Cytochemical Study of Various Phosphatase —Activities on the Capillary Walls of Rat Cerebral Cortex—

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Abstract

Normal adult Sprague-Dawley rats were used to demonstrate enzymatic activity on capillaries of the cortex. Fixation was performed by intracardiac perfusion of 2% glutaraldehyde in a 0.1 M cacodylate buffer of pH 7.2 with 8% sucrose. After perfusion for 2 min from 1.5 m in height, the brain was removed. Coronal slices (1.0 mm thickness) of the frontal cerebral cortex were fixed for an additional period of 10 to 30 min by immersion in the same fixative and then washed overnight in the same buffer solution. Frozen sections of 40 μ m in thickness were obtained from the washed tissues. The sections were immersed at 37°C for 30 min in the incubation media for acid phosphatase, alkaline phosphatase (ALPase), glucose-6-phosphatase, thiamine pyrophosphatase (TPPase), Mg^{++} -ATPase, Na^+ , K^+ -ATPase substituted for measuring K^+ -dependent p-nitrophenylphosphatase, 5'-nucleotidase (5'-Nase), and nucleoside diphosphatase (NDPase). Among them, ALPase, Mg^{++} -ATPase, TPPase, and NDPase demonstrated positive staining on the capillary walls when viewed with light microscope. The ultrastructural localization of these four enzymes on the capillary walls was investigated.

The cytochemical findings gave the following results. All four enzymatic activities were found on the abluminal surfaces of the endothelial cells and on the surfaces of the pericytes. Electron dense precipitations on the luminal surfaces of endothelial cells were infrequently seen and were considered an artefact. Intense reaction products of ALPase and Mg^{++} -ATPase were evenly distributed on the surfaces of the astrocytic processes in contact with the thick basal lamina. Heavy, dotted reaction products of TPPase and NDPase were found in the basal lamina.

The ultrastructural localization of ALPase, TPPase, Mg^{++} -ATPase, and NDPase were also briefly discussed in connection with cerebral microcirculation and the blood-brain barrier.

Key words: brain capillary, alkaline phosphatase, thiamine pyrophosphatase, Mg^{++} -ATPase, 5'-nucleotidase, nucleoside diphosphatase

Introduction

It is obvious that the function of brain capillaries in transporting substances from the blood to the brain requires some unique properties. An investigation of the localization of enzyme activity on the capillary walls, including the perivascular astrocytic processes, is necessary to elucidate the regulatory mechanism involved in this transport of substances.

There have been various reports regarding the ultrastructural localization of phosphatase activities on the capillary walls of the central nervous system^{13,15,21,22,24,31,35,36} and it has been thought that histochemical study of their distribution may clarify some aspects of the mechanisms occurring in the capillary walls, including the blood-brain barrier

and the enzymatic barrier.

Much of the physiological and biochemical research on the blood-brain barrier has dealt with metabolites which cannot normally be visualized. However, some indication of their movement can be inferred from the histochemistry of enzymes which transport compounds in the brain. The histochemical localization of alkaline phosphatase, Mg^{++} -ATPase, nucleoside diphosphatase, and thiamine pyrophosphatase on the cerebral capillary wall might be correlated with biochemical experiments. Therefore, we sought some anatomical correlation between the ultrastructural localization of these enzymes and the cerebral capillaries protected by the blood-brain barrier.

Materials and Methods

Adult Sprague-Dawley rats of both sexes were perfused through the ascending aorta with cold 2% glutar-

aldehyde and 8% sucrose in a 0.1 M cacodylate buffer (pH 7.2) for 2 min from 1.5 m in height under intraperitoneal Nembutal anesthesia. Next, 1 mm thick coronal slices of the frontal lobe were immersed in the same fixative for 30 min each for acid phosphatase, 5'-nucleotidase, alkaline phosphatase, thiamine pyrophosphatase, Mg^{++} -ATPase, and nucleoside diphosphatase histochemistry, and for 10 min each for glucose-6-phosphatase and Na^+ , K^+ -ATPase (p-nitrophenylphosphatase) histochemistry. Following fixation, the incubated tissues were washed overnight in a cold 0.1 M cacodylate buffer (pH 7.2) with 8% sucrose.

