Acetylcholinesterase activity in the frog neuromuscular junction observed at electron microscopic level after << vital >> histoenzymatic reaction

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Abstract: The aim of this work was to observe at electron microscopic level acetylcholinesterase (AChE) activity of frog neuromuscular junction revealed by an histoenzymatic reaction made on living tissues. In this purpose, Karnovsky's histochemical medium was used for the detection of AChE activity at concentrations ten times more diluted than in the original method. The medium was dissolved in Ringer solution containing citrate buffer, a chelating agent of cupric ions, and additional calcium chloride. Living nerve muscle preparation of the bullfrog was incubated in this histochemical Ringer solution. The muscle activity in response to electrical stimulation of motor nerve (0.1 Hz) was monitored by recording of muscular contraction. The incubation was not prolonged beyond 15 min, since a blockade of the neuromuscular transmission steadily occurred at that time. The tissue remained alive, however, since transmission was recovered by washing with fresh Ringer solution containing citrate buffer and additional calcium chloride. The primary histochemical precipitates endowed with oxidoreductase-like activity were revealed by secondary incubation with diaminobenzidine (DAB) and hydrogen peroxide. After osmification, osmium black of DAB gave fine localization of AChE activity in the synaptic cleft under the electron microscope.

Key words: Acetylcholinesterase; vital histoenzymatic reaction; frog neuromuscular junction, synaptic cleft; electron microscopic localization.

It is well known that acetylcholine (ACh) released into the synaptic cleft of the frog neuromuscular junction is quickly hydrolysed by AChE. The presence of this enzyme is very important to ensure the synaptic transmission and its localization contributes to understand how the synapses work. Koelle's copperthiocholine method¹⁾ and Karnovsky's << direct coloring >> thiocholine method²⁾ are the most widely used histochemical methods. The mechanism of the chemical reactions of both methods was examined by Tsuji³⁾ and Tsuji and Larabi.⁴⁾

Karnovsky's method was modified for sensitive localization of AChE activity at optical microscopic level by Tago *et al.*⁵⁾ These authors performed Karnovsky's histochemical reaction in a highly diluted medium and revealed a << peroxidase-like activity >> of a minute reaction product by means of DAB and H₂O₂. Recently, the mechanism of

Tago's reaction was examined by Tsuji⁶⁾ who modified the method to obtain fine localization of the enzymatic activity at electron microscopic level. The << peroxidase-like >> activity of the fine histochemical precipitate was considered to be in fact an oxidoreductase-like activity. Indeed, these precipitates are composed of cupric ferrocyanide and cuprous ferricyanide that are supposed to transform one in another by intramolecular oxidoreduction reaction of copper and iron ions. The oxidoreductase-like activity of the precipitates presumably derives from this intramolecular oxidoreduction. Indeed, Karnovsky's precipitates can be revealed by DAB alone⁷⁾ or DAB with H₂O₂.⁵⁾

To our knowledge, no vital method of histoenzymatic reaction was devised for the nervous tissue. Indeed, histochemical agents, in particular metallic ions, appear to be highly toxic⁸⁾ in comparison to organic dyes such as methylene blue.⁹⁾ However, in the case of AChE histochemistry, the << oxidoreductase-like >> activity of Karnovsky's precipitate revealed by DAB and H₂O₂ permits to reduce the concentration of histochemical agents.⁵⁾ Thus,

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as shown in this study, it became possible to perform a vital histoenzymatic reaction by modification of Karnovsky's reaction.

Materials and methods. Cutaneous pectoris muscles of bullfrogs (*Rana catesbiana*) were used for all the experiments. The preparations were pinned on the bottom of an experimental chamber containing 4.5 ml of Ringer solution (117 mM NaCl, 4.7 mM KCl and 1.8 mM CaCl_2). The bundle of motor nerves was electrically stimulated with a pair of silver chloride electrodes (supramaximal voltage: 3–7 V; 0.3 ms) at 0.1 Hz and the muscle contractions were monitored on a chart recorder (San-ei; Recti-Horiz) by isometric mechano-electric transducer (Nihon Kohden; TB-611T). In most cases, the normal tension of muscular twitch due to nerve stimulation was 3 g. In order to control the efficiency of the nerve stimulation, the muscle was stimulated directly by a single electrical pulse via a pair of silver wires fixed on opposite walls of the chamber (10–100 V, 3–10 ms), which provided equal or slightly more intense contractions than nerve stimulation.

For each experiment, the following solutions were prepared: 100 mM citrate buffer at pH 6.2, 100 mM CaCl_2 , mixture of citrate buffer (30 mM) and CaCl_2 (70 mM), 100 mM CuSO_4 , 100 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 100 mM acetylthiocholine iodide.

The histochemical medium was prepared in the experimental chamber by adding to 4.5 ml of Ringer solution: 0.5 ml of the mixture of citrate buffer and CaCl_2 , 15 μl CuSO_4 (0.3 mM), 2.5 μl $\text{K}_3\text{Fe}(\text{CN})_6$ (0.05 mM) and 9 μl acetylthiocholine iodide (0.18 mM). The final concentrations of cupric ions, ferric ions and acetylthiocholine iodide in the parenthesis correspond to one tenth of the concentrations currently used in the original Karnovsky's medium.

