Immunocytochemical Localization of Glutamate in Normal and Detached Cat Retina

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PURPOSE. Glutamate immunoreactivity in the mammalian retina has generally been observed using immersion fixation. The authors investigated glutamate immunoreactivity in the detached cat retina and reevaluated this activity in the normal retina using rapid fixation by perfusion.

METHODS. Unilateral retinal detachment was produced in cats by injecting 0.25% sodium hyaluronate into the subretinal space using a glass micropipet. The eyes were fixed by perfusion with a mixture of 1% glutaraldehyde and 4% formaldehyde 10 minutes and 60 minutes after detachment, and then they were examined by conventional light and electron microscopic immunocytochemistry.

RESULTS. In contrast to previous reports based on immersion fixation, the inner segment was not glutamate immunopositive in the normal retina. The inner segment showed intense glutamate immunoreactivity 10 minutes and 60 minutes after retinal detachment.

CONCLUSIONS. Glutamate immunoreactivity in photoreceptor inner segments may be a postmortem change induced by strong ischemia. Perfusion fixation is of critical importance when studying the immunocytochemical distribution of glutamate in the retina. (Invest Ophthalmol Vis Sci. 1998;39: 786-792)

Glutamate is generally accepted as the major excitatory neurotransmitter of the retina, and many immunocytochemical studies revealed the localization of glutamate in the vertebrate retina.1-12 Almost all these studies showed that photoreceptor inner segments, inner and outer plexiform layers, many ganglion cells, bipolar cells, and some amacrine cells are glutamate immunoreactive. Only one group of investigators who examined the goldfish retina reported that glutamate immunoreactivity was absent from the photoreceptor inner segments fixed by immersion. However, they eventually asserted that the photoreceptor inner segments were glutamate immunoreactive by showing positive glutamate immunoreactivity in the photoreceptor inner segments of the isolated retina incubated in an artificial aerobic environment.10,11 They also reported that glutamate immunoreactivity of the photoreceptor was extremely volatile. In all cases, the enucleated eyes were fixed by immersion after death.1-12 Therefore, the retinas examined in those studies may have undergone severe ischemia before fixation.

However, several investigators have reported that abundant glutamate is released from ischemic neural tissues, including the retina13-15 and that the process is followed by delayed neuronal death.16 Several immunocytochemical studies have reported that redistribution of some amino acid neurotransmitters occurs in the neural tissue after ischemia.17,18 We considered that the reported distribution of glutamate includes some postmortem changes and that the distribution of glutamate in the retinas that underwent ischemia may have been different from that of healthy retinas fixed rapidly before ischemia.

Retinal detachment is caused by the separation of the sensory retina from the retinal pigment epithelium as a result of the accumulation of subretinal fluid.19 The distribution of the retinal vessels in cats resembles that in humans and is confined to the brain retinal layers (the inner portion of the sensory retina).20,21 The cone and rod layer, the outer nuclear layer, and the greater portion of the outer plexiform layer are nourished by choriocapillaries through the retinal pigment epithelium. Thus, retinal detachment in the cat provides a good model of the ischemic retina in the neuroepithelial layer. It also provides the opportunity to examine the effect of ischemia on the distribution of glutamate in the retina.

The present study investigates the glutamate immunoreactivity of detached cat retinas as a model for ischemia in the neuroepithelial layer of the retina and reevaluates glutamate immunoreactivity in the normal cat retina by comparing the results obtained from healthy retinas fixed by perfusion with those fixed by immersion.

METHODS

Surgical Procedure

All animals in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We examined six adult cats (weighing approximately 3.0 kg each). Unilateral rhegmatogenous retinal detachment was produced in the upper nasal midperipheral retina in four cats. The operation proceeded essentially as...
A glutamate-specific antibody was prepared as described previously. Briefly, glutamate was coupled with rabbit serum albumin using glutaraldehyde, and a glutamate-glutaraldehyde-rabbit serum albumin conjugate was emulsified with an equal volume of complete Freund's adjuvant and was repeatedly injected subcutaneously into multiple sites on the back of a rabbit. The antibody was purified by affinity chromatography with glutamate immobilized on cellulose (GCL-2000m; Seikagaku Kogyo, Tokyo, Japan), as described previously. The specificity of the purified antibody was examined by a dot immunobinding assay. The purified antibody reacted significantly only with glutamate (Fig. 1).