The washed tissues were cut into 40 μm frozen sections and immersed in the following incubation media: 1) for acid phosphatase,¹⁰⁾ the sections were immersed in Gomori's medium at 37°C for 30 min. As a control, they were incubated either in the presence of 25 mM NaF or in the absence of the substrate (β -glycerophosphate); 2) for alkaline phosphatase (lead citrate method²⁴⁾), they were incubated at 37°C for 30 min. As a control, they were incubated either in the presence of 1.0 mM bromotetramisole or in the absence of the substrate (β -glycerophosphate); 3) for Mg^{++} -ATPase, the sections were incubated in the medium of Wachstein and Meisel⁴¹⁾ or by the lead citrate method of Ogawa and Mayahara²⁸⁾ at 37°C for 30 min. As a control, the reactions were performed either in the absence of Mg^{++} or of the substrate (ATP); 4) for Na^+ , K^+ -ATPase substituted for measuring K^+ -dependent p-nitrophenylphosphatase (lead citrate method²³⁾), the sections were incubated in the medium of Mayahara *et al.*²³⁾ at 37°C for 30 min; 5) for glucose-6-phosphatase,⁴⁰⁾ they were immersed in the medium at 37°C for 30 min; 6) for thiamine pyrophosphatase,²⁷⁾ they were incubated in the medium at 37°C for 30 min. The control experiments consisted of modifying the incubation medium either by omitting the substrate (thiamine pyrophosphate chloride) or by adding 25 mM NaF; 7) for 5'-nucleotidase,⁴¹⁾ the sections were incubated in the medium at 37°C for 30 min; 8) and finally for nucleoside diphosphatase,²⁷⁾ they were immersed at 37°C for 30 min. As a control, the sections were incubated in the absence of the substrate (sodium inosine diphosphate).

All the incubated sections were rinsed again in the buffer solution. For light microscopic histochemistry, they were immersed in 1% ammonium sulfide for 2 min. ALPase, Mg^{++} -ATPase, TPPase, NDPase, and 5'-Nase were studied with electron microscopic cytochemistry. The sections were postfixed in 1% osmium tetroxide for 60 min at 4°C, dehydrated in a graded series of ethanol, then in propylene oxide, and finally embedded in Epon 812. After ultrathin sectioning with

a LKB Ultratome III, the specimens were usually stained with uranyl acetate for 3 min, with lead citrate for 3 min, and observed at an accelerating voltage of 75 KV.

Results

I. Light microscopic observation (40 μm sections)

The activities of eight phosphatase enzymes were studied on the microvessels of the frontal cortex of the rat. Staining for acid phosphatase, glucose-6-phosphatase, 5'-nucleotidase, and Na^+ , K^+ -ATPase substituted for measuring K^+ -dependent p-nitrophenylphosphatase were not obtained on the capillary walls (Figs. 2A, B, C, D). On the contrary, staining for alkaline phosphatase, Mg^{++} -ATPase, thiamine pyrophosphatase, and nucleoside diphosphatase were obvious on the capillary walls (Figs. 1A, C, E, G). In the control experiments, there was no staining when the tissue sections were incubated in the following incubation media for alkaline phosphatase, in the presence of 1.0 mM bromotetramisole; for thiamine pyrophosphatase, in the presence of 25 mM NaF; for Mg^{++} -ATPase, in the absence of Mg^{++} and the substrate, and for nucleoside diphosphatase, in the absence of the substrate (Figs. 1B, D, F, H).

II. Electron microscopic observation

Ultrastructural observations were made for the four enzymes which had revealed remarkable staining on the light microscopic level. 5'-Nase was also studied.

Alkaline phosphatase: Electron dense reaction products were distributed discontinuously on the abluminal (contra-luminal) surfaces of endothelial cells. Precipitation on the luminal surfaces of endothelial cells was inconstant and was considered an artefact. Reactions were also noticed along both the luminal and abluminal membranes of the pericytes. Reaction products, stronger than those in any other structures, were distributed evenly or continuously on the surfaces of the astrocytic processes in contact with the thick basal lamina (Figs. 3A, B). In the control, no electron dense reaction product was found.

