Primate Rod and Cone Photoreceptors May Differ in Glucose Accessibility

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Purpose. Glucose is crucial for the function of retinal photoreceptors, other retinal neurons, and glial cells. Exogenous glucose can be extracted from the retinal and choroidal circulation, and endogenous glucose may be generated from breakdown of intracellular glycogen stores. Because glucose deprivation is a critical component of retinal ischemia, the authors sought to determine the sites of glucose entry into and generation within the retina.

Methods. The localization of the glucose transporter, GluT-1, and the brain and muscle isoforms of glycogen phosphorylase, GlyP, was studied by immunohistochemistry of adult human and monkey retinas.

Results. Brain glycogen phosphorylase (B-GlyP) immunoreactivity was found in cone, but not rod, photoreceptors. There was immunostaining of foveal and peripheral cones throughout the cytoplasm from the outer segment to the synaptic pedicle. Short wavelength ("blue") cones were positive for B-GlyP. Diffuse staining of the inner and outer plexiform and the nerve fiber layers did not resemble the distinct morphology of Müller cells. Immunoreactivity to muscle GlyP (M-GlyP) was confined to selected synaptic layers of the inner plexiform layer in monkey retina. Staining with antibody to GluT-1 demonstrated diffuse reactivity throughout the retina, including the blood–retinal barrier cells, retinal pigment epithelium, and vascular endothelium. Ultrastructural immunohistochemistry showed staining of rod and cone inner and outer segments.

Conclusions. These immunohistochemical studies indicate that rod and cone photoreceptors have the biochemical capability to transport exogenous glucose from the circulation. Only cones appear capable of using endogenous glycogen stores. These findings imply that cones could be more resistant to acute reductions in circulating glucose during hypoglycemia. However, during hypoxic insult, glycolysis and anaerobic glycolysis could result in increased production of intracellular lactic acid, potentially predisposing the cone to acidotic damage. Invest Ophthalmol Vis Sci. 1995;36:1259–1270.

The earliest metabolic studies performed on retina demonstrated a high rate of aerobic glycolysis and respiration.1 Since that time, many experiments have shown the importance of glucose metabolism in maintaining visual function.2–6 Glucose deprivation of retina was more damaging than hypoxia as measured by the recovery of light-evoked responses.7 Müller cells were damaged by glucose deprivation, although they were more resistant to injury than neural cells.8

There are two potential sources of glucose for the retina, an exogenous source from circulating glucose and an endogenous source from glycogen stores. Törnquist and Alm9 showed that 80% of the retinal glucose is derived from the choroidal circulation. Early experiments demonstrated the facilitated diffusion of glucose into retina.10 Recent investigation has demonstrated that facilitated glucose entry is accomplished by one of the family of glucose transporter proteins (GluT-1). GluT-1 is localized to sites of the blood–retinal barrier, that is, retinal vessel endothelial cells...
and the apical and basal surfaces of the retinal pigment epithelium.11-15

The localization and relative quantitation of glycogen deposits in the retina is complex because there are species differences, and glycogen stores are rapidly depleted by exposure of the retina to a variety of physiological stimuli.14 Retinal ischemia leads to loss of histochemically demonstrable glycogen within 30 to 45 minutes.15 This may be responsible for some of the variable results reported in the literature. Schabadasch and Schabadasch16,17 showed differences that depended on light and dark adaptation in the frog. They also made a strong point of the lability of glycogen to different types and times of fixation. In tissue prepared by freeze-drying, glycogen was reported to be in Müller cells and was found in cone inner segments in primates.18 In contrast to these light microscopic studies, quantitative electron microscopy of rat retina showed that the density of glycogen particles was greater in Müller cells than in neuronal cells, but, because the neuronal cells occupy 90% of the cytoplasm of the inner plexiform layer, the neurons contain approximately one third the total glycogen in this region.19

