

Original Article

Histochemical Demonstration of Purine Nucleoside Phosphorylase (PNPase) in Microglial and Astroglial Cells of Adult Rat Brain¹

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The histochemical localization of enzymes associated with purine nucleoside metabolism indicates that glial cells might participate in the regulation of these compounds in the central nervous system. In the present study we examined the histochemical localization of purine nucleoside phosphorylase (PNPase) in sections from adult rat brain. Some sections were also sequentially stained immunocytochemically for astroglial or microglial cells utilizing glial fibrillary acidic protein (GFAP) or OX-42 antibodies, respectively. Our observations showed that PNPase was restricted to glial cells, whereas neurons always remained negative. Brain sections stained for both PNPase and GFAP showed that the GFAP-positive astroglial cells were always PNPase positive. Other

PNPase-positive but GFAP-negative cells were also observed. These cells resembled microglial cells, and brain sections reacted for both PNPase and OX-42 confirmed this by showing that the major part of OX-42-positive microglial cells were PNPase positive. In these sections, the PNPase-positive but OX-42-negative cells present resembled astroglial cells. From our double staining experiments, we conclude that PNPase is present in both astroglial and microglial cells in normal adult brain. (*J Histochem Cytochem* 38:1535-1539, 1990)

KEY WORDS: Glial cells; Nucleosides; Histochemistry; Immunocytochemistry; Double labeling technique; Central nervous system; Hippocampus; Glial fibrillary acidic protein.

Introduction

Purine nucleosides (PN) and purine nucleoside phosphates (PNP) have been shown to participate in a wide variety of biological processes, including regulation of blood flow, cell growth, and immune reactions (Berne et al., 1979; Seegmiller, 1979; Fox and Kelley, 1978). In the central and peripheral nervous systems, PN and PNP play specific roles as neurotransmitters or modulators (Phillips and Wu, 1983; Phyllis et al., 1979). Histochemical studies of the cellular localization of enzymes involved in the metabolism of these substances suggest that glial cells might be involved in control of the PN and PNP levels in the nervous system. More specifically, ATPase, the enzyme that dephosphorylates ATP to ADP, has been reported to be present in the plasma membrane of microglial cells (Ibrahim et al., 1974; Sjöstrand, 1966). In addition, nucleoside diphosphatase (NDPase), the enzyme that dephosphorylates nucleoside diphosphates to nucleoside monophosphates, has been

histochemically localized to the microglial plasma membrane (Castellano et al., 1984a,b; Murabe and Sano, 1981,1982; Vorbrodt and Wisniewski, 1982) as well as to the endoplasmic reticulum and the nuclear envelope of oligodendrocytes and Schwann cells (Castellano et al., 1988,1989). 5'-Nucleotidase, the enzyme that dephosphorylates nucleoside monophosphates and releases nucleosides, has been demonstrated along the plasma membrane of oligodendrocytes, astrocytes, and microglial cells (Hickey and Kimura, 1988; Kreuzberg and Barron, 1978). Adenosine deaminase (ADA), which degrades adenosine to inosine, has also been demonstrated in glial cells, apparently oligodendrocytes (Schrader et al., 1987). Finally, in a recent report, purine nucleoside phosphorylase (PNPase), the enzyme that catalyzes the conversion of inosine to hypoxanthine, has been found in glial cells (Van Recepts et al., 1988). On the basis of their morphology, the PNPase-positive cells were identified as representing a subpopulation of astrocytes, although some of the PNPase-reactive cells could be microglial cells (Van Recepts et al., 1988). In the present study we examined the histochemical localization of PNPase in sections of rat brain. Other PNPase-stained sections were in addition stained immunocytochemically for the astroglial cell marker glial fibrillary acidic protein (GFAP) (Big-nami et al., 1972) or for microglial cells using the OX-42 antibody (Perry and Gordon, 1987).