Mg^{++} -ATPase: ① When the Wachstein and Meisel method⁴¹⁾ was employed, heavy, dotted electron dense reaction products occupied the basal lamina and some reactions appeared to be present on the adjacent glial cells. Some diffusions were found in the endothelial cells or on the luminal surfaces of the endothelial cells (Fig. 4A). In the cytochemical controls, the basal lamina did not contain electron dense precipitates. ② When the lead citrate method²⁸⁾ was used, finer and relatively continuous reaction products were obtained along the abluminal surface of the

Fig. 1 Light microscopic observations of phosphatase activities on the microvessels of rat brain cortex. A: Alkaline phosphatase; positive. $\times 312$. B: Control experiment of alkaline phosphatase in the presence of 1.0 mM bromotetramisole. $\times 312$. C: Mg^{++} -ATPase; positive. $\times 624$. D: Control experiment of Mg^{++} -ATPase, in the absence of the substrate. $\times 312$. E: Thiamine pyrophosphatase; positive. $\times 312$. F: Control experiment of thiamine pyrophosphatase, in the presence of 25 mM NaF. $\times 312$. G: Nucleoside diphosphatase; positive. $\times 624$. H: Control of nucleoside diphosphatase, in the absence of the substrate. $\times 312$.

Fig. 2 Light microscopic observation of other phosphatases on the microvessels of the rat brain cortex. A: Acid phosphatase; negative. $\times 312$. B: Na^+ , K^+ -ATPase (K^+ -dependent p-nitrophenylphosphatase); negative. $\times 312$. C: Glucose-6-phosphatase; negative. $\times 312$. D: 5'-nucleotidase; negative. $\times 312$.

endothelial cells, along both luminal and abluminal surfaces of the pericytes, and on the surfaces of astrocytic processes. No remarkable precipitation was noticed on the luminal surface of the endothelial

cells (Figs. 4B, C). In the control experiments, no reaction was seen.

Thiamine pyrophosphatase: Coarse electron dense reaction deposits were found along the abluminal

Fig. 3 Electron microscopic localization of alkaline phosphatase activity in the rat cerebral capillary wall. A: Low magnification; the reaction products are distributed evenly or continuously on the surface of the astrocytic process (*arrows*). Lumen of the capillary (lu). $\times 21,900$, Bar = $1\ \mu\text{m}$. B: High magnification of (A). Intense reactions are found on the surface of the astrocytic process (*arrows*) and on the abluminal surface of the endothelial cell (ed). Some precipitations seen on the luminal surface of the endothelial cell might reflect an artefact. Lumen of the capillary (lu). $\times 87,600$, Bar = $0.1\ \mu\text{m}$.

Fig. 4 Electron microscopic localization of Mg^{++} -ATPase activity in the rat cerebral capillary wall. A: With Wachstein and Meisel's procedure, heavy and dotty electron dense reaction products occupy the basal lamina (*arrows*). Some diffusions are seen in the endothelial cell (ed). Lumen of the vessel (lu). $\times 20,000$, Bar = $1\ \mu\text{m}$. B and C: With the lead citrate method, finer and relatively continuous reaction products are obtained along the abluminal surfaces of the endothelial cell (ed), on the surface of the pericyte (pc), and on the surface of astrocytic process (*arrows*). Lumen of the vessel (lu). B: $\times 40,500$, Bar = $0.1\ \mu\text{m}$, C: $\times 69,600$, Bar = $0.5\ \mu\text{m}$.

surfaces of the endothelial cells and along both the luminal and abluminal surfaces of the pericytes. Reactions were found in the basal lamina between the astrocytic processes and the pericytes. On the surfaces of the astrocytic processes there were dotted, patchy reactions (Figs. 5A, B). When the tissues were incubated in the incubation medium containing 25 mM NaF, the reactions were effectively inhibited.

