Identification of a G-protein in depolarizing rod bipolar cells

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Abstract
Synaptic transmission from photoreceptors to depolarizing bipolar cells is mediated by the APB glutamate receptor. This receptor apparently is coupled to a G-protein which activates cGMP-phosphodiesterase to modulate cGMP levels and thus a cGMP-gated cation channel. We attempted to localize this system immunocytochemically using antibodies to various components of the rod phototransduction cascade, including G (transducin), phosphodiesterase, the cGMP-gated channel, and arrestin. All of these antibodies reacted strongly with rods, but none reacted with bipolar cells. Antibodies to a different G-protein, Gαs, reacted strongly with rod bipolar cells of three mammalian species (which are depolarizing) and APB-sensitive. Also stained were subpopulations of cone bipolar cells but not the major depolarizing type in cat (h). Gβ antibody also stained certain salamander bipolar cells. Thus, across a wide range of species, Gαs is present in retinal bipolar cells, and at least some of these are depolarizing and APB-sensitive.

Keywords: Phosphodiesterase, cGMP-gated channel, Transducin, Arrestin, G-protein, Retina, Rod bipolar

Introduction
Synaptic transmission from photoreceptor to depolarizing bipolar cells is mediated by a glutamate receptor that binds the agonists 2-amino-4-phosphonobutyric acid (APB) (Sheils et al., 1981; Slaughter & Miller, 1981). This receptor appears to be coupled to a second messenger because the bipolar’s response is slow and runs down rapidly when the cell is dialyzed with a whole-cell patch electrode (Nawy & Jahr, 1990; Sheils & Falk, 1990), but not when electrical continuity is established via synaptin for-}

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there might be conservation of amino-acid sequences and thus immunological cross-reactivity. Cross reactions, furthermore, should be restricted to the depolarizing bipolar which termin-

ally large, and possibly exclusively, deep in the inner plex-i-

form layer (sublamina b) (reviewed by Sterling, 1990). Initially, we focused on cat retina where all of the types of bipolar cell that terminate in sublamina b have been identified (Cohen & Sterling, 1990). To determine the generality of our findings, the experiments were extended to other mammals and to tiger sal-

apatkan (one of the species in which the cGMP-dependent cur-

rent was reported in bipolar cells). In the text “retina” always refers to cat unless otherwise noted.

Materials and methods
Cats (4), deeply anesthetized by pentobarbital (40 mg/kg), were

encultured. Eyes were hemisectioned and fixed for 1 h in one of the following fixatives: 4% buffered paraformaldehyde with or

without 0.1% glutaraldehyde (0.1 M phosphate buffer, pH 7.3) or 4% buffered formaldehyde plus 0.1% glutaraldehyde (0.1 M PIPES buffer, pH 6.8). Eyes from cows (2), monkey (1),

Macaca mulatta, rat (2), and salamander (1) were treated similarly.

Immunocytochemistry
The cryo-cut was washed and immersed in 30% buffered sucrose

overnight. Retina was cut into small pieces, sectioned in a cryo-

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stat (8 µm), and placed on glass slides. Two different staining protocols gave the same results. In the first, all incubations were carried out at room temperature. Sections were permeabilized for 30 s with acetone, washed, reduced with 2 mg/ml sodium borohydride in phosphate-buffered saline (PBS), washed, blocked for 30 min in diluent containing normal goat serum (5%) and Tween 20 (0.05%) in PBS for 20 min, incubated in primary antibody in the same diluent for 2 h, washed, incubated in secondary antibody conjugated to fluorescent marker for 1 h, washed, and mounted in gelvatol.

In the second protocol, sections were blocked in diluent containing Triton X-100 (0.3%) plus normal goat serum (20%) in phosphate buffer for 30 min, incubated in primary antibody overnight at 4°C, and in secondary antibody for 2 h at room temperature. In some experiments, the second antibody was conjugated to HRP and visualized by diaminobenzidine reaction.

