Alpha Subunit of G\(_0\) Localizes in the Dendritic Tips of ON Bipolar Cells

NOGA VARDI*
Department of Neuroscience, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT
The metabotropic glutamate receptor (mGluR6), expressed by rod bipolar cells and ON cone bipolar cells, activates a trimeric guanine nucleotide-binding protein (G-protein) that ultimately closes a cation channel. The G-protein remains unidentified, but the alpha subunit of G\(_0\) (G\(_{0a}\)) has been suggested as a candidate because it is present in rod bipolar cells. However, the precise subcellular distribution of G\(_0\) within the rod bipolar cell, and its distribution among cone bipolar cells was not determined. This information is important in assessing the hypothesis that G\(_0\) couple mGluR6 to its effector. Here I report the distribution of G\(_0\) (alpha subunit) by immunostaining in several mammalian retinas. The overall distribution is conserved across mammalian species: strongest in the dendrites of ON bipolar cells, moderate in their somas, weak in their axons, and absent from their terminals. G\(_{0a}\) is also present in some amacrine somas and processes. In monkey fovea, where rods and rod bipolar cells are absent, G\(_{0a}\) is present in about half of the bipolar somas which occupy the upper tiers of the bipolar layer, and are therefore identified as ON cone bipolar cells. Ultrastructurally, in monkey and cat, G\(_{0a}\) is present in the dendritic tips of rod bipolar cells and ON cone bipolar cells, which are identified by their invaginating contacts. It is absent from OFF cone bipolar dendrites, which are identified by their flat contacts. It is also absent from axons entering the inner plexiform layer, and their terminals. In the primary dendrites, stain for G\(_{0a}\) mainly associates with the plasma membrane, but in the dendritic tips it is also present in the cytosol. Apparently, G\(_{0a}\) is expressed by the same bipolar cells that also express mGluR6, and is concentrated at the same subcellular location. Thus, G\(_{0a}\) could serve to couple mGluR6 to later stages of its signaling cascade.

Roughly three-fourths of mammalian retinal bipolar neurons, including all rod bipolar cells and half of the cone bipolar cells, depolarize to light increment and are termed “ON” cells (Cohen and Sterling, 1986, 1990; Martin and Grünert, 1992; Strettoi and Masland, 1995). The depolarization is due to the opening of a cation channel that remains closed in darkness by tonic release of glutamate. The transmitter binds to an identified metabotropic receptor, mGluR6, localized both to rod bipolar and ON cone bipolar dendritic tips (Nakajima et al., 1993; Nomura et al., 1994; Duvoisin and Vardi, 1996; Vardi and Morigiwa, 1997). This apparently triggers a phosphodiesterase (PDE)-dependent decrease in cGMP concentration, leading to channel closure (Nawy and Jahr, 1990; Shiells and Falk, 1990). It was natural to think that mGluR6 might employ the same elements as the phototransduction cascade: glutamate receptor → transducin → PDE → [cGMP] → close cGMP-gated channel, but immunostaining for these components (i.e., transducin, several isoforms of PDE, and the photoreceptor cGMP gated channel) proved negative (Wässle et al., 1992; Vardi et al., 1993).

To identify the G-protein in ON bipolar cells we have previously probed for different G-proteins and found that rod bipolar cells and certain cone bipolar cells express the alpha subunit of G\(_0\) (G\(_{0a}\); Vardi et al., 1993). However, using light microscopy, we could not resolve the subcellular localization of G\(_{0a}\) nor could we determine whether the stained cone bipolar cells were ON or OFF (Vardi et al., 1993). This fine tuning of the localization is important because expression of G\(_{0a}\) by the same cell compartments

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*Correspondence to: Dr. Noga Vardi, Department of Neuroscience, University of Pennsylvania, Philadelphia PA 19104.
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which express mGluR6 would raise the likelihood that Go mediates mGluR6 response. Here, using thin and ultra-thin sections, I show that Go is expressed in ON cone bipolar cells, most intensely where their dendritic tips invaginate the cone terminal. This expression pattern is conserved across four mammalian species and corresponds closely to the expression pattern of mGluR6 (Duvoisin and Vardi, 1996; Vardi and Morigiwa, 1997).