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Materials and Methods

Adult male Wistar rats (180–200 g) were anesthetized with sodium pentobarbital and perfused transcardially for 20 min with 4% paraformaldehyde and 5% sucrose in 0.1 M cacodylate buffer (pH 7.4). The brains were post-fixed in the same fixative for 2 hr at 4°C. Then, 30- μ m thick coronal brain sections, including the dorsal hippocampus, were cut on an Oxford vibratome and placed in cacodylate buffer (pH 7.4) with 7.5% sucrose for 2 hr at 4°C.

Double staining procedures combining histochemical staining for PNPase with immunocytochemical staining for GFAP or OX-42 were performed by following, in principle, the method described for combination of the histochemical demonstration of NDPase with GFAP immunocytochemistry (Castellano et al., submitted for publication).

To demonstrate PNPase, the vibratome sections were incubated for 30 min at 37°C in a medium containing 9.2 mg ribose-1-phosphate (Sigma, St Louis, MO; R-9381), 13.6 mg hypoxanthine (Sigma, H-9377), 6 ml distilled water, 3 ml 0.2 M Trizma buffer (Sigma, T-3128, pH 7.4), and 1 ml 1% lead nitrate. For control of the PNPase reaction, some sections were treated with 2% ammonium sulfide just after the incubation, and the distribution and density of the resulting brown-black reaction product evaluated.

For double staining for GFAP immunoreactivity, sections incubated for PNPase (without ammonium sulfide treatment) were washed in 0.05 M Tris-buffered saline (TBS), pH 7.4, with 1% Triton X100 (three times for 15 min), placed in 10% fetal calf serum (FCS) for 30 min at room temperature, and then incubated overnight with the primary rabbit anti-GFAP antibody (Dakopatts; Copenhagen, Denmark) in a 1:2400 TBS dilution with 10% FCS at 4°C. After being washed (three times for 15 min) in TBS including 1% Triton X100, the sections were incubated at room temperature for 60 min with an anti-rabbit Ig biotinylated antibody (Amersham; Poole, UK) at a 1:200 dilution in TBS with 10% FCS. After additional washing in TBS with 1% Triton X100 (three times for 15 min), the sections were incubated for 60 min at room temperature with a 1:70 dilution of avidin-labeled peroxidase (Sigma) in TBS with 10% FCS and then washed again in TBS with 1% Triton (twice for 15 min) and in TBS (twice for 15 min). Sections used for simultaneous demonstration of PNPase and OX-42 (a marker for microglia) were prepared as described above, except that the primary antibody was OX-42 (SerLab; Aarhus, Denmark) used in a 1:600 dilution, and the secondary antibody was an anti-mouse Ig-biotinylated antibody (Amersham), used at a dilution of 1:200.

For both immunostains, the peroxidase activity was demonstrated by immersing the sections in a 1-naphthol solution with 0.01% hydrogen peroxide for 15 min at room temperature. The naphthol solution was freshly prepared by dissolving 50 mg of 1-naphthol (Sigma, N-1000) in 0.5 ml ethanol and adding this, together with 10 ml 1% ammonium carbonate, to 89.5 ml of 0.05 M TBS (pH 7.4). Before the incubation and the addition of the hydrogen peroxide, the solution was filtered to remove the white precipitate. After the incubation for peroxidase, the sections were washed (three times for 10 min) in TBS, immersed in 2% ammonium sulfide for 2 min at room temperature to visualize PNPase reaction product, and washed again (three times for 10 min) in TBS. Thereafter, the sections were immersed in a solution of 0.05% Azur A in 0.05 M TBS, pH 8.0, for 30 min to give the immunoreaction product an intense blue color. Finally, after a wash in TBS, the sections were mounted on gelatin-coated slides, dehydrated in ethanol, and coverslipped with Dammar resin.

As controls for the PNPase staining, some sections were incubated in medium without hypoxanthine or/and ribose-1-phosphate. As control for the immunocytochemistry, some sections were processed without the primary antibody for GFAP or without OX-42.

