Phenylethanolamine N-Methyltransferase-Containing Neurons in Rat Retina: Immunohistochemistry, Immunochemistry, and Molecular Biology

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We sought to characterize in detail neurons in rat retina that contain phenylethanolamine N-methyltransferase (PNMT), the epinephrine biosynthetic enzyme. Cell bodies and processes of PNMT-containing neurons in retina were identified by immunohistochemistry. The coexistence of other catecholamine biosynthetic enzymes in the same cells was also investigated. Biochemical, molecular biological and immunochemical methods were applied to determine whether retinal PNMT is similar to the adrenal enzyme, since regulation of PNMT in retina and adrenal appears to be different. The results show that there are two types of PNMT-containing cells: those containing PNMT exclusively and those containing PNMT with two other catecholamine-synthesizing enzymes, tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC), but not dopamine β -hydroxylase (DBH). PNMT-only cell bodies are localized in the inner nuclear layer (INL) and the ganglion cell layer (GCL). Their processes are observed in outer and inner strata of the inner plexiform layer (IPL). Only a small fraction of PNMT neurons in INL also contain TH and AADC. These cells send their processes to the adjacent stratum of the IPL. Antibodies to bovine adrenal DBH, however, fail to localize DBH in any rat retinal cells. Immunochemical titration shows that PNMT from both retina and adrenal gland has the same immunoreactivity. Furthermore, a PNMT-cDNA probe hybridizes equally with PNMT-mRNA isolated from both the retina and the adrenal gland. These results indicate that PNMT is identical in these tissues.

The enzyme, phenylethanolamine N-methyltransferase (PNMT), which catalyzes the conversion of norepinephrine to epinephrine, has a wide substrate specificity, and requires S-adenosyl-L-methionine as a methyl donor. Since this enzyme has such a wide substrate specificity, numerous studies have focused on whether the enzyme exists as multiple forms (Joh and Goldstein, 1973; Lee et al., 1978; Park and Joh, 1985; Park et al., 1982); whether the enzyme in adrenal gland differs from that in other tissues (Park and Joh, 1985), such as brain and small intensely fluorescent cells (Eränkö and Härkönen, 1963), and whether the regulation of the enzyme differs in different tissues (Hadjiconstantinou et al., 1983; Pohorecky et al., 1973; Wurtman et al., 1968). Furthermore, in some brain areas, neurons stained for PNMT but not for the other enzymes of catecholamine biosynthesis (Ross et al., 1984; Ruggiero et al., 1985).

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The presence of PNMT in rat retina has been established (Hadjiconstantinou et al., 1983; Osborne and Nesselhut, 1983) and localized specifically to retinal neurons (Hadjiconstantinou et al., 1984; Foster et al., 1985). These reports demonstrated PNMT-like immunoreactivity in inner nuclear (INL), inner plexiform (IPL), and ganglion cell (GCL) layers of retina. However, these studies did not analyze in detail the neuronal processes within retina or the coexistence of other catecholamine-synthesizing enzymes, including tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), and dopamine β -hydroxylase (DBH). Furthermore, regulation of retinal PNMT appeared to be different from that of the adrenal enzyme (Hadjiconstantinou et al., 1983; Pohorecky et al., 1973; Wurtman et al., 1968), and thereby required that the biochemical characteristics of PNMT in rat retina also be investigated.

In the present report, we detail the immunohistochemical localization of PNMT in cell bodies and neuronal processes of specific layers of rat retina by means of the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1974). The presence of other catecholamine biosynthetic enzymes, including TH, AADC, and DBH, in the same cells was also investigated using a novel antibody double-labeling method with 125 I-labeled antibody and the PAP complex. In addition, a similarity of PNMT in retina and adrenal gland is demonstrated by immunochemical and molecular biological procedures.

Materials and Methods

Biochemical and immunochemical methods

Male Sprague-Dawley rats (Hilltop Laboratory Animals, Scottsdale, PA) weighing 200–225 gm were killed by decapitation. The eyes and adrenal glands were quickly removed, and the retinæ were dissected.

