HISTOCHEMICAL STUDY ON GUANYLATE CYCLASE ACTIVITY IN THE RETINA OF DEVELOPING RAT¹

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The histo- and cytochemical localization of guanylate cyclase activity was examined in the newborn rat retina during the first three postnatal weeks. The reaction was first observed on the 3rd postnatal day on the apical plasmalemma of the retinal pigment epithelium. The reaction was seen on the disk and plasmalemma of the premature short outer segments from the 10th day. From the 14th day, the enzyme localization seemed to be the same as shown in the adult rat. The reaction products were restricted to the apical plasmalemma of the retinal pigment epithelium and disk and plasmalemma of the tip of the outer segments.

To date it is generally accepted that in the rod outer segments Ca-ion and/or 3', 5'-cyclic guanosine monophosphate (cGMP) function as intracellular secondary messengers to control the Na-channels in the plasma membrane after bleaching of rhodopsin. Therefore, the enzyme activities of cGMP synthesis (guanylate cyclase) and degradation (cyclic nucleotide-phosphodiesterase) have been studied biochemically and physiologically, and the role of cGMP during the light-conductive mechanism has been discussed.

Based on this background, an *in-situ* study with the aim of fine structural localization of enzymatic activities linked to cGMP regulation in the outer segments is of particular importance for the interpretation of the light perceptive mechanism in the photoreceptor rod cells. The sites of guanylate cyclase (6) and cyclic nucleotide-phosphodiesterase (9) have already been studied utilizing newly developed cytochemical methods (2, 8). Histochemically, uniquely rich guanylate cyclase activity was found to be restricted to the tip of the outer segments and the microvilli of the retinal pigment epithelium (6). The aim of the present study is to elucidate the localization of this enzyme activity in the developing rat retina with special emphasis on the formation of outer segments.

MATERIALS AND METHODS

0 to 20-day-old Wistar Kyoto rats were used for this investigation. Under ether

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anesthesia, all the eyes were rapidly enucleated in the morning and cut into halves at the equator. The posterior halves were fixed in a cold mixture of 2% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 4% sucrose for 30 min. After fixation, they were washed in the same buffer for 2–4 hr at 4°C. They were cut into small pieces, and sectioned with the use of cryotome at approximately 20 μ m for light microscopic and 40 μ m for electron microscopic purposes.

The sections were incubated in the following incubation medium (2) for 60 min at 37°C; 80 mM Tris-maleate buffer with 250 mM sucrose, pH 7.4, 0.5 mM guanylyl imidodiphosphate (GMP-PNP), 2 mM theophylline, 3 mM MnCl_2 , 2 mM lead nitrate and 5% DMSO.

For light microscopy, the incubated sections were rinsed in distilled water, treated with 1% ammonium sulfide for 2 min and mounted on slide glass with glycerine jelly.

For electron microscopic observation, the sections were rinsed in cacodylate buffer (pH 7.4, with 8% sucrose) and immediately dehydrated via a graded ethanol series and propylene oxide, and embedded in Spurr's epoxy resin. Ultrathin sections were cut with an LKB Ultratome, and examined with a Hitachi HS-9 electron microscope without any poststaining.

For control purposes, the sections were incubated in the cytochemical medium without substrate, GMP-PNP.

RESULTS

Light microscopy: Although no particular reaction of guanylate cyclase was seen in the whole retina of the newborn rats, a very weak reaction was initially observed in the outer portion of the retina on the 3rd postnatal day. On the 6th postnatal day, the reaction increased and was recognized to localize between retinal pigment epithelium and photoreceptor cells (Fig. 1). From the 8th to 13th postnatal day, much higher reaction was revealed in the almost whole outer segments layer and retinal pigment epithelium (Fig. 2). Thereafter, the reaction was found in the tip of the outer segments and retinal pigment epithelium as previously observed in the adult rats (6).

In the control study, no remarkable reaction was localized when the substrate, GMP-PNP, was omitted from the cytochemical incubation medium.