Results

Brain sections stained exclusively for PNPase showed reactive cells

scattered in the gray and white matter (Figure 1). The histochemical reaction product was observed both in the nucleus and in the cytoplasm of the stained cells. Two types of stained cells were distinguished (Figure 3). One type had long, smooth processes and a round nucleus. The other had short, ramified processes and a small and often elongated nucleus. The cytoplasm and nuclei of neurons were always PNPase negative, and no stained cells were observed in the control sections incubated without substrate.

Brain sections reacted for both PNPase and GFAP immunoreactivity showed that the GFAP-positive cells were PNPase positive (Figures 2 and 4). In sections double stained for PNPase and GFAP immunoreactivity, the astroglial cells showed a strong blue immunocytochemical reaction of GFAP in the cell body and processes. The PNPase staining was observed as brown reaction product in the nucleus and sometimes in the surrounding cytoplasm, with a faint appearance in the otherwise blue processes (Figure 4). All GFAP-positive cells observed in the hippocampus and the corpus callosum also reacted for PNPase. All astrocytes in these areas were accordingly PNPase positive. There were, however, other PNPase-positive cells that were GFAP negative. These cells could in several instances be morphologically identified as microglial cells. They displayed strong PNPase staining of the nucleus and had short, ramified processes, which often were well delineated by the histochemical reaction (Figure 4).

Brain sections stained both for PNPase and OX-42 showed that the blue OX-42-positive cells, in general, exhibited strong brown PNPase staining of the nucleus (Figure 5). However, there were also some OX-42-positive cells that did not display PNPase staining. In the hippocampus, such OX-42-positive cells negative for PNPase were rare, but in the corpus callosum single-stained OX-42-positive cells were more frequent (Figure 6). In the sections reacted for OX-42 and PNPase, there were also PNPase-positive cells that were OX-42 negative, but these cells had a morphology similar to astroglial cells (see above).

Discussion

The enzyme PNPase converts inosine to hypoxanthine and ribose-1-phosphate and vice versa. The histochemical demonstration of PNPase is based on the release of phosphate from ribose-1-phosphate when the ribose ring is added to hypoxanthine to form inosine (Rubio et al., 1972). The released inorganic phosphate is captured in the incubation medium and precipitated as lead phosphate, which is then subsequently visualized for light microscopy by conversion to lead sulfide by addition of ammonium sulfide. Recently, we have shown that the histochemical demonstration of nucleoside diphosphatase (NDPase) can be combined with immunocytochemical staining of GFAP for simultaneous demonstration of microglial and astroglial cells in histological sections (Castellano et al., submitted for publication). In this study we used the same basic principle to combine the histochemical reaction for PNPase with the immunocytochemical staining for astroglial and microglial cells. Our observation that the PNPase positive cells in the adult rat brain are glial cells is in agreement with a previous report (van Reepst et al., 1988). By combining in this study the PNPase staining with immunocytochemical reactions for specific markers for astroglial and microglial cells, we can, however, unequivocally conclude that both