**MATERIALS AND METHODS**

Adult monkey (Macaca fascicularis), cat, rat, and rabbit were deeply anesthetized with pentobarbital (45 mg/kg). Following enucleation, the animal was killed by anesthetic overdose. All experiments involving animals were done in compliance with Federal regulations and University of Pennsylvania policy. Retinas were fixed by immersion in 4% paraformaldehyde + 0.01–0.1% glutaraldehyde in phosphate buffer (pH 7.3) containing 5% sucrose for 1 hour, rinsed in buffer, and soaked overnight in 30% buffered sucrose. For cryosections (6–10 µm), pieces of central retina were embedded in a mixture of two parts 20% phosphate buffered sucrose and one part Tissue Freezing Medium (Electron Microscopy Sciences, Fort Washington, PA). For Vibratome sections (50–100 µm), the retina was frozen and thawed, and cut into pieces which were embedded in 4% agarose.

**Immunocytochemistry**

Sections were stained for Go, according to a standard protocol: soak in 0.1 M phosphate buffer containing 10% normal goat serum, 5% sucrose, and 0.3% Triton X-100 (diluent); incubate in anti-Go (1:1,000–5,000 with diluent) overnight at 4°C; wash and incubate (3 hours) in anti-

Fig. 1. Optical sections (radial, ~2 µm) from confocal microscope show a conserved staining pattern for Go. Stain for Go in four species is strongest in the OPL. Bipolar dendrites (d) are clearly stronger than the somas (b). Stain in the IPL is weaker than in the OPL; in certain sections it appears diffused, in others, horizontal bands can be observed, especially in strata 1, 3, and 5 (arrows). Amacrine cell somas are typically weak (a) or unstained. OPL, Outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bars = 15 µm.
rabbit F(ab)2 fragment conjugated to indocarbocyanine (Cy3); mount in Vectashield (Vector Laboratories, Burlingame, CA). These sections were photographed on a confocal microscope (Leica, Allendale, NJ). Alternatively, staining was visualized by a 3,3’-diaminobenzidine tetrahydrochloride (DAB) reaction product which, in certain cases, was intensified by adding cobalt and nickel salts to the DAB solution (Adams, 1981).

For semi-thin sections (1 µm) and electron microscopy (85 nm), the above protocol was modified as follows: tissue was frozen and thawed three times prior to Vibratome sectioning; Triton X-100 was used at 0.1% with the pre-incubation medium; incubation in primary antibody was extended to 3 days. DAB reaction product was intensified by the gold-substituted silver-intensified peroxidase method (after Sassoe-Pognetto et al., 1994; modified from van den Pol, 1988). The tissue was then osmicated (1.5% osmium tetroxide, 60 minutes), stained with 1% uranyl acetate in 70% methanol (60 minutes), dehydrated in methanol, cleared in propylene oxide, and embedded in Epon 812. Ultrathin sections were mounted on formvar-coated slot grid and stained with lead citrate and uranyl acetate.

The antibody for G_{ox} was directed against the specific peptide “ANNLRGCGLY” located at the carboxy-terminus of the alpha subunit (gift from Dr. D. Manning). Its specificity is well established (Carlson et al., 1989; Law et al., 1991; Vardi et al., 1993). The antibody for calbindin was raised in mouse (Sigma, St. Louis, MO). The antibody for mGluR6 was directed against the carboxy-terminus of human mGluR6, and its detailed characteristic and staining pattern would be described elsewhere.

For electronic processing, negatives from light or electron microscopy were scanned at about 1,300 dots per inch (dpi). Digital images were cropped, enlarged, and contrast-enhanced with Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA). Final resolution was around 300 dpi. The figures were labeled with Adobe Illustrator.