The enzyme responsible for the breakdown of glycogen in tissue is glycogen phosphorylase (GlyP; EC 2.4.1.1), the rate-determining enzyme catalyzing glycogenolysis. GlyP activity is regulated by reversible phosphorylation and by allosteric and is intimately associated with glycogen particles.20 Using microdissection techniques, GlyP activity was highest in the photoreceptor outer and inner segments in mudpuppy retina21 and in the inner nuclear layer in rabbit retina, and it was lowest in the photoreceptor outer and inner segment layers in monkey and rabbit retina.22 There are three isozymes of GlyP (brain, muscle, and liver) that are expressed in mammals in a tissue-specific and developmentally determined fashion.23,24 A polyclonal antibody against the human brain isozyme has localized the protein predominantly to astrocytes in rat and monkey brain.25

Because glucose deprivation accentuates the neuropathology observed in retinal ischemia,8 it seemed reasonable to determine whether the enzymes necessary for exogenous and endogenous glucose access were present in all retinal cell types. Therefore, we examined immunoreactivity of antibodies to GluT-1 and the brain and muscle isozymes of GlyP in retina.

MATERIALS AND METHODS

Adult human eyes were obtained from the Sierra Eye and Tissue Bank (Sacramento, CA). Not more than 6 hours elapsed between the time of death and fixation of the human eyes. Adult rhesus (Macaca mulatta) and cynomolgus (Macaca fascicularis) monkey eyes were
FIGURE 2. (A) Immunoreactivity of B-GlyP in near foveal region of monkey retina. Henle’s fiber layer (H) was intensely stained. The OPL and IPL were diffusely stained. Some ganglion cell soma (arrow) were positive for B-GlyP. Bar = 100 μm. (B) Higher magnification of foveal region in monkey retina showed positive staining of inner segments, although outer segments were not obviously reactive. Bar = 25 μm.

obtained from the Regional Primate Center (Davis, CA) under conditions adhering to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The eyes were surgically enucleated and fixed immediately.

For light microscopy, a slit was made across the entire cornea, and the eye was immersed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 90 to 120 minutes, rinsed with buffer three times, dissected into appropriate-size pieces, infiltrated with 1.2 M acrylamide overnight, and frozen in Tissue-Tek OCT Compound (Miles, Elkhart, IN) in liquid nitrogen. Frozen sections of approximately 6-μm thickness were cut with an International Equipment Company cryostat at −20°C and stored in a humidified environment in the refrigerator until used.

Sections were treated with 3% hydrogen peroxide for 5 minutes to suppress endogenous peroxidase activity and were blocked with 0.5% to 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) with 1 mM MgCl₂ and 1 mM CaCl₂ (PBS M/C) for 15 minutes. Primary antibody, diluted in PBS M/C (sometimes containing 0.25% BSA), was reacted from 30 to 60 minutes at room temperature. After extensive rinsing, secondary antibody incubation was from 30 to 60 minutes at room temperature followed by buffer rinse. Immunoreactions were expanded using avidin-horseradish complex (ABC Kit; Vector, Burlingame, CA) for 30 minutes and developed with diaminobenzidine for 5 to 60 minutes.

Primary antibodies used were: GluT-1 (rabbit and-rat brain polyclonal from Calbiochem (La Jolla, CA), made against a peptide corresponding to carboxyl terminal residues 480 to 492); glial fibrillary acidic protein (rabbit anti-cow polyclonal; Dako, Santa Barbara, CA); brain or muscle glycogen phosphorylase (rabbit anti-human polyclonal); blue cone opsin (mouse monoclonal, OS-2; gift of Dr. Pal Röhlisch). Secondary antibody was biotinylated goat anti-rabbit immunoglobulin (IgG; Sigma, St. Louis, MO) (EY, Vector, San Mateo, CA). Secondary antibodies in the double labeling experiment were fluorescein conjugated anti-rabbit IgG (Sigma) and rhodamine conjugated anti-mouse IgG. Controls consisted of normal rabbit serum or PBS with 0.25%
FIGURE 3. (A) Staining of peripheral human retina with B-GlyP antibody. Cone cell cytoplasm was stained throughout the cell, and there was diffuse staining of the plexiform layers, as in the monkey retina. Bar = 50 μm. (B) Higher magnification showed the unstained nucleus of a cone cell and a lightly stained area in the synaptic pedicle that may have been the synaptic complex. Bar = 20 μm.