G-protein probes were rabbit antibodies raised to small peptides (10-17 residue). The peptide sequences had been deduced from the base sequences of the cloned α and β subunits and relative specificities were tested on Western blots using native recombinant forms of Gs, Go, Gq, and Gi. These antibodies and the sequences to which they were directed were: Gs, “common,” 1978, CCAGA/3/GKSTTVy; Gα15, DPVGGKQKRETTRTL; Gα13, Gα16, and Gα21, 2870, KNLKD/3/CGLG; Gqα1, 2919, CTPGAP/3/ESGKTPEL; Gqα15, 2347, CKETTEDQR/N/EKQ; Gqα13, 9072, ANL/3/RCCG/LY; Gqα16, and Gqα17, 0945, QGNN/3/KEYN/LV; all but 2347 and 0945 have been previously described (Carlon et al., 1989; Law et al., 1991).

The antibody to Gαq/α11α12 (PMc ID1, gift from Dr. Molday) was mouse monoclonal (Molday et al., 1990) diluted 1:20. Four different polyclonal antibodies raised in rabbit were used to detect PDE. One was produced against the whole enzyme (gift from Dr. N. Philp) and diluted 1:100 and three, also produced in rabbit, were specific to peptides α 740-755, γ 50-63 and γ 1-49 (gift from Dr. D. Takemoto; Takemoto et al., 1992), and diluted 1:25-30. The polyclonal antisem to arracin was made in rabbit (gift from Prof. Gery), and diluted 1:1000. All antibody to protein kinase C (PKC) was mouse monoclonal (clone MC5, Amersham, Illinois) diluted 1:100. All secondary antibodies were F(ab)2 fragments (Jackson Immuno Research Labs., Pennsylvania) diluted 1:50.

Results
Elements of the phototransduction cascade were not detected in bipolar cells
G-proteins are thought to be present in all cells, and this was easily demonstrated in the retina by applying antibody raised to a peptide sequence overlapping the G-1 GTP binding/hydrolysis domain (Carlon et al., 1989). Since this site is common to many G-proteins and highly conserved (Bourne et al., 1991), the antibody stained every cell, both neurons and glia, and also both synaptic layers (Fig. 1A). A similar result was obtained with antibody recognizing the β and β′ subunits which are similarly conserved (not shown). We then applied antibodies reactive for Gαq, Gα16, and to some extent Gα15 (Carlon et al., 1989), but which also recognize transducin (Gqα1). Stain was intense in the photoreceptors expected, and restricted entirely to that region (Fig. 1B).

Next, we applied antibodies raised to PDE. This stain, too, was intense in the photoreceptor outer segments but absent or weak elsewhere in the retina (Fig. 1C) as well as other species tested (salmon, rat, and monkey; not shown). When we applied antibodies raised to the Gαq/α11α12 channel (Molday et al., 1990), stain was intense in the photoreceptor outer segments but entirely restricted to them in cat (Fig. 1D) and in other species tested (salmon, rat, and monkey; not shown). Wissle et al. (1992) report a similarly negative result using the same

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**Fig. 1.** Cat retina stained for various components of the phototransduction cascade. (A) A, stained regions are dark (DAB); in B-D, stained regions are bright (Rhodamine). A: Antibody to conserved region of α subunit of G-protein (antisemur 1989). All retinal layers are stained. Scale bar = 20 µm. B: Antibody that recognizes Gα15 (G15/3), and subtypes of Gαq (Gq/3). Only outer segments are stained. C: Antibody to Gα9/α10 (whole enzyme). D: Antibody to Gαq/α11α12 channel. In B, C, and D, outer segments were stained but not bipolar cells. The light stain in the inner segment and the plexiform layers was mostly from a general background due to the use of polyclonal antibodies. In D, a monoclonal antibody was used and background is minimal. Scale bar = 20 µm. OS: outer segments; IS: inner segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layers; and GCL: ganglion cell layer.
antibody. Finally, we applied antibodies raised to arrestin, which is the 48 kDa protein involved in arresting further activation of G-protein by rhodopsin during deactivation of the transdu-
cation cascade. Arrestin is present, not only in photoreceptors, but also in tissues using metabotropic α and β-adrenoceptor mechanisms (Lowe et al., 1990). However, in retina staining by several polyclonal antibodies raised to arrestin was confined to the photoreceptors. Thus, antibodies to four elements con-
trolling the phototransduction cascade—G<sub>α</sub>, PDE, cGMP-gated sodium channel, and arrestin—failed to stain bipolar cells either in mammalian or salamander retina.