Nucleoside diphosphatase: Reaction products were mainly observed in the basal lamina, and especially intense reactions were noticed between the endothelial cells and the astrocytic end-feet. A definitive localization of this enzyme on the surface of the astrocytic process was not possible. The heavy, discontinuous

reaction granules on the luminal surface of the endothelial cells may have been artefacts (Figs. 6A, B). When the incubation medium omitted the substrate (sodium inosine diphosphate), no reaction products were found in the capillary walls.

5'-nucleotidase: Reaction products were very weakly positive in the basal lamina and on the astrocytic end-feet.

Discussion

A variety of phosphatases have been studied mainly at the level of light microscopy in vascular tissues of the central nervous system and of other organs. Of

Fig. 5 Electron microscopic localization of thiamine pyrophosphatase activity in the rat cerebral capillary wall. A: The coarse electron dense reaction products are observed along the abluminal surface of the endothelial cell (ed), on the surfaces of the pericyte (pc), and in the basal lamina (arrowheads). $\times 39,600$, Bar = $0.5 \mu\text{m}$. B: On the surface of astrocytic process there are dotted and patchy reactions (arrows). Lumen of the capillary (lu). $\times 49,500$, Bar = $0.1 \mu\text{m}$.

Fig. 6 Electron microscopic localization of nucleoside diphosphatase activity in the rat cerebral capillary wall. A: The reaction products are mainly observed in the basal lamina (arrows). Some precipitation granules on the surface of the endothelial cell (ed) may be due to artefacts. $\times 23,000$, Bar = $1 \mu\text{m}$. B: High magnification of (A). Pericyte (pc) and lumen of the capillary (lu). $\times 44,000$, Bar = $0.5 \mu\text{m}$.

phosphatase activities, alkaline phosphatase and Mg^{++} -ATPase activities have been most intensely studied in the nervous system. Much less attention has been paid to other phosphatases such as thiamine pyrophosphatase and nucleoside diphosphatase. To our knowledge, neither thiamine pyrophosphatase nor nucleoside diphosphatase activity in the cerebral capillaries has been studied by means of ultrastructural cytochemical methods.

The ultrastructure of capillaries in the central nervous system has been studied by a number of investigators.^{5,18,25} Dahl⁵ described the precise

ultrastructure of the capillary. The endothelium of the brain capillary forms a complete, continuous layer with no fenestration. The walls of capillaries may contain pericytes surrounded by basement membranes. Membranous contact between cytoplasmic processes of the pericytes and the endothelium, similar to the myo-endothelial junctions of the arterioles, are rarely seen. In capillaries without pericytes, the basement membrane is the only structure which separates the endothelium from the surrounding cortical parenchyma. According to the observations of Maynard *et al.*,²⁵ the basement membrane may be said to be shared by

both the endothelium and the sheath of the astrocytic feet. The capillaries are incompletely sheathed (approximately 8.5% complete) by the watery end-feet of the astrocytes. Other cortical elements, including nerve perikarya, fill in the discontinuities. Vertebrate blood capillaries were ultrastructurally classified by Bennett *et al.*⁴⁾ The capillaries of the brain possess a characteristic structure showing a complete continuous basement membrane, no fenestration or perforation of endothelial cells, and a complete pericapillary cellular investment which is composed of glial processes or end-feet between parenchymal cells and capillaries.

The primary importance of brain capillary endothelial cells and astrocytic end-feet has been emphasized in the maintenance of the blood-brain barrier. Tight junctions between endothelial cells preclude movement of large molecules from the capillary lumen to the extracellular space, and vice versa. Reflecting their specific transport role, brain endothelial cells have an increased number of mitochondria, as compared to other capillaries.¹⁴⁾