Pairs of retinæ were homogenized in 300 μ l of 5 mM potassium phosphate buffer, pH 7.0, containing 0.2% Triton X-100, and a pair of adrenal glands was homogenized in 2 ml of the same buffer. Homogenates were centrifuged at $9750 \times g$ for 10 min. The resulting supernatant was used for PNMT assay, protein determination (Lowry et al., 1951), and immunotitration. PNMT activity was determined by a method described elsewhere in detail (Park and Joh, 1984; 1985). In essence, the reaction mixture contains 0.5 mM D,L-phenylethanolamine, 10 μ M S-(methyl- 3 H)-adenosyl-L-methionine, 150 mM Tris-Cl buffer, pH 8.6, and tissue extract containing 10 μ g BSA in a final volume of 100 μ l. All other assay procedures were carried out as described elsewhere (Park et al., 1982). The procedures for production of antibodies to PNMT, TH, DBH, and AADC, and the determination of their specificity have been described in detail previously (Jaeger et al., 1983; Joh and Ross, 1983; Park et al., 1982; Teitelman et al., 1985). The method of Park et al. (1982) was used for the immunotitration of PNMT.

Detection of PNMT mRNA using PNMT cDNA probe

Tissues were frozen immediately following dissection. Total RNA was isolated by the guanidine thiocyanate lysis procedure of Chirgwin et al.

Figure 1. Immunochemical titration curves for rat retinal (O) and adrenal (●) phenylethanolamine N-methyltransferase. Immunochemical titration was carried out by adding increasing amounts of antibodies to 50 μ l of crude extract from both tissues, respectively. The total volume was adjusted to 70 μ l with preimmune serum. The mixture was allowed to stand for 1 hr at room temperature with occasional shaking, and then for 16 hr at 4°C. Antigen-antibody precipitate was removed by centrifugation, and a 50 μ l aliquot of the supernatant was assayed for enzyme activity. Note that the titration curves for both tissues are parallel, indicating that the enzymes from both sources are immunochemically comparable.

(1979) and fractionated from cellular components by CsCl density gradient centrifugation (Glisin et al., 1974). Poly-A⁺ RNA was prepared by oligo-dT cellulose chromatography (Aviv and Leder, 1972).

RNAs were denatured in 8% formaldehyde, 60% formamide, plus 10 mM sodium phosphate, pH 6.6, at 55°C for 15 min (Maniatis et al., 1982) and bound to nitrocellulose in 10 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate) on a Schleicher and Schuell filtration manifold by the procedure of Thomas (1980). Hybridization was performed in 5 $\times$ SSC containing 1 $\times$ Denhardt's solution, 100 μ g/ml *E. coli* tRNA, 0.1% SDS, 10 μ M PIPES, pH 6.5, and 33% formamide according to Wahl et al. (1979).

PNMT-10 (740 base pair) cDNA (Baetge et al., 1983) was nick-translated (Rigby et al., 1977) to a specific activity of 5×10^8 cpm/ μ g DNA; 10^7 cpm was added to each hybridization at 42°C for 18–24 hr. Filters were washed in a solution containing 10 mM EDTA, pH 8, 0.1% SDS, and 0.05% sodium pyrophosphate to a stringency of 0.1 $\times$ SSC at 45°C. Autoradiography was performed at –80°C in the presence of a Cronex intensifying screen.

Immunocytochemistry and autoradiography

For immunocytochemistry, the animals were anesthetized with pentobarbital (50 mg/kg, i.p.); the eyes were then removed and immersed in 0.1 M PBS solution. The retinæ were dissected and fixed for 5 min in a 3.75% solution of acrolein in 0.1 M PBS, postfixed for 1 hr in a 2% solution of formaldehyde in 0.1 M PBS, and left overnight in a 30% sucrose solution. On the following day, 16- μ m sections of the tissues were made with a cryostat microtome and mounted on gelatin-coated glass slides.