Antibodies

For light microscopic examination, the retinas were embedded in paraffin. Sections (5 μm) were mounted on albumin-coated slides, deparaffinized in xylene, and soaked in 0.3% H2O2 in methanol for 5 minutes. The sections were hydrated through a graded alcohol series and were washed twice in NaCl-TB (0.15 M Tris-HCl buffer containing 500 mM NaCl, pH 7.6) for 5 minutes each. The sections were incubated in 1% skim milk diluted in NaCl-TB at room temperature with fresh 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Chemical Institute, Kumamoto, Japan) that served as the chromogen (20 mg in 100 ml NaCl-TB that contained 0.01% H2O2). After washing in PBS, the sections were treated with 0.01% H2O2. The sections were rinsed several times in PBS and were blocked for 60 minutes with 3% bovine serum albumin (BSA) in PBS (3% BSA-PBS, a blocking solution) in a humidified chamber. Controls were incubated with preimmune rabbit serum diluted 1:500. Antigen-antibody reactions were visualized using peroxidase-anti-peroxidase. Purified antibody immunostained only Glu (arrowhead).

Immunogold Immunocytochemistry for Electron Microscopy

The retinas were postfixed with 1% osmium tetroxide in 0.1 M phosphate-buffered saline (PBS), pH 7.4. After washing in PBS, the retinas were dehydrated in a series of graded ethanol and acetone and then were embedded in an epoxy-based medium (Quetol 812; Nissin Electron Microscopy, Tokyo, Japan). After specific areas of interest were identified on toluidine blue-stained semithin sections, the blocks were trimmed to the appropriate dimensions, and ultrathin serial sections (80-nm thick) were collected on nickel grids (150 mesh square). An ultramicrotome (Ultracut; Reichert, Wein, Austria) was used for semithin and ultrathin sectioning.

All steps proceeded at room temperature. Sections were immersed in 1% sodium metaperiodate for 30 minutes to oxidize the osmium, and this was followed by a wash in PBS. The sections were blocked for 60 minutes with 3% bovine serum albumin (BSA) in PBS (3% BSA-PBS, a blocking solution) in a humidified chamber. Excess blocking solution was removed, and the sections were incubated in antigu glutamate antibodies for 14 hours and washed in PBS. Antigu glutamate antibodies were diluted to 0.1 μg/ml in 1% BSA-PBS. The sections were incubated with a rabbit antigu glutamate antibody (1:100 with NaCl-TB for 1 hour in a moist chamber at room temperature. Controls were incubated with preimmune rabbit serum diluted 1:500. The sections were incubated for 10 minutes at room temperature with fresh 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Chemical Institute, Kumamoto, Japan) that served as the chromogen (20 mg in 100 ml NaCl-TB that contained 0.01% H2O2). The sections were rinsed several times in NaCl-TB between each step. To identify the immunonegative structures of the retina, some neighboring sections were stained with methylene blue.

Immunohistochemistry for Light Microscopy

For light microscopic examination, the retinas were embedded in paraffin. Sections (5 μm) were mounted on albumin-coated slides, deparaffinized in xylene, and soaked in 0.3% H2O2 in methanol for 5 minutes. The sections were hydrated through a graded alcohol series and were washed twice in NaCl-TB (0.15 M Tris-HCl buffer containing 500 mM NaCl, pH 7.6) for 5 minutes each. The sections were incubated in 1% skim milk diluted in NaCl-TB for 30 minutes at room temperature, then with an antibody against glutamate (0.1 μg/ml NaCl-TB) for 16 hours, and then by goat antirabbit immunoglobulin G conjugated with horseradish peroxidase (Medical & Biological Laboratories, Nagoya, Japan) diluted 1:100 with NaCl-TB for 1 hour in a moist chamber at room temperature. Controls were incubated with preimmune rabbit serum diluted 1:500. The sections were incubated for 10 minutes at room temperature with fresh 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Chemical Institute, Kumamoto, Japan) that served as the chromogen (20 mg in 100 ml NaCl-TB that contained 0.01% H2O2). The sections were rinsed several times in NaCl-TB between each step. To identify the immunonegative structures of the retina, some neighboring sections were stained with methylene blue.
Protein A coupled to 15-nm colloidal gold particles (EMPAG15; British BioCell International, Cardiff, UK) at a dilution of 1:40 in 1% BSA-PBS and then were washed in PBS. After a rinse in double-distilled water, the grids were dried thoroughly and counterstained with uranyl acetate and lead citrate.