BSA. For the GlyP antibody reactions, preimmune serum was used as a control.

Although the B-GlyP antibody has been described,36 a brief description of the M-GlyP antibody follows. A 13-residue peptide, corresponding to cysteine coupled to the 12 carboxyterminal residues of human M-GlyP,23 was synthesized by the Protein Structure Laboratory (University of California, Davis). The N-terminal cysteine of the peptide was coupled to keyhole limpet hemocyanin as described.25 Preimmune serum was collected, and rabbits were immunized intramuscularly and intradermally with 100 μg of keyhole limpet hemocyanin-peptide suspended in 1 ml of 0.1 M sodium phosphate, pH 6.8, and mixed with 1.0 ml of incomplete Freund's adjuvant (Sigma) and 5 mg of Mycobacterium tuberculosis (Difco, Detroit, MI). Immune serum collected at 6 weeks was used for antibody characterization and immunohistochemistry (Gorn FA, unpublished, 1994).

Tissue processing for electron microscopy was similar to that for light microscopy, except that monkey eyes were fixed in 4% paraformaldehyde + 1% glutaraldehyde, rinsed in buffer overnight at 4°C, dehydrated in a graded ethanol series, and infiltrated with LRWhite resin (EMS, Fort Washington, PA). Blocks were polymerized overnight at 60°C, and sections were cut at 70 nm and supported on 200 mesh platinum copper grids.

Grids were incubated in PBS M/C with 1.5% normal goat serum in the blocking buffer for 30 minutes. Primary antibody (diluted 1/1000 in PBS M/C + 1 mg/ml BSA) was reacted for 1 hour, rinsed thoroughly, and followed by gold conjugated anti-rabbit IgG (15 nm, Amersham, Little Chalfont, UK) for 30 minutes. After rinsing, the antibody was poststained with 2% aqueous uranyl acetate for 5 minutes.

RESULTS

Glycogen Phosphorylase

Monkey eyes were obtained surgically and fixed immediately so that alteration of immunoreactivity from ischemia or autolysis was less likely than it was with human retina, but the results were similar in human specimens and in specimens from rhesus and cynomolgus monkeys. Immunoreactivity to brain glycogen phosphorylase antibody showed intense staining of cone cells (Fig. 1). The ellipsoid (mitochondria rich) and the myoid portions of the inner segment were stained, but myoid staining was more intense. Some profiles of cone outer segments were clearly stained. This stain-
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FIGURE 4. (A) Monkey retina stained with antibody to B-GlyP showed staining of all cones in the field. Bar = 20 μm. (B) The same section stained with antibody to blue cone opsin showed that the outer segment of only the center cone of the field was positive. Bar = 20 μm.

ing was not artifactual, appeared to be confined to the cone outer segment, and was not in the cone sheath of the interphotoreceptor matrix. Reaction deposit was present in the proximal cytoplasm of the cone cells, neatly outlining the nucleus, filling the cell to the synaptic ending. Immunoreactivity was diffusely present in the outer plexiform layer, inner plexiform layer, and nerve fiber layer. Except for some intensely positive ganglion cell soma in peripheral and near foveal regions, nuclei of the retina were not stained. The retinal pigment epithelium contained dark melanin granules, and examination at high magnification did not show B-GlyP immunoreactivity in the cytoplasm. The rod cells were not stained by B-GlyP antibody.

The pattern of positive cone staining to B-GlyP was present in the central (Fig. 2) and the peripheral retina of monkey and human retinas. Ellipsoid and myoid portions of the inner segment were stained in foveal cones, but the outer segments were not heavily immunoreactive in this region. The positive reactivity of all the cone cytoplasm resulted in intense staining of Henle's fiber layer, the axons of the cone cells (Fig. 2). In the human peripheral retina (Fig. 3), cones but not rods were intensely immunoreactive. There was diffuse staining of the outer and inner plexiform layers, but the nerve fiber layer was not as intensely stained as in the monkey retina. The human near foveal region had the same staining as the monkey (data not shown).