**RESULTS**

Overall distribution of G_{ox} is conserved across mammalian species

The overall staining pattern for G_{ox} was observed in thin optical sections (~2 µm) obtained by confocal microscopy (Fig. 1, see also Fig. 2A). The most intense stain was in the outer plexiform layer, in bipolar dendrites approaching the rod and cone terminals. Primary dendrites of some bipolar cells were stained and so were their cell bodies, but their axons crossing to the inner plexiform layer were not. The inner plexiform layer contained relatively weak and diffuse staining. Horizontal bands at strata 1, 3, and 5 were seen in every species tested, but they were not evident in every section (Vardi et al., 1993). This overall pattern was similar in all the species examined (rat, cat, rabbit, and monkey). Closer study was conducted in monkey and cat to determine whether bipolar axon terminals are stained, and which bipolar types express G_{ox}.

**G_{ox} is absent from bipolar axon terminals**

Epon sections (1 µm) in cat and monkey showed that stain was localized to initial axons close to the somas (arrowheads in Fig. 2), but it gradually disappeared as the axons approached the inner plexiform layer (arrows in Fig. 2). At the electron microscope level, axons could be recognized by their radial orientation and the inclusion of microtubules. In the inner plexiform layer, all axons were unstained. Occasionally I observed staining in axons ap-
proaching the inner plexiform layer, but the stain dis-appeared once the axon crossed into the layer (Fig. 3A).
Bipolar terminals, which were recognized by their synap-tic ribbons, were never stained. Figure 3B and C shows two examples of unstained bipolar axons terminating in the ON sublamina. The terminals were surrounded by stained processes of amacrine cells, indicating that the antibody penetrated this region. Large dendrites, presumably of ganglion cells, were unstained (Fig. 2A).

**Gαo is expressed in macaque ON cone bipolar somas**

To observe the distribution of Gαo in cone bipolar somas without the confusing presence of rod bipolar cells, I first examined macaque fovea. There, because rods are absent, all bipolar cells connect to cones. In the outermost tier of the inner nuclear layer, which is occupied by horizontal cells, immunostain for Gαo was negative. But just beneath this layer, stain was observed in bipolar somas that occupy the upper one to two tiers of the bipolar layer (Fig. 4A). The lower one to two tiers of the bipolar layer were unstained as were, still deeper, the Müller cell somas.

Foveal ON and OFF bipolar somas had been identified by tracing their axons through serial sections to the ON and OFF strata of inner plexiform layer (Calkins et al., 1994). ON and OFF somas are equally numerous: ON cells occupy the upper tiers; OFF somas occupy the lower tiers (Fig. 4B). A comparison of Figures 4A and B shows that Gαo staining is associated with the ON somas.
Fig. 4. Half of the cone bipolar somas in monkey retina stain for Goα and lie high in the INL. A: A Vibratome section (50 µm) of fovea immunostained for Goα. Rods and rod bipolar cells are absent. Cone bipolar dendrites (arrowheads) terminate in a straight line beneath the cone terminals. Stained somas (+) occupy the upper 1.5 tiers of bipolar cells. This location corresponds to the ON cells in B. B: Electron micrograph through fovea showing location of bipolar somas which were traced to sublamina a (the OFF sublamina) and b (the ON sublamina) (Calkins et al., 1994). The OFF somas (−) are pale, the ON are dark (+); (micrograph courtesy of P. Sterling). H, Horizontal cells; M, Müller cells. C: Parafovea (50 µm Vibratome section) immunostained for Goα. Stained somas (+) occupy the upper two tiers of bipolar somas, corresponding to about half of the bipolar somas shown in D. Staining in the outer plexiform layer (OPL) includes cone bipolar dendrites that terminate subjacent to cone pedicles, and rod bipolar dendrites (arrowheads) that pass between the cone pedicles to terminate in rod spherules. D: Parafovea (50 µm Vibratome section) immunostained for the alpha subunit of the GABAα receptor (antibody bd24, Fig. 9A from Vardi and Sterling, 1994). This stain reveals the sub-layering within the inner plexiform layer (INL). Bipolar somas (three to four middle tiers) are stained. The layers are: H, horizontal cells; B, bipolar cells; M, Müller cells; A, amacrine cells.
In the parafovea, somas stained for Go
a
 occupy the upper two tiers of the bipolar stratum (Fig. 4C). In this region, cone bipolar somas plus a few rod bipolar somas occupy about four tiers total in the middle of the inner nuclear layer. This is seen by immunostain for the alpha subunit of the GABA
A
 receptor which identifies all bipolar cells (Fig. 4D; Vardi and Sterling, 1994). Thus, in parafovea, too, about half of the cone bipolar somas contain Go
a
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It would be desirable to test every bipolar cell type for presence of Go
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, but the specific antibodies that identify most cell types are raised in rabbit. Since the antibody for Go
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 was also raised in rabbit, double labeling for those and Go
a
 prove to be extremely difficult and unreliable. One type of OFF bipolar cell (diffuse bipolar cell type 3, or DB3), however, can be identified by immunoreactivity for calbindin with an antibody raised in mouse (Gruenter et al., 1994). This cell type did not colocalize with Go
a
 (Fig. 5).

