Vardi and Morigiwa, 1997; Vardi et al., 2000), and the same G-protein α subunit (α_{o1}) and γ subunit ($G\gamma_{13}$) (Huang et al., 2000). Because $G\alpha_{o2}$ is expressed only in a subtype of the ON bipolar cells, it may differentially contribute to shaping response kinetics. (2) $G\alpha_{o2}$ could couple different receptors to different biochemical cascades. $G\alpha_{o1}$ and $G\alpha_{o2}$ differ in the C-terminal domain that is generally involved in specific interaction with the receptor and the effector (Conklin et al., 1996). An example for effector specificity is given by Helisoma neurons, in which α_{o2}, but not α_{o1}, inhibits Ca^{2+} channels (Man-Son-Hing et al., 1992). An example for receptor specificity is given by rat pituitary GH3 cells, in which G_{o1} inhibits the Ca^{2+} channel by coupling muscarinic receptor, whereas G_{o2} inhibits this channel by coupling somatostatin receptors (Kleuss et al., 1991; Chen and Clarke, 1996; Degtiar et al., 1997). In rat retina, rod bipolar cells and a subset of cone bipolar cells express, in addition to mGluR6, the metabotropic glutamate receptors mGluR1 and mGluR5 (Koulen et al., 1997). Therefore, it is possible that G_{o2} couples these receptors to a second-messenger cascade. Because these receptors typically regulate intracellular [Ca^{2+}] (via PLC pathway) (Abe et al., 1992) and because intracellular [Ca^{2+}] is critical for adaptation in rod bipolar cells (Shiells and Falk, 1999; Nawy, 2000; Berntson and Taylor, 2000), G_{o2} may contribute to adaptation. (3) G_{o2} may be involved in some aspects of development. Expression patterns of G_{o1} and G_{o2} during development suggest that it mediates several processes in development (Strittmatter et al., 1990; Rouot et al., 1992; Duc-Goiran et al., 1999). Thus, similar function may apply also in retina. It is important, however, to note that whatever the function in development of G_o is, it is not crucial for gross morphology because all of the α_o knock-outs ($G\alpha_o-/-$, $G\alpha_{o1}-/-$, and $G\alpha_{o2}-/-$) have normal brain and retinal morphology (Valenzuela et al., 1997; Jiang et al., 1998; Dhingra et al., 2000; this study).

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