Although it appeared that all cones were immunoreactive to the B-GlyP antibody, we thought we would be unable to determine if blue cones were staining because they represent a small proportion (roughly 10%) of the total cone population. Therefore, we performed double-labeling studies localizing the OS-2 antibody to blue cone opsin and the brain glycogen phosphorylase antibody (Fig. 4). The blue cones were also positive for B-GlyP, which was distributed similarly in all cone types.

It can be seen in Figures 1, 2, and 3 that immunostaining for brain glycogen phosphorylase did not resemble the morphology of the Müller cell, extending from the inner to the outer limiting membrane. The fiber baskets of the Müller cell, processes extending past the outer limiting membrane into the interphotoreceptor space, were clearly not stained by antibody to B-GlyP. The reactivity of the outer and inner plexiform layers may, in part, be caused by staining of Müller cell processes in these layers.

We did not observe immunoreactivity to the muscle isozyme of GlyP in human retinas. In monkey retina, a railroad track pattern in the inner plexiform layer was observed (Fig. 5).

Results in our controls were usually negative (Fig.
Figure 5. Immunoreactivity of M-GlyP in monkey retina showed railroad track pattern in the inner plexiform layer, suggestive of layering of synaptic endings. Bar = 100 μm.

6), but occasionally we saw nonspecific, generalized, background staining in random specimens. This may have been caused by some cross-reactivity of the antiserum with other proteins, as seen in the preimmune serum (Fig. 7). Western blot analysis of monkey retina reacted with antibody to the B-GlyP antibody demonstrated one principal band at 97 kd and a faint band that may be the GlyP native dimer. The molecular weight of the B-GlyP monomer is 97 kd. 25

Two of six human specimens revealed insulin-dependent diabetes mellitus, but we saw no change in the staining pattern of B-GlyP (data not shown).

Glucose Transporter

Monkey retinas showed generalized staining of the cytoplasm of most retinal cells, but not the nuclei (Fig. 8a). Human retinas showed a similar pattern. Cells that form the blood-retinal barrier were positive for GluT-1, the retinal pigment epithelium, and the retinal vessel endothelial cell.

Light microscopy revealed that in the more peripheral retina, rod inner and outer segments were clearly immunoreactive but cones appeared to lack staining (Fig. 8b), although there may have been a thin rim of stain at the surface of the cone inner segments (Fig. 8c). However, the foveal cone inner segments were intensely stained in monkey (Fig. 8a) and human retinas (data not shown). We hypothesized that this apparent difference was an artifact caused by the thickness of the sections under examination as well as the difference in size of the cone outer and inner segments. If GluT-1 were present in the outer plasma membrane, as reported by Hsu and Molday, 28 and not in the cytoplasm of the cone, sections thicker than cone diameter would contain the full cone cell. The entire plasma membrane would be present in this section, and GluT-1 would be more readily visualized. Because the foveal cones were approximately 2 μm in diameter, the 6-μm-thick sections used would contain several foveal cones within one section. In contrast, the wider cone inner segment of the peripheral retina (approximately 8 to 9 μm) would result in only a portion of one cell in any given section. If GluT-1 were only in the outer plasma membrane, a thin rim of staining would be seen that might not be apparent because of the resolution of the light microscope. Therefore, we performed electron microscopic immunohistochemistry to localize GluT-1 subcellularly.

Results are seen in Figure 9. Gold particles indicating the presence of GluT-1 immunoreactivity were seen in rods and cones in inner and outer segments. They were found predominantly within the cytoplasm and not on the plasma membrane, as hypothesized. The numbers of gold particles per unit area of the ellipsoid was the same in rods and cones in the peripheral retina—15.68 ± 1.98 and 15.50 ± 2.60 particles per μm², respectively. Foveal cones had a lower density of GluT-1-immunoreactive gold particles—5.34 ± 0.88 particles per μm². However, the preservation of the inner segments of the foveal cones was less good than that of peripheral rods and cones (compare Figs. 9b and 9c with Fig. 9d), so it may be that the lower density of gold particles in the foveal cone ellipsoids is caused by swelling of the cytoplasm. As can be appreciated in Figure 9a, the myoid portion of the photoreceptor cells were distorted with the fixative used; therefore, we did not attempt to quantitate gold particles in these areas. The fiber baskets of the Müller cells were particularly rich in GluT-1 immunoreactivity (Fig. 9e).