The sections were washed in Tris-buffered saline (TBS, 0.1 M Tris, pH 7.6) exposed first to 0.25% Triton X-100 in TBS for 15 min, then to goat serum diluted 1:30 in TBS for 30 min, and incubated overnight at 4°C with antibodies (diluted 1:500 with 0.1 M TBS) to TH, AADC, DBH, or PNMT. The sections were then washed with TBS, incubated with a 1:50 dilution of goat anti-rabbit IgG (Miles-Yeda), rinsed with

Figure 2. Autoradiographic determination of phenylethanolamine N-methyltransferase mRNA by dot blot hybridization using ³²P-labeled PNMT cDNA probe. Total and poly-A⁺ RNAs were prepared from rat retina, adrenal gland, and liver as described in Materials and Methods. Adrenal (5 μ g) and retinal (50 μ g) total RNAs and 0.5 μ g each of adrenal, retinal, and liver poly-A⁺ RNAs were bound to nitrocellulose. The presence of PNMT-mRNA was detected by hybridization to a nick-translated PNMT-cDNA insert.

TBS, and exposed for 30 min to PAP diluted 1:100 in TBS. The bound PAP was visualized by reaction with either 0.013% 3,3'-diaminobenzidine (DAB, Aldrich Chemical Co.) or 0.043% 4-chloronaphthol (Sigma) and 0.01% hydrogen peroxide. The sections processed with DAB were dehydrated and mounted with Permount; those reacted with 4-chloronaphthol were mounted with a solution of glycerine and water (9:1 vol/vol).

The double-labeling of PNMT and TH was carried out by a modification (Pickel et al., unpublished observations) of the Larsson and Schwartz method (1977). The sections were first incubated overnight with antibodies against TH (diluted 1:30,000), then for 45 min with a 1:50 dilution (TBS + 1% goat serum) of a solution of donkey anti-rabbit IgG labeled with ¹²⁵I (specific activity, 5–20 μ Ci/ μ g, Amersham). The following day the sections were rinsed six times in TBS and exposed for 24 hr to a 1:500 dilution of antibodies against PNMT. Finally, the sections were sequentially incubated with a 1:50 dilution of goat anti-rabbit IgG for 30 min and to a 1:100 dilution of PAP. The bound PAP complex was visualized by incubating the sections with 0.013% DAB and 0.01% of hydrogen peroxide. After the DAB reaction, the sections were air-dried overnight and dipped in Ilford L-4 photographic emulsion (diluted 1:1 with water), air-dried, and incubated in a light-tight box containing a drying agent (Drierite) at 4°C. After 1 to 2 weeks, the sections were developed for 2 min at 16–17°C in Kodak D-19 developer, rinsed in water, fixed in Kodak Ektaflo (diluted 1:3) for 8 min, dehydrated, and mounted in Permount.

Results

Rat retinal PNMT is an active enzyme, with a mean specific activity of 9.97 ± 0.569 (mean $\pm$ SEM) pmol/mg protein/15 min ($n = 10$) in the crude extract. Rabbit antibodies raised against bovine adrenal PNMT cross-react with both rat retinal and adrenal PNMT in an immunochemical titration experiment (Fig. 1). The slopes of titration curves for PNMT from both tissues are parallel (Fig. 1), indicating that the enzyme is immunochemically similar in both tissues.

In order to determine whether PNMT is synthesized *in situ* in retinal cells, the presence of the mRNA for PNMT is examined by hybridization to a PNMT-cDNA probe. Dot blot hybridization, as shown in Figure 2, indicates that PNMT-mRNA is present in retinal cells, although the intensity of hybridization of the probe to retinal PNMT-mRNA is lower than that of adrenal mRNA. PNMT-mRNA is detected in both total and poly-A⁺ RNA from both tissues. However, the intensity of hybridization to retinal RNA is much stronger than that observed in RNA prepared from liver—a tissue which does not express PNMT—thereby indicating the presence of specific PNMT-mRNA in retinal cells.