**RESULTS**

**Light Microscopic Findings in the Normal Retina**

Figure 2 shows glutamate staining in the normal retina fixed by perfusion and immersion. In the retina fixed by perfusion, many ganglion cells, bipolar cells, and amacrine cells were glutamate immunoreactive. The inner and outer plexiform layers were also glutamate immunoreactive. However, the inner and outer segments of the photoreceptors and the outer nuclear layer did not seem to be glutamate immunoreactive (Fig. 2A). In the retina fixed by immersion, many ganglion cells, bipolar cells, and amacrine cells were as glutamate immunoreactive as those fixed by perfusion. The inner plexiform layer was highly glutamate immunoreactive,
whereas the outer plexiform layer was less so than that of the retina fixed by perfusion. The photoreceptor inner segments were moderately glutamate immunoreactive (Fig. 2B). Glutamate immunoreactivity in the inner segment was the most striking difference between retinas fixed by perfusion and those fixed by immersion.

Light Microscopic Findings in the Detached Retina

Figure 3 shows glutamate staining in the detached retina fixed by perfusion. Although retinal thickness increased as a result of retinal edema after detachment, the profile of glutamate staining in the inner retina was similar to that in the normal inner retina. In the photoreceptor inner segments, however, glutamate immunoreactivity was evident at the edge of retinal detachment and gradually increased in proportion to the height of detachment (Fig. 3A). In the moderately detached retina, the photoreceptor inner segments were glutamate immunoreactive (Fig. 3B). The section shown in Figure 3 is the retina fixed 10 minutes after detachment. The distribution of glutamate staining in retinas fixed 10 minutes and 60 minutes after detachment was the same (data not shown).

Electron Microscopic Findings

At the electron microscopic level, the distribution of immunogold labeling in the photoreceptor layer was basically the same as that at the light microscopic level. In the normal retina fixed by perfusion, the level of labeling in the outer and inner segments was not above background (Fig. 4A). In the normal retina fixed by immersion, the inner segments were moderately labeled, whereas the outer segments were weakly labeled (Fig. 4B). In the detached retina fixed by perfusion, the labeling pattern in the outer and inner segments was similar to that in the normal retina fixed by immersion, but the inner segments were more intensely labeled (Fig. 4C). Although rich in mitochondria, the immunogold label in the inner segments was not exclusively localized to these organelles.
**DISCUSSION**

Most immunochemical studies of the retina have investigated the retina fixed by immersion, possibly because perfusion fixation may expand blood vessels in the retina, which can lead to morphologic changes in the inner retina. The present study showed that this kind of change was small in the inner retina and was not found in the outer retina, nor was it found in the inner segments of the photoreceptor. In contrast, glutamate immunoreactivity in the photoreceptor inner segment differed significantly between perfusion and immersion fixation. The staining pattern in the inner segments fixed by immersion was similar to that in the detached retina fixed by perfusion. This observation suggests that photoreceptor inner segments are glutamate immunoreactive only under acute anoxic conditions.