Discussion

Although it is difficult to make definitive statements concerning biochemical functionality based on enzyme localization by immunohistochemical techniques, the results of this study imply a difference between rods and cones in primate retinas with regard to glucose availability.
Cone photoreceptors of primate retina appear to have access to glucose extracted from the circulation as well as glucose derived from the hydrolysis of glycogen stores, based on the presence of the brain isozyme of glycogen phosphorylase within the cone cytoplasm. In contrast, rods do not express either the brain or muscle isozyme of GlyP, indicating that rod photoreceptors have limited glycogenolytic activity. This suggests that primate cones, but not rods, may be transiently protected from hypoglycemia—for example, during insulin-induced hypoglycemia. Interruption of the choroidal circulation causes the disappearance of glycogen from the cone myoid within 20 minutes, presumably because of the activity of the B-GlyP found there. Kuwabara and Cogan stated that glycogen disappears from unfixed retinas within 3 to 6 hours of death or removal of the eyes, and they suggest that it is metabolically degraded, not simply solubilized by fixatives. Glycogen is not demonstrable in rabbit retina after 30 to 45 minutes of ischemia. Although these experiments induce both hypoglycemia and hypoxia, the loss of glycogen is known to be dependent on the activity of glycogen phosphorylase. For example, the genetic diseases of Hers' and McArdle's syndromes prove that glycogen cannot be degraded unless glycogen phosphorylase is present.

The lability of glycogen stores is undoubtedly caused by glycogen phosphorylase, which can be rapidly activated within milliseconds by the calcium-dependent stimulation of phosphorylase kinase. Matschinsky demonstrated that after only 1 minute of ischemia, the activity of glycogen phosphorylase more than doubles in all layers of rabbit retina. Glycogen can turn over rapidly with little net accumulation. For example, the overall content of glycogen in adipose cells is small, but the turnover rate is rapid. A similar phenomenon may occur in cone outer segments, which have not been shown to be positive for glycogen stores but were immunoreactive for B-GlyP in our study. GlyP may be a better indicator of potential glycogenolysis than the histologic presence of glycogen. Because Hsu and Molday have shown the presence of several enzymes of the glycolytic pathway in rod and cone outer segments, perhaps these cell compartments are more metabolically active than usually envisioned. The location of glycogen phosphorylase in human and monkey retina corresponds closely with the localization of glycogen stores, as described by Mizuno and Sato.

Brain glycogen phosphorylase immunoreactivity is found in all cone types of the retina, including the short wavelength cone (S-cone). It is known...
that these cones constitute approximately 10% of the total cone number, have some properties that are different from the red and green cone types, and are sometimes considered rod like. This S-cone pathway loses sensitivity in several disease states, including diabetes, retinitis pigmentosa, and glaucoma, but it is unknown if this is caused by alterations in the S-cone photoreceptor itself or in postreceptor sites. Acute changes in circulating glucose in patients with diabetes affect S-cone pathway functions but not functions of red and green cones. Therefore, we thought it possible that blue cones did not contain GlyP and could not react to transient hypoglycemia by the use of glycogen stores. Because our results show that S-cones do have B-GlyP, the explanation for the sensitivity of this pathway to changes in circulating glucose levels must be sought elsewhere. One may conjecture that a condition such as hypoxia, which could result in increased anaerobic glycolysis and produce lactate and a proton, is deleterious to blue cones, which may have a lower buffering capacity in the form of carbonic anhydrase.

