EXTRALYSOSOMAL LOCALIZATION OF ACID PARA-NITROPHENYLPHOSPHATASE ACTIVITY IN THE DEVELOPING RAT CEREBELLAR CORTEX

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Using para-nitrophenyl phosphate as substrate, extralysosomal localization of acid phosphatase activity was investigated in the developing and adult rat cerebellar cortex, particularly in the Purkinje (P) cells. Light microscopic observations revealed that acid para-nitrophenylphosphatase (acid NPPase) activity appeared increasingly in the P cell and molecular layers throughout their postnatal development. Under an electron microscope, the acid NPPase activity was found in the cisterns of nuclear envelope and endoplasmic reticulum of the P cells, in addition to the lysosomal activity. In the synapses on the P cells, the acid NPPase activity was predominantly localized in the presynaptic membrane of such parallel fibers. The activity of nonlysosomal acid NPPase was inhibited by fluoride (0.1 mM) and CuSO_4 (1.0 mM), but not by L(+)-tartrate (10 mM). The present results are discussed in a possible relationship to an activity of phosphotyrosine protein phosphatase in the developing rat cerebellar cortex.

Acid phosphatase activity, using β -glycerophosphate as substrate, has been well localized in the lysosomal organelle population (1). On the other hand, extralysosomal localization of acid phosphatase has been also described in various organs, and most of the activity was found with other substrates, such as naphthol phosphate, para-nitrophenyl phosphate (NPP) and phenyl phosphate, rather than glycerophosphate (1, 16, 21). It has been suggested that polymorphism of acid phosphatases reflects an unique metabolic function for each of the enzymes (4, 7).

Recently, there is increasing evidence that certain acid phosphatases, termed low-molecular-weight acid phosphatase, are closely associated with some protein phosphatases (5, 13, 24, 27). The acid phosphatase activity is characterized by an use of NPP as substrate rather than β -glycerophosphate. The protein phosphatases, purified from mammalian tissues such as brain, liver, small intestine, bone and placenta, were biochemically characterized to be specific to phosphotyrosyl proteins (5, 13, 20, 27). As a counterpart of protein tyrosine kinases, phosphotyrosine pro-

tein phosphatase (PTPPase) can regulate the level of phosphorylation of proteins (12, 26). Multiple PTPases in the brain suggest that protein phosphorylation on tyrosine residues is closely associated with the neural function (3, 8, 11, 20, 24).

Previously, the nonlysosomal acid paranitrophenyl phosphatase (acid NPPase) activity has been reported in some mammalian tissues such as kidney, bone and epidermis (14, 16, 19). Miyayama *et al.* (16) demonstrated that the acid NPPase activity in the mouse kidney was localized in the plasma membrane of the tubule epithelial cells, in addition to the intracytoplasmic activity of the endoplasmic reticulum. Ohno *et al.* (19) described that the acid NPPase activity in the rat epidermis was that of phosphoprotein phosphatase.

In an attempt to characterize the nonlysosomal acid phosphatases with respect to a possible involvement of PTPases in the nervous tissue, the histochemical and cytochemical localizations of acid NPPase activity in the developing and adult rat cerebellar cortex, particularly in the Purkinje (P) cells were investigated in the present study.

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MATERIALS AND METHODS

Twenty-five rat pups of the Jcl: Wistar, ranging from postnatal day (PD) 5 to 20, and three adult male rats were used in the present study. Animals were anesthetized with sodium pentobarbital and were briefly perfused with saline through the left cardiac ventricle, followed by cold 30 mM PIPES-buffered (pH 7.3) fixative containing 0.5–1% glutaraldehyde (GLA) and 2% paraformaldehyde (PFA). After a 5 min perfusion, the cerebellum was quickly removed and placed in each fixative for an additional 25 min. The cerebellar vermis was dissected, rinsed with 30 mM PIPES buffer solution containing 8% sucrose, and cut into 40 μ m sections by using a Microslicer (DKM-1000, DOSAKA EM Ltd., Kyoto). The tissue slices were then incubated in the medium for acid NPPase (16) for 30 min at 37°C. The incubation medium consisted of 100 mM sodium acetate buffer (pH 6.0), 3.0 mM NPP (sodium salt), 3.6 mM lead nitrate and 8% sucrose at final pH 5.6–6.0. For control experiments, the tissue slices were also incubated in a substrate-free medium or in a medium in which β -glycerophosphate was substituted for pNPP as substrate. In addition, effects of sodium fluoride (0.1–5.0 mM), L(+)-tartrate (5–10 mM), cupric sulfate (1.0 mM), sodium metavanadate (0.1 mM) and ammonium molybdate (0.1 mM) on the acid NPPase activity were examined. In another control experiment, some sections were incubated in the medium for the demonstration of glucose-6-phosphatase (G-6-Pase) activity (30). After incubation, sections for light microscopy were rinsed with a buffer solution, treated with a 1% yellow ammonium sulfide solution and mounted in gelatin. Similarly, the incubated sections for electron microscopy were rinsed with a buffer solution, postfixed with 2% osmium tetroxide in the PIPES buffer, dehydrated in a series of graded ethanol solutions, treated with propylene oxide, and embedded in Spurr's epoxy resin.