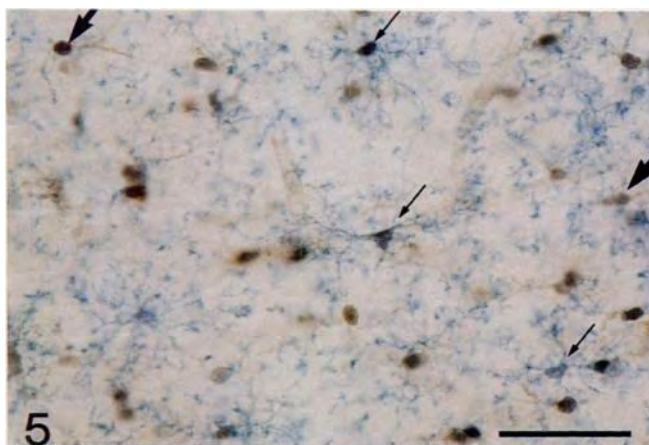
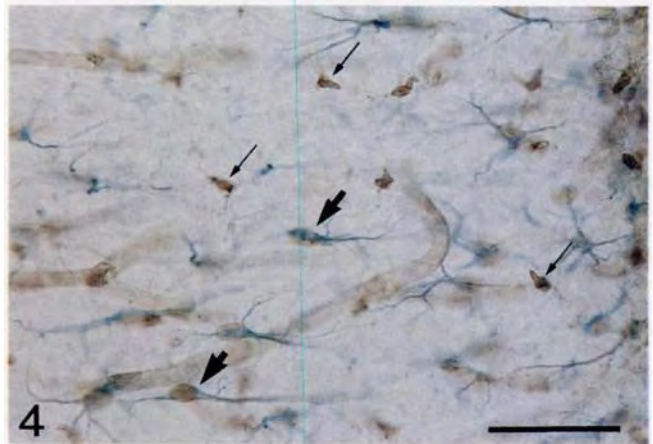
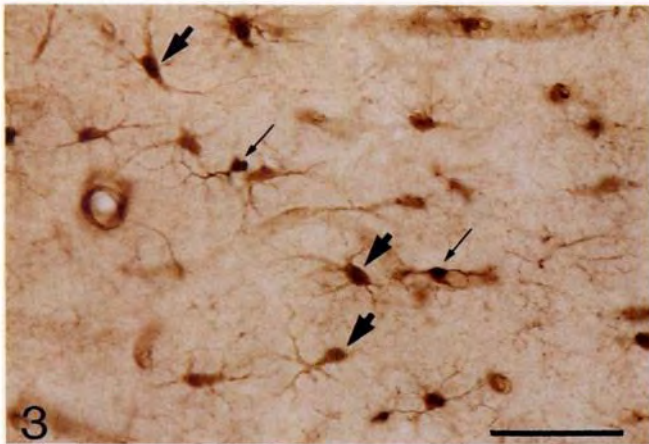
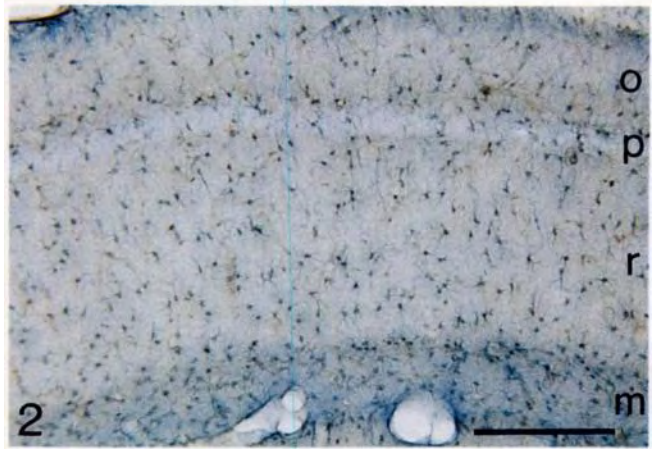
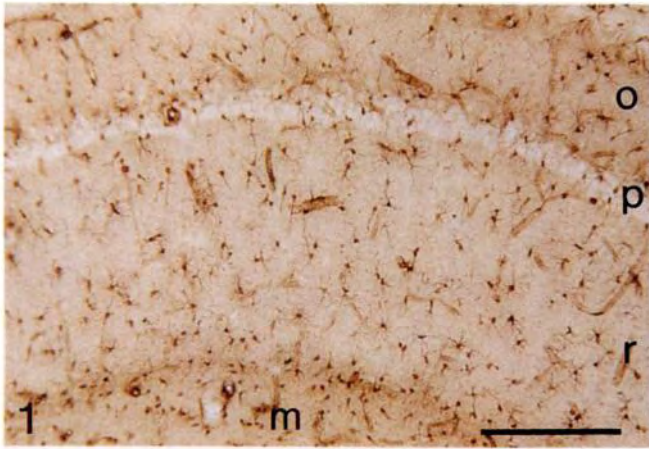


Figure 1. Overview of hippocampal CA1 area stained histochemically for PNPase. o, stratum oriens; p, stratum pyramidale; r, stratum radiatum; m, stratum moleculare. Bar = 200 μ m.