Staining of retina with the muscle isozyme of GlyP is limited to a railroad track pattern in the inner plexiform layer of monkey retina. This probably represents the presence of this isozyme at specific synaptic contacts of certain cell types. Uptake of 2-deoxyglucose is found in a laminar pattern in the inner plexiform layer of rat (Wilson DJ, personal communication, 1994). Striated patterns of synaptic contacts are present in the inner plexiform layer of primate retina. Linear deposition of glycogen is seen in the inner plexiform layer of frog. Perhaps there are unique synaptic connections obligatorily dependent on glycogenolysis as an energy source. Although we are unaware of synapses dependent on glycogenolysis, it is known that vasoactive intestinal peptide and other (but not all) neurotransmitters can stimulate glycogenolysis in cerebral cortical slices. Ames et al have interpreted their studies to suggest that synaptic transmission (at least in the rabbit retina) is dependent on glycolysis rather than oxidative phosphorylation as an energy source. The presence of glycogen phosphorylase in the plexiform layers indicates that glycogen could be used in these layers as a source of glucose for glycolysis.

Our ultrastructural immunohistochemical demonstration of the GluT-1 transporter is the first in human retina. All the retinal cells are immunoreactive for Glut-1 in the human and monkey retina with the antibody used in this study. This is different from the sparse immunostaining reported in rat retina that was specific for the barrier cells of the retina—the retinal pigment epithelium and vascular endothelial cells. However, Harik et al remark that human and rat eyes stain similarly except that, in the human, "... neural elements, especially photoreceptors" were immunoreactive to antibodies to GluT-1. GluT-1 is present in bovine and chicken photoreceptor outer segment plasma membranes. Two recent studies have shown a much more widespread distribution of GluT-1 immunoreactivity in retinal cells, although they differ in some details from each other and from this study.

Although our light microscopic studies suggested a difference in staining of the foveal and nonfoveal cones, electron microscopic immunohistochemistry demonstrated that foveal and nonfoveal cones contained GluT-1. Although foveal
FIGURE 8. (A) Foveal slope of monkey retina. Immunoreactivity of GluT-1 was present in all retinal cells but not in their nuclei. GIS = cone inner segment; H = Henle’s fiber layer; GC = ganglion cell layer. Bar = 100 μm. (B) Parafoveal monkey retina. The nuclear layers were not immunoreactive to GluT-1 antibody, nor was the nerve fiber layer (NFL), but the plexiform layers were positive. The cone inner segments appear nonreactive, but Henle’s fiber layer was intensely stained. Bar = 100 μm. (C) Higher magnification of human parafoveal region showed apparent lack of staining of GluT-1 antibody in cone inner segments. Bar = 20 μm.

cones had a significantly lower density of gold particles representing sites of GluT-1, because of the relative swelling of this region compared to the peripheral retina, we were unsure whether this difference was of physiological significance. GluT-1 has been reported on the plasma membrane of both rod and cone outer segments. The electron microscopic results show gold particles predominantly in the cytoplasm of the photoreceptor inner segments. It is known, in cultured cells, that GluT-1 proteins can translocate from a cytosolic location to the plasma membrane under various stress conditions. This may provide an explanation for the disparity in results of our immunohistochemical studies and the biochemical analysis of Hsu and Molday. Our electron microscopic studies revealed only that foveal and nonfoveal cones were immunoreactive to the GluT-1 antibody used. Whether there is a differential distribution between active forms in the plasma membrane and cytosolic (therefore, presumably, inactive) forms will have to be the subject of a more exhaustive study of immunolocalization using a variety of fixatives, antibodies, and physiological conditions. There are few studies that deal with the in vivo regulation of GluT-1.

The difference between the rod and cone localization of B-GlyP in primates may reflect a different need for glucose in these two cell types or, as Macaluso et al state, “...a marked difference in metabolic mechanisms...” Although many studies have shown the dependence of retinal function on glucose, there is little information on differences between rods and cones. This leads us to question whether there are differences in energy requirements between rods and cones. In a general sense, one may think that the energy requirements of cones are greater than those of rods because, although rods and cones maintain a current in the dark, rods do not function in bright light whereas
cones continuously function and adapt to varying light levels. Cohen has noted that mitochondria are more prevalent in cone inner segments than in rods and calculated that the energy requirements of cones might be approximately 15 times greater than those of rods. This requires further study.

**Key Words**

glucose, glycogen, rods, cones, short wavelength cones, glycogen phosphorylase, glucose transporter, ischemia

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**References**