During histochemical incubation (15–20 min), muscular contractions evoked by nerve stimulation were monitored. After incubation the preparation was washed either with Ringer solution or Ringer solution containing the mixture of citrate buffer and CaCl_2 . The preparations were fixed (30 min) in 2.5% glutaraldehyde dissolved in Ringer solution. After washing with Ringer solution, the oxidoreductase-like activity of the histochemical precipitates was revealed (15 min) with DAB (0.05%) and H_2O_2 (0.01%) dissolved in Ringer solution. The staining of the motor endplates by DAB was controlled under optical microscope. For electron microscopic observations, the preparations were osmified and embedded in Araldite by a conventional procedure. Ultrathin sections were observed without counterstaining under JEOL 100 CX electron

microscope at 80 kV.

Results and discussion. Citrate buffer is necessary for preventing undesirable interaction between cupric and ferricyanide ions, thus stabilizing Karnovsky's medium.⁶⁾ However, when we added citrate buffer (10 mM) to Ringer solution the neuromuscular transmission was blocked instantaneously, while the muscle contraction elicited by strong field stimulation remained unaffected. The inhibitory action of citrate buffer was thought to come from its ability to chelate calcium ions, thus reducing calcium concentration in the synaptic area. In order to avoid this side-effect on neuromuscular transmission, we reduced the concentration of citrate buffer and increased that of CaCl_2 by adding 0.5 ml of the mixture of citrate buffer (30 mM) and CaCl_2 (70 mM) to 4.5 ml of Ringer solution. Thus, in this modified Ringer solution, the final concentrations were: citrate buffer 3 mM and CaCl_2 8.8 mM. By addition of this mixture the muscle contractions increased in strength from 3 g to 4.5 g and returned to the initial level of 3 g within 3 minutes. In this case the blockade of the neuromuscular transmission due to citrate buffer was avoided.

The addition of CuSO_4 (0.3 mM) and $\text{K}_3\text{Fe}(\text{CN})_6$ (0.05 mM) before acetylthiocholine iodide to modified Ringer solution blocked the neuromuscular transmission gradually within a few minutes (4–5 min). However, at that time the direct electrical stimulation of the muscle still remained efficient. Cupric ions, in spite of the presence of the citrate buffer which forms with them a stable complex, appear as the main inhibitory agent, since potassium ferricyanide alone had less inhibitory effect.

When acetylthiocholine iodide (0.18 mM) was added together with the cupric and ferricyanide ions in the histochemical medium, the inhibitory effect of the metal ions was reduced. In these conditions the complete blockade of the transmission occurred only after 15–20 min of incubation. At that time, we ended histoenzymatic reaction. It was considered that R-SH radical of thiocholine iodide which appeared during the enzymatic hydrolysis of acetylthiocholine iodide, has electrochemically reduced a part of cupric and ferricyanide ions respectively into cuprous and ferrocyanide ions. This resulted in the formation of insoluble precipitates which do not have an appreciable inhibitory effect on the neuromuscular transmission.

After the blockade of neuromuscular transmission, the nervous tissue still remained alive. Indeed, a recovery of the transmission was observed when the preparations were washed with Ringer solution containing citrate buffer and additional CaCl_2 during 10 min. Washing in Ringer

solution without citrate buffer did not allow to restore neuromuscular transmission. This suggests that citrate buffer suppressed the blocking effect of cupric ions, probably through its ability to chelate these ions. Indeed, previous analyses showed the inhibitory effects of the heavy metal ions on synaptic transmission,⁸⁾ suggesting that also cupric ions could powerfully inhibit the neuromuscular transmission.

After histochemical reaction, the tissue was fixed by glutaraldehyde, washed and treated by DAB-H₂O₂ in

order to reveal oxidoreductase-like activity of the minute precipitate. Under optical microscope, we observed motor endplates stained by DAB. Although DAB staining seemed at first sight to be limited to the superficial layers of the preparation, the staining was observed in the depth of the tissue, when muscle fibers were separated each other. In this case, most, if not all, endplates were stained (Fig. 1 A and B).

When the preparations were observed by electron microscopy, a fine localization of DAB precipitates was

Fig. 1. Histochemical localization of AChE activity in the frog motor endplate obtained by << vital >> histoenzymatic reaction followed by glutaraldehyde fixation and DAB-H₂O₂ staining. After separation of muscle fibers, the histochemical staining of DAB precipitate was observed in all the depth of the muscle layers. A) Separated muscle fibers with motor endplates at low magnification (×80). B) High magnification of the region marked by an asterisk in A) (×510).

Fig. 2. Electron microscopic observation of the << vital >> histochemical reaction of AChE activity, revealed by DAB-H₂O₂. DAB precipitate is localized in the synaptic cleft delimited by the pre- and postsynaptic membranes. Synaptic vesicles are satisfactorily preserved. AZ: active zones, JF: junctional folds, SV: synaptic vesicles (×66,000).

observed in the synaptic cleft between pre- and postsynaptic membranes. The synaptic vesicles in the motor nerve terminal were satisfactorily preserved (Fig. 2).

The histochemical staining of the endplates located in the depth of the tissues demonstrate the effective penetration of histochemical medium in most endplates of the preparation. Thus, we can assume that the neuromuscular transmission blockade due to the histochemical agents was not limited to superficial muscle layers of the preparation, and that the deep muscle layers of the preparation underwent the influence of the inhibitory effect of the histochemical medium.

To our knowledge, the present work provides the first << vital >> histoenzymatic reaction of AChE activity which was revealed in a second step by DAB and H₂O₂ using the oxidoreductase-like activity of the histochemical precipitates.

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