G-protein in bipolar cells is G<sub>α</sub>.

Failing to find G<sub>α</sub> in bipolar cells, we applied a panel of anti-
bodies with demonstrated specificity for other G-protein spe-
cies. The antibody to G<sub>α</sub>, stained all structures but only faintly. Antibody to G<sub>α1</sub> stained ganglion cell axons (as reported by Hirino et al., 1990), and antibody to G<sub>α2</sub> stained Muller cells in a pattern to be reported elsewhere. However, none of these antibodies stained the bipolar cells or structures in either plexi-
form layer.

Antibody to G<sub>α</sub> (without distinction between G<sub>α1</sub> and G<sub>α2</sub>) strongly stained certain bipolar neurons and weakly and rarely stained certain amacrine cells and was absent from photorecep-
tors, inner plexiform layer, and Muller cells. Stain was intense in both the outer and inner plexiform layers (Fig. 2A). There was no stain when the primary antibody was omitted, and pre-
absorption of the primary antibody by the peptide to which it was directed (ANNL.RGCGGYL) reduced staining markedly (Figs. 2B and 2C). Preabsorption with a different peptide (NPE-
KLEFYNV) did not affect the stain (Fig. 2D). The G<sub>α</sub> antibody exhibits a weak cross-reactivity with G<sub>α1</sub> (as determined by Western blotting with purified sub-
unit). We tested the possibility that the G<sub>α</sub> stain (Fig. 2) repre-
sents G<sub>α1</sub>. This was accomplished by comparing the G<sub>α</sub> stain to that obtained from a G<sub>α1</sub> antibody which by West-
ern blotting exhibits a substantially stronger reactivity for G<sub>α1</sub>. The G<sub>α</sub> did not stain rod bipolar cells (Fig. 1B). Therefore, we conclude that the “G<sub>α</sub>like” immunological staining genuinely represents the distribution of G<sub>α</sub>. The distribution of G<sub>α</sub> was generally similar in other mammalian retinas (rat, monkey, and cow) and in salamander (Fig. 3). When staining intensity was reduced, three bands were evident in the inner plexiform layer: strata 1 and 5 stained intensely, stratum 3 weakly (not shown); although the preferential staining of strata 1 and 5 are evident in Fig. 2 and to some extent in Figs. 3A and 3C.

G<sub>α</sub> is localized to rod bipolar and certain cone bipolar cells.

Bipolar neurons in cut retina, indeed in all mammalian studied, collect chemical synapses exclusively either from rods or from cones (Cajal, 1972). The bipolar population in cat is about equally divided between rod bipolar and cone bipolar cells. For example, in the central area there are about 30,000 rod bipolar and about 36,000 cone bipolar cells per mm<sup>2</sup> (Sterling et al., 1988; Cohen & Sterling, 1990; Gregorath et al., 1990). Cajal (1972) originally recognized rod bipolar cells because their den-
\-drites ascend through the layer of cone pedicles to form sprays of extremely fine terminals within the layer of rod spherules. We now also know the soma position of the rod bipolar cell and that it distributes most densely of all bipolar types. The distri-
bution of G<sub>α</sub> showed these very features: presence in fine ter-
\-minals in the layer of rod spherules and in densely packed bipolar somas high in the inner nuclear layer (Figs. 2 and 4). These signatures of the rod bipolar cell were observed also in other mammalian retinas to which we applied the G<sub>α</sub> antibody. The monkey retina showed particularly clearly the extension of stained dendritic terminals into the rod spherule layer (Fig. 3C).