Figure 2. Overview of hippocampal CA1 area double stained for PNPase (brown) and the astroglial marker GFAP (blue). Abbreviations as in Figure 1. Bar = 200 μ m.

Figure 3. PNPase staining of stratum radiatum of CA1, showing stained microglial cells (thin arrows) and astrocytes (thick arrows). Bar = 50 μ m.

Figure 4. Double staining of stratum radiatum of hippocampal CA1 for PNPase (brown) and GFAP (blue). Note astroglial cells positive for both PNPase and GFAP (thick arrows), and other PNPase-positive but GFAP-negative microglial cells (thin arrows). Bar = 50 μ m.

Figure 5. Double staining of stratum radiatum of hippocampal CA1 for PNPase histochemistry (brown) and OX-42 immunoreactivity (blue). Note double-stained microglial cells (thin arrows) and PNPase-positive but OX-42-negative astroglial cells (thick arrows). Bar = 50 μ m.

Figure 6. Double staining of corpus callosum for PNPase histochemistry (brown) and OX-42 microglial immunoreactivity (blue). Note double-labeled microglial cells (thin arrows) as well as OX-42-positive but PNPase-negative microglial cell (double-headed arrow). Thick arrow points to PNPase-positive, presumed astroglial cell. Bar = 100 μ m.

astrocytes and microglial cells are PNPase-positive cells. In addition, the combination of the histochemical reaction for PNPase with immunocytochemical detection of the microglial cells by the OX-42 antibody enabled us to distinguish two subpopulations of microglial cells, one PNPase positive and one PNPase negative. The brain microglial cells might accordingly be heterogeneous, at least with regard to their purine nucleoside metabolism or the actual stage of this.

The functional significance of the presence of PNPase in astroglial and microglial cells, but not in neurons, cannot be explained at present. However, accumulating evidence suggests that after injury or in different pathological stages both glial cell types are involved in immune and repair reactions, where they (e.g., in relation to the immune system) may be induced to act as antigen-presenting cells (Hayes et al., 1987; Frank et al., 1986; Woodroffe et al., 1986; Hickey et al., 1983,1985). Seen in this perspective, the involvement of astroglial and microglial cells in the metabolism of purine nucleosides agrees with studies showing that the immune response is regulated by the levels of these compounds (Seegmiller, 1979; Fox and Kelley, 1978). In humans, a deficiency of PNPase is thus known to lead to an important dysfunction of the immune system (Seegmiller, 1979).

Along with a possible regulation of immune and repair reactions in the CNS, glial control of purine nucleoside/purine nucleoside phosphatase levels in the extracellular space could constitute a mechanism by which the cytotoxic effects attributed to some of these compounds (Fox and Kelley, 1978) are prevented. It is also known that purinergic terminals release adenosine and adenosine nucleoside phosphates (Phillips and Wu, 1983; Burnstock, 1979). Glial cells might therefore be involved in the inactivation and turnover of these neuromodulators or neurotransmitters through enzymes which, by working in cascade, would degrade these substances. This suggestion agrees with the present results, showing the presence of PNPase in microglial cells, and with previous reports demonstrating the presence of ATPase, NDPase, and 5-nucleotidase in the microglial plasma membrane (Hickey and Kimura, 1988; Schrader et al., 1987; Murabe and Sano, 1982; Vorbrodt and Wisniewski, 1982; Kreutzberg and Barron, 1978; Ibrahim et al., 1974; Sjöstrand, 1966).

From our observations, we conclude that all GFAP-positive astroglial cells and the major part of OX-42-positive microglial cells in the normal adult rat brain are PNPase positive. Further studies dealing with the cellular distribution and possible changes of PNPase in the developing or injured brain may help us to further understand the role of purine nucleosides and purine nucleoside phosphates in the nervous system.

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