Go
a
 is expressed in dendritic tips that invaginate the cone terminal

The next step was to determine which bipolar dendritic tips stain for Go
a
. ON bipolar dendritic tips invaginate the cone terminal to receive input from the synaptic ribbon, whereas OFF bipolar dendritic tips simply abut the basal surface of the terminal to form “flat” or “basal” contacts (Raviola and Gilula, 1975; Stell et al., 1977; reviewed by Kolb and Nelson, 1995). In the fovea, some ON dendrites also form basal contacts (Calkins et al., 1996), but outside of this region, there are sufficient invaginating sites to accommodate all ON bipolar dendrites (Chun et al., 1996). Consequently, the segregation between ON-invaginating and OFF-basal contacts in the periphery is unambiguous. Therefore, I studied the monkey retina at 3–6 mm eccentricity.

In semi-thin sections, stain for Go
a
 formed rows of dense puncta that appeared to invaginate the base of the cone terminal (Fig. 6A). This is distinct from the appearance of flat bipolar cells whose dendrites have a flattened arbor (Gruenter et al., 1994). In ultra-thin sections, stain was clearly observed in the invading dendritic tips located just beneath the synaptic ribbon (Fig. 6B). Between the invading dendrites, beneath the base of the cone pedicle, there was always an unstained region (Fig. 6B,C). This region is known to be occupied by dendrites of OFF bipolar cells forming flat contacts. The flat contacts, as well as the outline of the dendrites, are difficult to see because it was necessary to aim for complete staining, and this required the addition of a detergent. However, the unstained dendrites are certainly OFF because a similar staining pattern was obtained also for mgluR6: the invading dendrites are stained, and the dendrites beneath the cone pedicle are unstained (Fig. 6D, see also Vardi et al., 1998). Staining for Go
a
 in cat retina also showed that invading dendrites were stained while those forming basal contacts were unstained (Fig. 7).

Go
a
 at the dendritic tip is in the cytosol

Commonly, the G-protein links to its membrane-bound receptor by attaching to the membrane via a lipid tail. Upon activation, the alpha subunit of the G-protein dissociates into the cytosol to trigger subsequent events in a cascade (Simon et al., 1991; Casey, 1995). In the present experiments, since the antibody was directed against the alpha subunit, I carefully assessed its fine localization.