Immunohistochemical localization of PNMT in retinal cells shows that a majority of PNMT-positive cells contain only PNMT, whereas the other catecholamine biosynthetic enzymes, TH, AADC, and DBH, are not detected in the same cells. The cells containing PNMT only are found in the inner portion of the INL (Figs. 3A, B, E, and 4A). Their average diameters range

Figure 4. Camera lucida drawings of rat retina demonstrating distributions of amacrine and ganglion cell staining for the catecholamine-synthesizing enzymes, PNMT (*A*) and TH and AADC (*B*). Processes derived from PNMT-labeled, but not TH- or AADC-labeled, amacrine and ganglion cells contribute primarily to a band in the inner stratum of the IPL. In contrast, TH and AADC neurons send their processes primarily into the outer stratum.

from 8 to 12 μm . A small population of PNMT-only cells is also found in the GCL, as shown in Figures 3*A*, *B*, and 4*A*. PNMT-positive perikarya in the INL and GCL produce a dense band of interlacing processes within an inner stratum of the IPL (Figs. 3*A*, *B*, and 4*A*). A lighter band of PNMT-positive varicosities derived from cells of the INL is, in turn, distributed in the outer stratum of the IPL (Figs. 3*A* and 4*A*).

Double-labeling immunohistochemical staining demonstrates that only in the INL are some neurons positive for PNMT, TH, and AADC, but not for DBH (Figs. 3*A*, *C*, *D*, and 4*B*). These cells give rise to fibers extending to the outer stratum of the IPL (Figs. 3*A*, 4*B*).

Discussion

The presence of epinephrine and the activity of the epinephrine-synthesizing enzyme, PNMT, have been detected in rat and bovine retina (Hadjiconstantinou et al., 1983; Osborne and Nesselhut, 1983). Regulation of retinal PNMT differs from that of the adrenal enzyme: In response to hypophysectomy, the activity of adrenal PNMT decreases drastically (Pohorecky et al., 1973; Wurtman et al., 1968), while retinal PNMT activity remains unchanged (Hadjiconstantinou et al., 1983). This phenomenon may be due either to physiological differences between the tissues or to different molecular forms of the enzyme known

Figure 3. Different classes of PNMT neurons in adult rat retina localized by immunohistochemistry and autoradiography. *A*, Photomicrograph of a histological section sequentially stained with antibodies against TH and PNMT, visualized by autoradiography and the PAP technique, respectively. Note the presence of neurons containing only PNMT in the GCL (short-tailed arrows) and in the INL (long-tailed arrows). These cells are labeled only by the brown precipitate of diaminobenzidine. Other cells, indicated by thick arrows, contain both TH and PNMT, since they are double-labeled by silver grains and diaminobenzidine. Bar, 14 μm . *B*, Light-microscopic photomicrograph of adult rat retina, illustrating the location of "PNMT-only" neurons. The section, stained with 4-chloronaphthol, clearly shows the different layers of the retina and the location of the stained perikarya in the GCL and INL. Both types of PNMT-only neurons send processes to the IPL. Bar, 14 μm . *C*, Immunohistochemical localization of AADC in histological sections of rat retina. The neurons containing AADC are, by location and size, identical to those containing TH and PNMT, but different from the PNMT-only neurons. Bar, 14 μm . *D*, High-magnification photograph illustrating the presence of both TH and PNMT, visualized by silver grains and diaminobenzidine precipitate, respectively, in perikarya and processes of some retinal neurons of rat. Bar, 4 μm . *E*, High-magnification photomicrograph of a PNMT-only neuron from the INL of adult rat retina. A histological section of retina was sequentially incubated with antibodies against TH and PNMT and the bound antibodies were visualized by autoradiographic and PAP techniques, respectively. Note that the neuronal perikarya and processes contain only PNMT, as indicated by the diaminobenzidine precipitate. Other processes, in the same cell layer, are labeled by silver grains.

to exist (Joh and Goldstein, 1973; Lee et al., 1978; Park and Joh, 1985; Park et al., 1982). It is important to establish whether retinal PNMT is comparable to adrenal enzyme in its biochemical characteristics.