It is possible that certain microvessel enzymes have a specific function in the mechanism of the blood-brain barrier. Van Gelder³⁹⁾ stressed, in his study on the blood-brain barrier, that in addition to anatomical barriers, metabolic processes had to play an important role in preventing a variety of different compounds from penetrating into the central nervous system. Katzman¹⁴⁾ thought of an enzymatic barrier in the brain capillary endothelium where, for at least aminobutyrate and L-DOPA, transport between blood and brain tissue was retarded by enzymatic degradation of these substances. Torack *et al.*³⁶⁾ described that the distinct localization of a high energy producing enzyme, in the basement membrane such as ATPase, controlled capillary permeability. Histochemical studies on the distribution of γ -aminobutyric acid (GABA)-ketoglutarate transaminase in the mammalian central nervous system suggest that the barrier may in part be a reflection of high GABA-transaminase activity in the wall of cerebral vessels and in the cells lining the cerebrospinal fluid spaces.^{37,38)} Joó and Csillik¹²⁾ revealed that butyrylcholinesterase activity was present only in the cytoplasm of endothelial cells of brain capillaries in areas that were defended by the blood-brain barrier, and supposed that the blood-brain barrier function was correlated with the hydrolysis of some biologically active choline ester participating in the pinocytotic process. By biochemical and histochemical procedures, Orlowski *et al.*²⁹⁾ found that γ -glutamyl transpeptidase was localized in the endothelial cells of cerebral capillaries and that this enzyme might function in the transport of some amino acids across the barrier.

There have been some reports that, at the light microscopic level, various phosphatases are localized on the walls of brain capillaries. Bartoniček and Lojda²⁾ investigated the activity of various nucleoside phosphatases on the capillaries of different species; viz. rat, mouse, guinea-pig, and golden hamster. Among them, the rat cerebral capillaries showed the strongest activities of 5'-nucleotidase, ATPase, and thiamine pyrophosphatase on the endothelial cells. In brain capillaries, Becker *et al.*³⁾ were unable to specify whether the staining for ATPase activity was in endothelial cell membranes or basement membranes, or both. In arterioles, the cell membranes of endothelial and muscle cells stain darkly for this enzyme activity. Landers *et al.*¹⁶⁾ found ATPase activity in capillary endothelial cells and pericytes. Manocha²⁰⁾ observed that ATPase activity was more pronounced in the neuropil and the blood vessels compared to that in the neurons.

In our experiment, light microscopic histochemistry for 5'-nucleotidase showed the slightest staining on the capillary walls. Torack and Barrnett³⁵⁾ found that the capillaries in the cortex were strongly active with ATP, CTP, or IDP as substrates, whereas no activity was obtained with AMP as 5'-nucleotidase. The capillaries and blood vessels elsewhere in the brain were not as rich in 5'-nucleotidase activity as they were in ATPase.

In their cytochemistry of 5'-nucleotidase in the brain capillary, Torack and Barrnett³⁵⁾ found no activity in the basement membrane with inosine diphosphatase as a substrate while Mg^{++} -ATPase was consistently found. Kreutzberg *et al.*¹⁵⁾ observed that typical cerebral pericapillary astrocytic footplates possessed 5'-nucleotidase activity with coarse reaction products in their plasma membranes. These reaction products were generally greater on the tissue side of the basement membrane than on the capillary side. According to our observations, weak 5'-nucleotidase activity was localized in the basal lamina and on the astrocytic processes. Orlowski *et al.*²⁹⁾ biochemically measured the activity of 5'-nucleotidase, alkaline phosphatase, and γ -glutamyl transpeptidase in bovine brain cortex fractions which consisted mainly of fragments of capillaries or of large vessels branching out into capillaries: for 5'-nucleotidase, 1.5 units; alkaline phosphatase, 2.2 units; and γ -glutamyl transpeptidase, 0.82 units (one unit represented the amount of enzyme that catalyzed the formation of 1 μ mol of the product per 30 min per milligram of protein).

On the microscopic level, alkaline phosphatase staining of the walls of the capillaries in the brain has been reported by a number of researchers using various histochemical methods.^{1,8,9,16,17,19,22,32,33)} Walls of

capillaries in the rat brain of about 6–9 μm in luminal diameter showed alkaline phosphatase activity, but capillaries of less than 5 μm in luminal diameter demonstrated no activity, and no enzyme activity was seen in the walls of venules or veins.²²⁾