In the primary dendrites of both rod bipolar and cone bipolar cells, stain was largely associated with the plasma membrane (Fig. 8A, see also Fig. 6A). The width of the stain which was associated with the membrane was about...
Fig. 6. Cone bipolar dendrites that stain for Go form invaginating contacts and unstained dendrites form flat contacts (monkey). A: Semi-thin section through monkey parafovea shows stained bipolar somas in the inner nuclear layer and puncta in the outer plexiform layer. Outer puncta are rod bipolar dendritic tips (rb). Inner puncta are cone bipolar dendritic tips (cb) organized in a row beneath cone pedicles. The finger-like appearance of these puncta are typical of invaginating dendrites. Note also that the stain circumscribes the primary dendrite, and is not present in its cytosol. B–D: Electron micrograph through the outer plexiform layer (OPL) at ~4 mm eccentricity. B,C: Staining for Go. D: Staining for mGluR6 is provided for comparison. To obtain complete staining of Go expressing dendrites, it was necessary to use Triton X-100. Consequently, it is difficult to see the outlines of the dendrites. Nonetheless it is clear that stain for Go, like stain for mGluR6, is present in cone bipolar dendritic tip (ib) which invaginate the pedicle (CP) at a ribbon synapse. Stain is absent from the region just below the cone base (thickness of about 200 nm), where flat bipolar terminate (short arrows). r, Synaptic ribbon; h, horizontal cell terminal.

Fig. 7. Cone bipolar dendrites that stain for Go form invaginating contacts (cat). A,B: Electron micrographs from adjacent sections through cat cone pedicle near area centralis show stained cone bipolar dendrites (ib1, ib2) associated with synaptic ribbons (arrowheads). A dendrite forming a basal contact (thin, short arrow) is unstained. h, Horizontal cell terminal.
150 nm. This could represent either diffusion of the DAB reaction product from the plasma membrane inwards, or genuine staining of submembranous material. Unlike the primary dendrites, at the very tip or where the dendrites just penetrates the rod terminals, stain was localized both to the membrane and to the cytosol (Fig. 8A,B). The presence of stain deep in the cytosol can not be explained by diffusion because some of these dendrites are large (−700 nm) whereas diffusion of the reaction product is 150 nm or less. An additional example is shown in Figure 8C where two fine dendrites (−300 nm) run horizontally. The dendrite above (closer to the tip) is fully stained while the one below stains mainly near the plasma membrane. This differential subcellular distribution suggests that the alpha subunit is associated with the membrane in primary dendrites, and present in the cytosol at the tip of the dendrites.

**DISCUSSION**

*Does Goα mediate the mGluR6 response?*

I show here that Goα is present in about half the cone bipolar somas, i.e., the fraction corresponding to ON cells; it is present in the upper tiers of bipolar somas, where ON cells reside; and it is absent in OFF diffuse bipolar cell type 3 (DB3). Furthermore, the dendrites which contain Goα invaginate the cone terminal, as ON cells are known to do, and the dendrites which do not contain Goα terminate at the base of the cone, as OFF cells are known to do. This Goα positive subset of bipolar neurons is the same as that which expresses mGluR6 (Duvoisin and Vardi, 1996; Vardi and Morigiwa, 1997).

Within a bipolar cell, Goα localizes most strongly to the same subcellular compartment as mGluR6, i.e., the dendritic tip. There, Goα is present on the plasma membrane, in close proximity to mGluR6. Some Goα is also present in the cytosol of the dendritic tip, as would be expected if this trimeric G-protein had been partially activated at the moment of fixation. Goα distributes more broadly in the cell than does the mGluR6 receptor which localizes strictly to the dendritic tip (Nomura et al., 1994; Masu et al., 1995; Duvoisin and Vardi, 1996; Vardi and Morigiwa, 1997). This fits the pattern seen in photoreceptors: rhodopsin localizes strictly to the outer segment, while its G-protein, transducin, distributes more broadly, to the inner segment and even to the synaptic terminal (Brann and Cohen, 1987; Philip et al., 1987). Therefore, Goα has a distribution consistent with the hypothesis that it couples mGluR6 to the signaling cascade. Since no other G-protein has been found in the subset of bipolar cells which express Goα (Vardi et al., 1993; Vardi and Studer, 1994), Goα is currently the only candidate. Goα has been claimed to be present in certain bipolar cells, but not in rod bipolar cells (Peng et al., 1997).