For electron microscopy, thin sections were prepared using a Reichert-Jung OmU₄ ultramicrotome. These sections were either unstained or stained with only uranyl acetate, and examined under a JEM 1200 EX (80 kV) electron microscope.

RESULTS

The P cells have located in their final position in the cerebellar cortex at PD5 and have initiated the dendritic growth into the molecular layer. Under a light microscope, acid NPPase activity was found in the developing P cell and thin molecular layers of the

cerebellar cortex at PD5 (Fig. 1a). In the P cells, granular activity of acid NPPase, perhaps lysosomal activity, was observed in their somal cytoplasm. With thickening of the molecular layer accompanying the dendritic growth of P cells, the activity of this enzyme was increased and became intense in the molecular layer (Figs. 1b, c). The most intense activity was seen in the deep portion of the molecular layer, which corresponded to the condensed area of newly-formed parallel fibers (Figs. 1b, c). The distribution pattern of the activity in the cerebellar cortex at PD20 was comparable to that in the adults (Figs. 1c, d). The acid NPPase activity in the granular layer appeared at PD10 and the activity was observed to be gradually localized in the presumptive synaptic glomeruli during the following ages. When the materials were incubated in the substrate-free medium, the acid NPPase activity was not detected in any structure of the cerebellar cortex (Fig. 1e).

Under an electron microscope, the acid NPPase activity was localized in the cisterns of endoplasmic reticulum and nuclear envelope of the developing and mature P cells, as well as in the lysosome and Golgi apparatus (Figs. 2a, b). In the molecular layer, the activity was observed in the axonal plasma membranes of parallel fibers rather than in the dendritic plasma membrane of P cells (Fig. 2c). In the synapses on the P cells, the acid NPPase activity was predominantly demonstrated in the presynaptic membrane (Fig. 2c). At PD5, a small number of parallel fibers were seen among the short and voluminous dendrites of P cells in the thin molecular layer. The axolemmal activity of acid NPPase at this age was localized on some axons, probably corresponding to the parallel and/or climbing fibers, near the P cells (Fig. 3a). With advancing postnatal ages, the axolemmal activity in the parallel fibers was increased, and almost all parallel fibers exhibited the activity on the plasma membrane, including the presynaptic membrane, by PD20 (Fig. 3b). In the granular layer, the acid NPPase activity was observed in the plasma membranes of the granular cell soma and their processes projecting to the synaptic glomerulus, as well as in their cisterns of endoplasmic reticulum and nuclear envelope (Fig. 4a). Macrophages, which appeared in the meninx, showed a dense and diffusible reaction of this enzyme in the cytoplasm (Fig. 4b).

In control experiments, sodium fluoride (NaF) over a concentration of 1.0 mM clearly inhibited the acid NPPase activity in the cerebellar cortex. NaF at the concentration of 0.1 mM also inhibited the enzyme activities of the endoplasmic reticulum and nuclear envelope of neuronal cells, but some activity remained

FIGS. 1a-e. Light micrographs of the acid NPPase activity in the developing (a-c) and adult (d) rat cerebellar cortex and control experiment in substrate-free medium (e). The acid NPPase activity has been observed in the P cell (P) and thin molecular (Mo) layers at PD5 (a) and the activity appeared increasingly in the thickening molecular layer (Mo) with advancing postnatal days (b, PD12, c, PD20 and d, adult). e) In the substrate-free medium, no activity was found in the cerebellar cortex (PD12). EG: external granular layer, G: (internal) granular layer. a-c and e. $\times 290$, d. $\times 220$.

in lysosomes and Golgi apparatus of the P cell soma and the meningeal macrophages (Figs. 5a, b). The axolemmal activity of acid NPPase in the molecular layer was clearly inhibited by NaF (Fig. 5c). On the other hand, L(+)-tartrate at the concentrations of 5–10 mM appeared to inhibit the acid phosphatase activity in the lysosomal organelle population in the P cells and meningeal macrophages, but did not affect the nonlysosomal activity of acid NPPase in the cerebellar cortex (Figs. 6a-c). Cupric sulfate (CuSO_4) at the concentration of 1.0 mM greatly decreased the acid NPPase activity in the cerebellar cortex, especially in the molecular layer (Fig. 7a). Sodium metavanadate (0.1 mM) and ammonium molybdate (0.1 mM) had no valuable inhibitory effect on the acid NPPase activity (Figs. 7b, c). The substitution of β -glycerophosphate for NPP as substrate lead to a great decrease of the acid phosphatase activity, especially that in the molecular layer (Fig. 7d).