A few studies have been reported concerning the ultracytochemical demonstration of alkaline phosphatase activity in cerebral microvessels.^{22,24,31)} The investigators observed enzymatic activity in the plasma membranes of the endothelial cells, along the basal lamina, on the surfaces of astrocytic processes in contact with the thick basal lamina of the capillary,^{22,24,31)} and on the membranes of pericytes.^{22,24)} From these findings, alkaline phosphatase might be involved in the control of the transport mechanism across capillary walls and glial cell membranes, *i.e.*, of the blood-brain barrier.^{22,24)}

In their ultrastructural localization of Mg^{++} -ATPase, Joó *et al.*¹³⁾ and Torack and Barrnett³⁵⁾ noted that reaction products were confined mainly to the basal lamina of the capillary wall. The plasma membranes of the glial foot processes abutting on the basal lamina contained a slight, spotty reaction.³⁵⁾ Within the limits of Wachstein and Meisel's method which employs lead nitrate as a precipitating reagent, our findings, as well as those of Torack and Barrnett³⁵⁾ and Joó *et al.*,¹³⁾ demonstrated that the electron dense reaction products were heavily distributed in the capillary wall, but also that some unwanted precipitations were inevitable in the structures. The lead citrate method for Mg^{++} -ATPase histochemistry²⁸⁾ was more useful because finer and more delicate localizations were obtained along the abluminal surface of the endothelial cells, on the surface of the pericytes, and on the surface of the astrocytic processes without any unwanted precipitation in the tissue.

Except for a brief description of nucleoside diphosphatase activity by Torack and Barrnett,³⁵⁾ the ultrastructural localization of thiamine pyrophosphatase and nucleoside diphosphatase in the capillaries of the central nervous system has never been reported. Nucleoside diphosphatase reaction products were found in the pericapillary region of mammothrophic hormone producing cells in the adenohypophysis of lactating rats, and a few clumps of electron dense deposits were seen along the luminal surface of the endothelium.³⁴⁾ The basement membrane between the secretory cells of the rat anterior pituitary gland and the adjacent blood capillaries showed many reaction products of nucleoside diphosphatase with scant patches of reaction products usually found at the luminal surface of the capillary endothelial cells.³⁰⁾

In spite of our negative histochemical results for Na^+ , K^+ -dependent p-NPPase by using the lead

citrate method of Mayahara *et al.*,²³⁾ Mršulja and Mršulja,²⁶⁾ using Ernst⁷⁾ and Guth and Albers¹¹⁾ procedures, found that the site of K^+ -dependent p-NPPase was associated with the vascular endothelium and neuropil. Since Na^+ , K^+ -ATPase has been biochemically found to be present in isolated brain capillaries,¹³⁾ a thorough cytochemistry of Na^+ , K^+ -ATPase in this tissue on the electron microscopic level is still required.

Similar to Landers *et al.*,¹⁶⁾ our findings revealed no staining for acid phosphatase activity on the wall of cerebral capillaries, but it is probable that electron microscopic cytochemistry for this enzyme could demonstrate activity on the lysosomes. In brain capillary endothelium, the lysosomes are concentrated and their large size and apparently high level of acid phosphatase activity suggests that lysosomes might be involved in the mechanism of the blood-brain barrier.³⁾

Light microscopic histochemistry of glucose-6-phosphatase was negative on the walls of cerebral capillaries in our experiment. Ultrastructural cytochemistry for this enzyme is still required because there is biochemical data which demonstrates 4.2 times greater glucose-6-phosphatase activity than in the brain parenchyma, and the glucose transport system through the capillary endothelial cell under the existence of glucose-6-phosphatase.²⁶⁾

Conclusions

Our findings support the suggestion that the phosphatase enzymes localized in the walls of cerebral capillaries, including the astrocytic processes, form a barrier against the entrance of exogenous phosphatic compounds into the central nervous system. The activity of phosphatases in the basal lamina of the capillaries point to an active transport of metabolites.²⁾ The studies reported here indicate that cerebral capillary endothelium and pericapillary astrocytic end-feet are metabolically active tissues with regard to certain phosphatases, such as alkaline phosphatase, Mg^{++} -ATPase, thiamine pyrophosphatase, and nucleoside diphosphatase. However, clearer and more direct biochemical evidence has to be accumulated for the relationship between the activity of phosphatases and the blood-brain barrier to be proved.

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