Fig. 2. A: Antibody to G<sub>α</sub>. Staining is intense in the synaptic layers (OPL, IPL), and present also in rod bipolar and some cone bipolar and amacrine somas. B: Control: primary antibody omitted from the incubation. C: Control: primary antibody was preabsorbed with its target peptide (30 μg/ml) before primary incubation. All staining in the OPL, INL, and IPL was eliminated; ganglion cell layer displayed some nonspecific stain. D: Control for preabsorption procedure: primary antibody incubated with a different peptide (50 μg/ml) did not block staining (cat, rhodamine).
Polynk (1941) described the “mop” bipolar that forms this pattern, and this cell is agreed to collect exclusively from rods (Boycott & Dowling, 1969; Grünter & Martin, 1991).

Although it was clear that many rod bipolar cells harbor G_{0}, it was impossible to be certain that all of them do; nor could we tell whether certain cone bipolar types might also be stained. Therefore, we used the fact that antibody to certain isoforms of protein kinase C (PKC) labels the rod bipolar cell population completely and exclusively (Gefrerath et al., 1990; Grünter & Martin, 1991; Negishi et al., 1983). Sections were treated simultaneously with antibody to PKC (coupled indirectly to Texas red) and with antibody to G_{0} (coupled indirectly to fluorescein isothiocyanate). There was dramatic conjugation of the two stains. Fig. 5A shows the rod bipolar population photographed for PKC, and Fig. 5B shows the same region photographed for G_{0}. Every cell that is positive for PKC is also positive for G_{0}; therefore, the rod bipolar cell is certainly the major cell type containing G_{0}.

A few cells are positive for G_{0} but negative for PKC (boxes in Fig. 5). These cells, with somas at middle levels of the bipolar layer, are roughly one-tenth as numerous as the rod bipolar cells. Dentrites positive for G_{0} and negative for PKC could be seen in color micrographs of the outer plexiform layer, just beneath the cone pedicles. Thus, it is likely that these G_{0} positive cells are cone bipolar neurons. Given their numbers, they probably represent a single type, possibly b_{4}, a type with axon arbor in stratum 3 and soma in the middle of the bipolar layer (Cohen & Sterling, 1990).

G_{0} and PKC differ in subcellular distribution

Looking for clues to function, we noticed carefully the subcellular locations of these probes. Staining for PKC was intense, in the soma, especially the supranuclear region, and in the proximal dendrites, but markedly weaker in the distal dendritic tips (Fig. 5A). Some PKC has been localized to the tips by electron microscopy (Gefrerath et al., 1990; Grünter & Martin, 1991). Stain for G_{0} was present in the proximal dendrites, but was also intense in the distal tips (Fig. 5B). A double exposure in color showed the proximal dendrites as yellow, indicating that both PKC (red) and G_{0} (green) were present. The distal tips were green indicating that mainly G_{0} was present. The rod bipolar axon terminals in sublamina A stained intensely for both PKC and G_{0}. The fewer levels of PKC in the distal dendritic terminals was consistent with the finding that PKC inhibitors do not affect the current evoked by APB (Yamashita & Wässle, 1991).