Artifactual glutamate immunoreactivity in the inner segments fixed by immersion may be explained by the penetration rate of the fixative. The immersion fixative is generally composed of glutaraldehyde alone or a glutaraldehyde/paraformaldehyde mixture because glutaraldehyde is critically important when fixing glutamate. However, glutaraldehyde is thought to penetrate tissue more slowly than other fixatives. To our knowledge, the penetration rate of fixative in the retina has not been studied. A study of the liver has shown that the rate of glutaraldehyde and paraformaldehyde penetration was calculated by the following formula: $d = K/\sqrt{t}$, in which $d$ = depth of penetration (mm), $K$ = a constant specific for the fixative, and $t$ = time (hours). In this formula, the $K$ values for glutaraldehyde and paraformaldehyde are 0.34 and 2.0, respectively. The average retinal thickness of the cat retinas ranges from 0.145 to 0.150 mm. Thus, glutaraldehyde requires 10 minutes to reach the photoreceptor of the retina from the inner surface by immersion fixation, whereas paraformaldehyde can reach the photoreceptor in only 20 seconds. This calculation concretely shows that glutaraldehyde penetrates the tissue slowly, although the value is still approximate. It is entirely possible that retinal ischemia alters glutamate immunoreactivity in the photoreceptor inner segment before the outer retina is sufficiently fixed by glutaraldehyde. Our results are consistent with this because they demonstrate that the inner segment of the detached retina is glutamate immunoreactive within 10 minutes of detachment.

We used a mixture of 1% glutaraldehyde and 4% formaldehyde as a fixative. Previous studies have generally applied a higher concentration of glutaraldehyde and a lower concentration of formaldehyde. However, Marc and coworkers reported glutamate immunoreactivity at 0.5% glutaraldehyde concentration. Furthermore, they reported that
Glutamate immunoreactivity in Cat Retina

Glutamate immunoreactivity is equally preserved between 0.5% and 2.5% glutaraldehyde, regardless of formaldehyde content. Similarly, Aoki and coworkers reported that a glutaraldehyde concentration of 1% was effective in diminishing the background-staining intensity. Thus, the fixative mixture used in the present study likely did not result in any adverse effects on the preservation of glutamate immunoreactivity.

The reason the photoreceptor inner segments are glutamate immunoreactive under ischemic conditions remains unclear. However, there are two possible explanations. Glutamate efflux to the extracellular space as a result of ischemia can be followed by glutamate uptake by the inner segments. Photoreceptor inner segments in the isolated Macaca monkey retina accumulate glutamate, as demonstrated by autoradiographic localization. Rauen and coworkers showed immunocytochemically that photoreceptor cone inner segments in the rat retina possess a glutamate transporter, GLT-1, that takes up glutamate intracellularly. The difficulty with this explanation is in the concentration of extracellular glutamate. According to findings of microdialysis studies, the concentration of glutamate on the rabbit retinal surface increases to approximately 1.7 times the control level during the first 10 minutes of ischemia, whereas that in the CA1 field of the hippocampus increases to approximately 15 times the control level during the first 5 minutes of ischemia.

The other explanation is that the disturbance of the glutamate metabolism as a result of ischemia influences glutamate distribution in the retina. Glutamate production in the vertebrate retina is sustained through at least three major pathways involving the following enzymes: aspartate aminotransferase (AAT); glutamate dehydrogenase; and phosphate-activated glutaminase. These enzymes have been studied in the rat retina. In the mitochondria-rich inner segments, AAT activity was not evident in the outer segments. AAT exists in mitochondrial and cytoplasmic forms. Mitochondrial AAT is correlated with the functions of AAT in energy metabolism in the tricarboxylic acid cycle. Another study showed that among all retinal elements, the highest activity of glutamate dehydrogenase and phosphate-activated glutaminase was localized in the photoreceptor inner segments in which neurotransmission does not occur.

These results suggest that energy metabolism involving glutamate is active in the inner segments. Free cytoplasmic glutamate may not be so abundant in the metabolic pathway under normal conditions, and disruption of the cycle and an alteration in the equilibrium of the enzymes as a result of ischemia may lead to glutamate accumulation in the cytoplasm.

In the present study, little glutamate immunoreactivity was observed in the inner segments of photoreceptors under normal metabolic conditions. Studies of the retina will help further understanding of retinal ischemia because glutamate immunoreactivity can be considered a parameter of the degree of the retinal anoxic condition, including retinal detachment. Furthermore, the effects of therapeutic drugs on ischemia can be estimated using glutamate immunoreactivity. Thus, perfusion fixation will be critically important to avoid the artificial redistribution of glutamate.

References