When the tissue materials from the 12 day-old rats were incubated in a medium for G-6-Pase activity, the enzyme activity was positive in the P cell soma and the stem dendrite in the molecular layer, but no activi-

ty was seen in other elements of the molecular layer (Fig. 8a). Electron microscopic observations showed that the G-6-Pase activity was localized only in the cisterns of rough endoplasmic reticulum and nuclear envelope (Fig. 8b).

DISCUSSION

The present study shows that the developing and mature P cells in the rat cerebellar cortex exhibit the nonlysosomal activity of acid phosphatase in their nuclear envelope and endoplasmic reticulum, whose activity is found with NPP as substrate rather than β -glycerophosphate. In the synapses on the P cells, the acid NPPase activity is localized in the axonal plasma membranes including the presynaptic membrane. The acid NPPase activity in extralysosomal sites is sensitive to NaF and CuSO_4 , but not to L(+)-tartrate. Ng and Tam (18) have reported that total activity of acid phosphatase in the cerebellum was highest on day 45 of postnatal development, and that around 70–80% of the acid phosphatase activity remained in the presence of 200 mM tartrate.

FIGS. 2a-c. Electron micrographs of the acid NPPase activity in the perikaryon of a P cell (a and b) and the molecular layer (c) of 12 day-old rat cerebellar cortex. In the cytoplasm of P cell, the activity was found in the cisterns of nuclear envelope and endoplasmic reticulum, as well as in the lysosome (Ly) and Golgi apparatus (G). Nu: nucleus of the P cell. The acid NPPase activity in the molecular layer was observed in the plasma membranes, including the presynaptic membranes (arrows), of such parallel fibers (pf). a. $\times 12,500$, b. $\times 22,700$, c. $\times 38,000$

Histochemical localization of the NPPase activity at an alkaline pH has been described in a relation to the demonstration of Na^+ , K^+ -ATPase activity (10, 15, 17, 22, 29). The alkaline NPPase activity, which can not be considered as Na^+ , K^+ -ATPase activity, had been demonstrated in the cisterns of nuclear envelope and endoplasmic reticulum of the neuronal cells (10, 17). Ueno *et al.* (28, 29) had described that,

in the guinea pig retina, the K^+ -independent alkaline NPPase activity in such intracytoplasmic elements was a cytoplasmic marker of Müller cells rather than that of the neuronal cells. On the other hand, the alkaline NPPase activity in the synaptic membranes and the postsynaptic density of the neuronal cells has been considered as that of Na^+ , K^+ -ATPase (10, 17, 22). Non-specific alkaline phosphatase activity, using β -

FIGS. 3a-b. Electron micrographs of the acid NPPase activity in the molecular layer. a. Axolemmal activity in the parallel (pf) and/or climbing fibers at PD5. The axons with acid NPPase activity were localized near the P cells (P). mG indicates a migratory granular cell. $\times 22,500$ b. Acid NPPase activity in the superficial portion of the molecular layer at PD20. The activity was densely found among the closely packed parallel fibers (pf) and on the presynaptic membranes of parallel fibers on the P cell spines. $\times 12,000$ Arrows indicates the acid NPPase activity in the presynapses.

FIGS. 4a-b. Electron micrographs of the acid NPPase activity in the 12 day-old rat cerebellum. a. Acid NPPase activity in the granular layer. In addition to the activity in the cisterns of the nuclear envelope and endoplasmic reticulum, reaction products were rather observed on the plasma membranes of granular cell soma (GC) and their cytoplasmic processes (arrowheads). Sg shows an axon terminal in the synaptic glomerulus. $\times 15,000$ b. Acid NPPase activity in a meningeal macrophage (Mc). The macrophage contained numerous granular depositions of the acid phosphatase activity. Cap: capillary in the meninx. $\times 10,000$

FIGS. 5a-c. Effect of NaF (0.1 mM) on the acid NPPase activity. a. Light micrograph of the cerebellar cortex of a 20 day-old rat. Almost all the activity in the cerebellar cortex was inhibited by NaF. Arrows indicate the remnant of the lysosomal acid phosphatase activity in the meningeal macrophages. $\times 290$ b. Electron micrograph of P cell cytoplasm (PD20). NaF inhibited the activity in the endoplasmic reticulum (arrows), but some activities were observed in lysosome (Ly) and Golgi apparatus (G). $\times 22,400$ c. Electron micrograph of the molecular layer (PD12). NaF clearly inhibited the axonal activity of acid NPPase. Asterisks indicate the axonal terminals on the P cell spines. $\times 37,000$