Discussion

In these experiments, probes applied to several components of the photoreceptor cascades (G_{0}, PDE, cGMP-gated channel, and arrestin) stained strongly the photoreceptor outer segments in species from salamander to monkey. This shows that (1) the antigenicity of these components survived our procedures for fixation and tissue preparation and (2) these components are well enough conserved to cross-react reliably with antibodies from different species. The inner retina was consistently negative; none of these antibodies reacted with bipolar cells or other neural elements in any species. This confirms the finding of Wässle et al. (1992) who found no staining in cat and rat inner retina for the cGMP-gated channel using the same antibody. Thus, we found no evidence for a homologue of the phototransduction pathway, as proposed by Naus and Jahn (1990) and by Shells and Falk (1990). Since these physiological studies strongly favor the existence of such a system, one might imag...
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Fig. 4. Antibody to Gα, Distal tips of bipolar dendrites (arrow) stain intensely in OPL at the level of rod spherules. Several cone pedicles observed under DIC optics are outlined. Rod spherules fill the region from the outer surfaces of the pedicles to the inner surfaces of the photoreceptor somas (ONL). Two spherules (*) are shown with central dark slots. These are the stained tips of rod bipolar dendrites (arrows). 1 μm radial section, DAB.

Fig. 5. A: Antibody to PKC (visualized with Texas red). B: Same section also stained with antibody to Gα (visualized with FITC). The antibodies colocalize in rod bipolar cells. Caudal somas in mid-INO (bomed) intensely stained for Gα but not for PKC (bomed). These are probably cone bipolar cells. Antibody to PKC stains axonal endings intensely and the dendritic endings runadly. In contrast, Gα stains the dendritic endings intensely and apparently less so the axonal endings.

intact that it does exist, but diverges so sharply at the molecular level from the photoreceptor transduction system that it fails to cross-react with our probes. On the other hand, Hirano and MacLeish (1991), recording from salamander depolarizing bipolar cells, find a highly negative reversal potential for APB which is consistent with its being coupled to a cGMP-gated sodium channel.

In contrast to our negative results for components of the phototransduction cascade, immunoreactivity for a different G-protein (Gβγ) is present in a major class of depolarizing, APB-sensitive bipolar cells (rod bipolar). This staining is conserved across mammals (rat, cat, cow, and monkey) and is present also in salamander bipolar cells (which are not exclusive for rods). Peptide absorption controls suggest that the staining reflects genuine Gβγ. Similar staining to Gβγ has been reported previously (Yasuhama et al., 1987a, b; Lad et al., 1987) but the cellular and subcellular localization was not determined. The fine dendritic terminals that innervate rod spherules stain intensely (Fig. 4). Gβγ is in the right place to be modulated by the APB receptor. On the other hand, no experiments as yet actually demonstrate coupling of Gβγ to the APB receptor. Furthermore, staining for Gβγ is not confined to depolarizing bipolar cells but is also present in subliminals a where it may well be localized in hyperpolarizing bipolar cells and/or amacrine cells. Finally, Shih and Fark (1992) suggest that the G-protein modulated by the APB receptor is sensitive to both persis and chelation toxins, sensitivities which have not been reported for Gα.

In cat, Gα staining is also present in one cone bipolar type, probably Gβγ, which like rod bipolar cells has been postulated to carry low temporal frequencies (Cohen & Sterling, 1990). The other cone bipolar types innervating sublimina a, apparently including the most numerous type, b, (known to be depolarizing, Nelson & Kell, 1983), did not stain. Therefore, different types of depolarizing bipolar cell within the same retina exhibit different chemical pathways.

Gα is also present in the rod bipolar axon terminal, there in conjunction with intense immunoreactivity to PKC. An association between Gα and PKC has been observed in other brain regions (Worley et al., 1986). Since the main direct synaptic input to this axon terminal is GABAergic (Chun & Wissel, 1989; Freed et al., 1987; Pourcho & Owczarzak, 1989), one could imagine a system linking these components to control gain at the rod bipolar output. For example, a GABA receptor might activate Gα, thereby stimulating hydrolysis of phosphatidyl inositol to yield IP3 and diacylglycerol as exhibited in bipolar axon terminals (Peirce et al., 1991), and ultimately to increase Ca2+ and PKC activity. Data supporting Gα as an activator of phospholipase C metabolism are quite limited, so control of rod bipolar output might instead be related to direct regulation of Ca2+ or K+ channels. Thus, reports such as the present one, simple begin, for circuits whose anatomical structure has been largely determined, the dissection of their corresponding chemical architectures.

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