In our studies, the specific activity of rat retinal PNMT is approximately 1% of the adult rat adrenal enzyme. However, this is four times the activity reported by Hadjiconstantinou et al. (1983) and Cohen and Hadjiconstantinou (1984). The differences between our results and those of others are probably due to differences in the enzyme assay conditions employed. In the present study, the concentrations of the substrates, *S*-adenosyl-L-methionine and *D,L*-phenylethanolamine, were 10 μ M and 0.5 mM, respectively, which represent optimal concentrations for the 15 min assay for enzyme activity. In contrast, suboptimal concentrations of both substrates (0.84 μ M of *S*-adenosyl-L-methionine and 0.1 mM of *D,L*-phenylethanolamine) have been used in other studies (Cohen and Hadjiconstantinou, 1984; Hadjiconstantinou et al., 1983).

The immunochemical properties of PNMT in the retina and adrenal gland are similar (Fig. 1). In addition, dot blot hybridizations using a specific PNMT-cDNA probe (Fig. 2) strongly indicate the biosynthesis of PNMT in the retinal neurons in which PNMT immunoreactivity is found. Northern blot analysis (data not shown) reveals hybridization of the PNMT-cDNA probe to a single band in both retinal and adrenal poly-A⁺ RNAs, but not in liver, further confirming the results obtained by dot blot hybridization. This establishes that the localization of the PNMT enzyme in the retina must be attributable to translation of endogenous PNMT mRNA in this tissue. Previous findings (Hadjiconstantinou et al., 1983; Pohorecky et al., 1973; Wurtman et al., 1968) of a difference in response of the adrenal and retinal enzymes to hypophysectomy may be due to the physiological differences between the tissues, but not to the different forms of the enzyme.

The localization of PNMT-containing cell bodies in the INL and GCL in the present study is in good agreement with data of previous studies (Foster et al., 1985; Hadjiconstantinou et al., 1984) using the indirect immunofluorescent staining procedures; however, they are in contrast to the findings of PNMT perikarya in the IPL by Hadjiconstantinou et al. (1984). In our study, displaced PNMT-containing cell bodies were never found in the IPL. Processes from PNMT-cell bodies in the INL were consistently observed in the outer (minor) and inner (major) layers of the IPL (Figs. 3A, B, and 4A), as has been reported by others (Foster et al., 1985; Hadjiconstantinou et al., 1984). However, we are the first to report that the processes from the GCL cells are also present in the inner stratum of the IPL (Figs. 3B and 4A). Staining methods may account for the differences between our results and those of others. Whereas others have employed an indirect immunofluorescent method, we have used the PAP technique, which shows a much clearer localization of PNMT-containing processes.

Our study also indicates that there are two types of PNMT-immunoreactive cells in the rat retina: PNMT-only cells and those containing PNMT and the other catecholamine biosynthetic enzymes, TH and AADC. Cells that contain only PNMT are probably similar to the cells Foster et al. (1985) described earlier, in which TH and PNMT do not coexist. Using sequential double-labeling of the same section, we found that some cell bodies in the INL are clearly stained with TH and AADC antibodies (Figs. 3A, C, D, and 4B). These cell bodies send their processes only to the outer stratum of the IPL, and the processes are also stained with TH and AADC antibodies. The absence of DBH in the latter cells is interesting. The probable explanation is that the content of DBH is too low to detect by immunohistochemistry. An alternative explanation is that the lack of DBH immunostaining is due to the low cross-reactivity of rat DBH with anti-bovine DBH antibodies. Since anti-bovine

DBH antibodies have been successfully used to localize other DBH-containing cells in rat brain (Armstrong et al., 1982), the most probable explanation is that the DBH content in these retinal cells is exceedingly low. However, the possibility that DBH is not expressed in these cells should not be ruled out. This alternative is supported by our recent findings of a large population of neurons in hypothalamus containing PNMT but not the other catecholamine biosynthetic enzymes (Ross et al., 1984; Ruggiero et al., 1985).

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