Some biochemical evidence supports the hypothesis. First, when purified mGluR6 is mixed with Goα or transducin, L-2-amino-4-phosphonobutyrate (L-AP4), a specific agonist of mGluR6, activates Goα 18-fold more strongly than transducin (Weng et al., 1997). This demonstrates that mGluR6 can interact with Goα, with relatively high affinity. Second, in a retinal homogenate L-AP4 suppresses ADP-ribosylation of a G-protein by pertussis toxin but not by cholera toxin (Kikkawa et al., 1993). This fits the known pattern of toxin sensitivity for Goα (Gilman, 1987). On the other hand, the dogfish G-protein that mediates the ON response is sensitive to both toxins, a pattern resembling transducin (Shiells and Falk, 1992). This might be a
genuine species difference, given the gap between elasmo-branch and mammal, or it could reflect the fact that G_{on} has a cholera toxin site (Kaziro et al., 1991; Simon et al., 1991), which might be exposed by an agonist-induced conformational change (Rens-Domiano and Hamm, 1995).

**Other metabotropic receptors probably couple to G_{on}**

The number of known G-protein-coupled receptors far exceeds the number of G-protein alpha subunits, and a single type of alpha subunit commonly couples a number of receptors to their effectors (Raymond, 1995). For example, the octaery G-protein, G_{on}, is present in the octaery cilia, where it mediates olfaction, but it is also present in other brain tissue (Hervé et al., 1993) including the retina (Vardi and Studer, 1994). Even transducin, thought to be specific for photoreceptors, has now been shown to couple with the D4 dopamine receptor in mouse mesencephalic neuronal cells (Yamaguchi et al., 1997). Similarly, G_{on} is not unique to ON bipolar cells, but is also expressed in amacrine cell processes which do not express mGluR6. These cells, however, do express several other types of metabotropic receptors (Akazawa et al., 1994; Hartveit et al., 1995; Duvoisin et al., 1995; Koulén et al., 1997), so they might be coupled by G_{on}. In rat, rod bipolar cell dendrites (but apparently not cone bipolar cells) express, in addition to mGluR6, mGluR1, and mGluR5 (Koulén et al., 1997), and conceivably these also could couple to G_{on}. Certain bipolar axon terminals express mGluR7 and also the metabotropic GABA_{B} receptor (Maguire et al., 1989; Heidelberg and Matthews 1991; Okamoto et al., 1994; Brandstätter et al., 1996). However, since G_{on} is absent from bipolar axon terminals, it could not serve these receptors.

**What might link G_{on} to the cGMP-gated channel?**

G_{on} is extensively expressed in the brain, and it is clearly involved in a large variety of second messenger cascades involving a vast number of effector molecules. However, which molecules might serve as effectors is unknown. In the mGluR6 cascade, the activated G-protein (probably G_{on}) should eventually modulate cGMP concentration (Nawy and J ahr, 1990; Shiells and Falk, 1990; Yamashita and Wässle, 1991; de la Villa et al., 1995; Euler et al., 1996). It was initially hypothesized that the G-protein activates PDE (Nawy and J ahr, 1990; Shiells and Falk, 1990), but the PDE isoform could not be identified (Vardi et al., 1993).

In Xenopus oocytes, transfected G_{on} activated phospholipase C (PLC) to regulate Ca^{2+} dependent Cl− current (Hille, 1992; Blitzter et al., 1993). Thus, an alternative scheme for the mGluR6 cascade could be that G_{on} activates PLC to produce inositol tri-phosphate (IP3). This would raise internal Ca^{2+} concentration, which would activate a calmodulin-sensitive PDE. The PDE could then modulate cGMP concentration (Dabin et al., 1995). Several components of this cascade were found in rod bipolar cells (IP3 receptor: Peng et al., 1991; PLC: Ferreira and Pak, 1994; the message for a calmodulin-sensitive PDE: Dabin et al., 1995). Further, whole cell recordings show that L-AP4 seems to activate PLC via G_{on} or G_{on} in rod bipolar cells (Feigenspan and Bormann, 1994). Since L-AP4 is not an agonist for mGluR1 and mGluR5, this effect could not be mediated by these receptors. This supports the involvement of PLC in the mGluR6 cascade.

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**LITERATURE CITED**


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