Staining of acetylcholinesterase activity of the enteric nervous system by means of intracardiac perfusion of the histochemical medium: Restricted localization of the extracellular enzymatic activity

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Abstract: Histochemical staining of acetylcholinesterase (AChE) activity of rat enteric nervous system was studied using intracardiac perfusion of modified Karnovsky's histochemical medium. The optical and electron microscopical observation disclosed the AChE activity in extra-somatic and extra-axonal space in both the myenteric and submucous plexuses. Absence of staining of intracellular AChE is due to impermeability of the plasma membrane to the histochemical medium during the intracardiac perfusion. Possible physiological significance of the extracellular AChE is discussed.

Key words: Acetylcholinesterase; enteric nervous system; histochemical reaction; intracardiac perfusion.

Introduction. Cholinergic synaptic transmission employs acetylcholine (ACh) as a neurotransmitter. Acetylcholinesterase (AChE) is the enzyme of rapid hydrolysis of ACh and contributes to terminating the action of ACh. The histochemistry of AChE was mostly performed on fixed nervous tissues and revealed intense activity of AChE in the soma (perikaryon) of different types of neurons. Due to weak sensitivity, the extra-somatic and extra-axonal activities of AChE were only faintly stained or almost neglected in the classical histochemistry.

An improved highly sensitive histochemical method demonstrated AChE activity not only in the neural perikaryon but also on the fine neurites of the central nervous system (CNS). This sensitive method employs highly diluted Karnovsky's medium. Because of low concentration of the histochemical medium, quantity of the primary precipitate was also minute and invisible, but its presence could be revealed using peroxidase-like or oxido-reductase-like catalytic activity of the precipitate by means of diaminobenzidine (DAB)-H₂O₂.

This improved method gave us an idea to reduce toxicity of the cupric ions of the histochemical medium in order to perform histochemical reaction of AChE on the living neuromuscular junction. A fine staining of the neuromuscular AChE has been obtained by this histochemical method. We undertook to apply this method to the neural ganglia. In the present study, the histochemical medium for staining AChE activity was applied to the rat by means of intracardiac perfusion, so that staining could be performed in a semi-vital condition. The enteric nervous system (ENS) was successfully stained.

Materials and methods. Intracardiac perfusion of histochemical medium with acethiocoline iodide. Deeply anesthetized rats were perfused with 100 ml of the modified Krebs solution (117 mM NaCl, 4.7 mM KCl and 2.5 mM CaCl₂) for 3 min, and 500 ml of modified and undiluted Karnovsky's histochemical medium for AChE activity, containing 3 mM CuSO₄, 0.5 mM K₃Fe(CN)₆ and 1.8 mM acetylthiocholine iodide dissolved in a mixture of 450 ml of the Krebs solution and 50 ml of 0.1 M citrate buffer pH 6.1, 15 min, followed by 300 ml of fixative containing 4% paraformaldehyde, 0.25% glutaraldehyde, 0.25% glutaraldehyde dissolved in the Krebs solution, 10 min. Segments of the ileum were cut out. Preparations of longitudinal muscle bearing the
Fig. 1. Staining of AChE activity in the rat enteric nervous system after intracardiac perfusion of modified Karnovsky's medium. Contour of the neuronal somata and nerve strands was positively stained. (A) Myenteric plexus. (B) Submucous plexus. (C) Deep muscle plexus. Scale bar, 50 μm.
Fig. 2. Electron micrographs of rat myenteric plexus and submucous plexus after staining of AChE activity with intracardiac perfusion of modified Karnovsky's medium. (A) Staining of extra-somatic space of myenteric neuron (arrows). Extracellular space of longitudinal muscle cells was also stained (arrowheads). (B) Staining of extrasomatic space of submucous neurons (arrows). (C) Extra-axonal AChE activity. Extracellular space of circular muscle cells was also partially stained. cm: circular muscle; lm: longitudinal muscle; n: nucleus. Scale bars, 1 μm for A-C.
myenteric plexus layer and the submucous layer containing the submucous plexus were isolated in the Krebs solution, and treated for 10 to 15 min with diaminobenzidine tetrahydrochloride (DAB), 4 mg/10 ml Krebs solution, to which 0.01% H$_2$O$_2$ was added. The control of the cytochemical reaction was done by omitting the enzymatic substrate. In this case the reaction was negative. When 10$^{-5}$ M iso-OMPA, an inhibitor of nonspecific cholinesterases was added, the staining was not changed.

Electron microscopic observation of the histochemical reaction. A part of preparations was osmified, dehydrated and embedded in Araldite according to the conventional procedure. The ultrathin sections were observed with JEOL 100 CX electron microscope at 80 kV without counterstaining.

Results. Histochemical reaction through intracardiac perfusion. Although the undiluted Karnovsky’s medium provided “direct-coloring” of the nervous tissues within several minutes in vitro, the same undiluted medium did not provide the “direct-coloring” of the enteric nervous tissue in situ after intracardiac perfusion. The products of primary histochemical reaction were, however, sufficient to be visualized by the further treatment with DAB and H$_2$O$_2$ within 5–10 min. This is equivalent to most of our modified histochemical reactions, performed in vitro with 10 times diluted modified Karnovsky’s medium and revealed with DAB and H$_2$O$_2$ within 5–10 min. It is therefore reasonable to suppose that the concentration of the histochemical medium at the tissue level after the perfusion is about 1/10 of that of the undiluted perfusate.

Observation with the optical microscope. Fig. 1 shows the histochemical staining for AChE activity in the myenteric plexus (A) and submucous plexus (B). The AChE activity was observed to be limited to the contour of the neuronal soma but not found inside of the soma in both plexuses. Thick nerve strands of the primary plexus, the secondary and tertiary fine plexuses of the myenteric plexus and those in submucous plexus were positive for the AChE reaction (Fig. 1A and B). However, the extra-axonal localization of AChE was difficult to be confirmed in the optical microscopy due to the restricted dimension of the neurites (See electron microscopic observation). Varicose-like structure of the deep muscle plexus was finely stained in the circular muscle layer (Fig. 1C).

Observations with the electron microscope. Extracellular AChE activity observed with the optical microscope was confirmed electron microscopically in the rat myenteric (Fig. 2A) and submucous plexuses (Fig. 2B). The extra-axonal localization of AChE activity was demonstrated (Fig. 2C). In addition, extracellular space of the longitudinal (Fig. 2A) and circular (Fig. 2C) muscle cells was stained as well.

Discussion. Contrary to the intense somatic (perikaryon) staining in the classical histochemistry of AChE performed on the fixed enteric nervous tissues, somata of the enteric neurons were not stained with cardiac perfusion of the histochemical medium. Electron microscopic observation of the histochemical reaction on fixed neurons revealed the AChE staining not only in the endoplasmic reticulum in somata but also in synaptic and extracellular space both in the CNS and the ENS. Thus, it is possible that our exclusive extracellular staining is due to the impermeability of the plasma membrane to the histochemical agents in semi-vital condition.

It has been presumed that somatic AChE is not directly involved in the cholinergic transmission. In addition, a release of dendritic AChE was observed and the released enzymatic molecules appear to modulate neuronal excitability. Indeed we recently demonstrated ultrastructural basis for exocytotic release of dendritic AChE in the CNS. A question arises if only the extraneuronal AChE would play a role for cholinergic transmission in synaptic and diffuse area, the latter being hypothetically proposed in the CNS.

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