Electron microscopy: Initial electron-opaque reaction products of guanylate cyclase activity was found in the cytoplasmic side of apical plasmalemma (microvilli) of retinal pigment epithelium from the 3rd to 9th postnatal day (Fig. 3). In this period, the outer segments were not developed from the photoreceptor cells. From the 10th postnatal day, some short outer segments were produced from photoreceptor cells. The reaction products were seen on the disk membranes of the outer segments together with the microvilli of retinal pigment epithelium (Fig. 4). After the 10th postnatal day, the outer segments developed and elongated. The reaction precipitates were localized on the disk membranes of the whole outer segments. The reactions on the microvilli of retinal pigment epithelium were still marked (Fig. 5). From the 14th to the 20th day, the localization of guanylate cyclase activity was almost the same as shown in the adult rats. The reaction products were distributed on the disk membranes and plasmalemma of the outer segments. This reaction was evident in only the tip of the elongated outer segments. In addition, although the reaction products

FIG. 1. Light micrograph of guanylate cyclase activity in the rat retina on the 6th postnatal day. Note the reaction between retinal pigment epithelium and photoreceptor cells (arrow indicated). $\times 400$

FIG. 2. Light micrograph of the enzyme activity on the 13th postnatal day. An intense reaction was seen in the almost whole outer segments and retinal pigment epithelium (arrow indicated). Compare the intensity of reaction with that in Fig. 1. $\times 400$

FIG. 3. Electron micrograph of guanylate cyclase activity on the 8th postnatal day. Reaction products were localized mainly in the cytoplasmic side of apical plasmalemma (arrows) of retinal pigment epithelium (PE). $\times 25,000$

FIG. 4. Electron micrograph of guanylate cyclase activity on the 10th postnatal day. Together with intense reactions on the apical villi of retinal pigment epithelium, reaction was seen on the disk membranes of the immature outer segments (arrows). $\times 12,500$

FIG. 5. Electron micrograph of the activity on the 13th postnatal day. Reactions on the apical villi of retinal pigment epithelium were still marked (arrows). Note the developed and elongated outer segments (OS) showing reaction products.

FIG. 6. Electron micrograph of the activity in the tip of the outer segments on the 20th postnatal day. The reaction products were distributed on the disk and plasma membranes of the outer segments (OS). Weak reaction was seen on the apical villi of retinal pigment epithelium (arrows). $\times 50,000$

were seen on the microvilli of the retinal pigment epithelium, the intensity of the reaction was slightly reduced from that observed in the premature period described above (Fig. 6).

The result of the control study was similar to that examined under light microscopy. No reaction was seen under electron microscopy.

DISCUSSION

Although numbers of studies on the development of the outer segments have been reported, most authors seem to have concentrated solely on the fine structural difference through the post natal periods and have made no attempt to correlate the functional developments. As previously mentioned, cGMP plays a role as second messenger during the phototransduction process which occurs in the outer segments. Therefore, the localization of guanylate cyclase activity which produces cGMP from GTP might be a good marker to examine some functional development of the outer segments.

In several morphological studies, it was mentioned that the outer segments including disk membranes were formed at least by the 10th postnatal day in the rat (1, 4, 5). In our study, the premature flat outer segments with several disk membranes were observed on the 10th postnatal day. This result offered us the particularly important possibility that the disk membranes possessed guanylate cyclase activity just after formation. Previous morphological studies concerning the origin of the disk lamellar membranes indicated that two completely different situations exist in the rod outer segments. An invagination of the outer segment plasma membranes was reported (7), but, on the other hand, it was counterclaimed that these lamellar membranes could be traced to the small vesicles or smooth endoplasmic reticulum (5). In the cytochemical studies of guanylate cyclase activity, however, remarkable reactions could be observed, not on the small vesicles or endoplasmic reticulum but on the plasmalemma of the outer segments during the initial postnatal weeks and also in the adult stage (6). This might be an interesting result, when we consider the origin of the disk membranes.

Our histochemical observations showed another interesting result. First, although in the initial stage, guanylate cyclase activity was localized on the whole outer segments, it was restrictedly localized only on the tip of the outer segments after their elongation from the 14th postnatal day as seen in the outer segments of adult rats. In the study of electroretinograms of a developing rat during the 12th to 14th postnatal day, although minimal or incomplete responses could be recorded on the 12th or 13th day, all wave components became same as that of the adult rat (5). This coincidence is also interesting for the interpretation of the role of cGMP as a phototransduction second messenger and the special location of guanylate cyclase in the mature outer segments.

Secondly, the reactions on the microvilli of the retinal pigment epithelial cells was already observed on the 3rd postnatal day. From the morphological viewpoint, the retinal pigment epithelial cell was reported to differentiate earlier than other retinal cells, and to reveal the mature feature of the plasmalemma already in the prenatal stage (1, 3, 5). However, it should be emphasized that the guanylate cyclase activity appeared at much later stage. This is also important for consideration of biological

cell development of the retinal pigment epithelium. Thus, we would like to conclude that our histochemical results might be evidence showing the difference in the functional and morphological developmental features of these retinal components.

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