FIGS. 6a-c. Effect of L (+)-tartrate (10 mM) on the acid NPPase activity. a. Light micrograph of the cerebellar cortex of a 20 day-old rat. There was no significant decrease of the acid NPPase activity in the cerebellar cortex. Note a decrease of the activity in the meninx (arrowheads). $\times 290$ b. Electron micrograph of the P cell cytoplasm (PD12). Tartrate appeared to inhibit the acid phosphatase activity in the lysosome (Ly) and Golgi apparatus (G), but did not inhibit that of the endoplasmic reticulum (er). $\times 14,000$ c. Electron micrograph of the molecular layer (PD12). The acid NPPase activity in the presynaptic membrane was not inhibited by tartrate (arrows). $\times 45,000$

FIGS. 7a-d. Control experiments for the acid NPPase activity. a. 1.0 mM CuSO_4 . The acid NPPase activity in the cerebellar cortex was greatly inhibited (adult). b. 0.1 mM metavanadate. There was no decrease of the acid NPPase activity in the cerebellar cortex (PD12). c. 0.1 mM molybdate. There was no distinct inhibitory effect on the acid NPPase activity (PD12). d. Substitution of β -glycerophosphate for NPP as substrate. When β -glycerophosphate was used as the substrate, a distinct activity of acid phosphatase was observed in the P cell soma and their large stem dendrites (PD12). a-c. $\times 290$, d. $\times 220$

FIGS. 8a-b. G-6-Pase activity in the 12 day-old rat cerebellar cortex. a. Light micrograph of the G-6-Pase activity in the soma and stem dendrites (arrows) of the P cells. $\times 290$ b. Electron micrograph of the G-6-Pase activity in the P cell cytoplasm. The activity was found only in the cisterns of nuclear envelope (arrowheads) and endoplasmic reticulum (er), but not in the Golgi apparatus (G). $\times 11,000$

glycerophosphate as substrate, had also been demonstrated in the presynaptic membrane of the rat spinal cord (32). Since the acid NPPase activity could not be observed on the postsynaptic density and the acid phosphatase activity using β -glycerophosphate as substrate was negative in the axonal plasma membrane of the cerebellar cortex, the acid phosphatase activity in such axonal plasma membranes, including the presynaptic membrane, is characterized to be specific to NPP as using substrate at acidic pH.

Most of the acid NPPase activities, which are closely associated with PTPases, are insensitive to fluoride and L (+)-tartrate, while they are sensitive to vanadate and molybdate (5, 20, 27). Recently, Singh (24) showed that an acid NPPase from the bovine brain was also sensitive to fluoride, as well as the osteoblastic acid NPPase (13, 14). In addition, the PTPases, from a variety of tissue cells, have been identified as cytosolic (5, 20, 24, 27) or membrane-associated (2, 25, 26) entities. As shown in the pre-

sent histochemical study, we failed in demonstrating the cytosolic activity of acid NPPase and the inhibition of the activity with vanadate and molybdate. Chen and Chen (4) reported that certain activity of cytoplasmic acid phosphatase was inhibited by glutaraldehyde, which is usually used in tissue fixation. Thus, it should be considered that in our present application such a fixation procedure may result in demonstrating a limited activity of the acid NPPases.

It has been well known that the brain contains undefined acid and alkaline phosphatase activities (18). In the cerebellar cortex, the alkaline phosphatase activity is high in the proliferative external granule cells during the early postnatal development and the activity disappears from the cortical neurons in adult life (23, 32). In contrast, the acid NPPase activity in the rat cerebellum appears increasingly throughout the postnatal development and a major site of positive nonlysosomal activity is the axonal plasma membranes, including presynaptic membrane, of the parallel fibers in the molecular layer. Singh (24) reported that the activity of acid NPPase, which has a PTPase activity, was highly sensitive to micromolar concentrations of Ca^{2+} . Previously, we have reported that the plasma membrane activities of Ca^{2+} - and Mg^{2+} -ATPases in the P cells and granular cells were proportionally increased throughout postnatal development (33). The appearance of such ATPase activities in the newly-formed parallel fibers is especially well coincident to that of the acid NPPase activity in the axonal plasma membranes of the parallel fibers, including the presynaptic membrane, throughout the cerebellar development. The present results may provide evidence that acid NPPase may play a significant role in establishing and/or regulating the neuronal communications between the cortical neurons in the cerebellum.

In conclusion, although we have no direct evidence that the acid NPPase activity is that of PTPase, the present study shows that the developing P cells and granular cells in the cerebellar cortex may contain several types of nonlysosomal acid NPPases. It is considered that the present results of acid NPPase activity, particularly the axolemmal activity in the developing synapses between the cortical neurons may be recalled by advancing studies of protein phosphorylation and dephosphorylation, which can involve in regulating various cellular activities such as cell growth, differentiation and/or neural transmission (6, 9).

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