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13 Microwave-Assisted Cytochemistry

Accelerated Visualization of Acetylcholinesterase at Motor Endplates

John P. Petrali and Kenneth R. Mills

INTRODUCTION

Acetylcholinesterase (AChE) is the modulating enzyme of cholinergic systems. Study of its morphological distribution and its pathophysiological disposition following disease or exposure to anticholinesterase compounds such as pesticides, organophosphates, and chemical (chem) warfare nerve agents, has been through the use of a multitude of specific cytochem reactions which use metal-capturing methods of thiocholine, following hydrolysis of acetylthiocholine by AChE. The first of these methods to be recognized and used in many investigations was the Koelle technique (Koelle and Friedenwald, 1949). This *in situ* procedure used copper (Cu) as a capturing metal which without any further treatment yielded dense precipitates localized at sites of enzyme activity. These precipitates could be visualized at the light-microscopy and electron microscopy (EM) level. The empirical reaction for the primary cytochem Cu- capturing reaction is as follows:

Acetylthiocholine iodide + AChE → Thiocholine iodide + Acetate

Thiocholine iodide + Cu sulfate → Cu thiocholine iodide

*The opinions or assertions herein are the private views of the authors, and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. In conducting this research, the investigators adhered to the "Guide for Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources National Research Council."

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The intermediate white precipitate, Cu thiocholine iodide, could be darkened by sequential incubation with a variety of secondary reactants, such as, potassium ferricyanide (Karnovsky and Roots, 1964), sodium sulfide/silver nitrate (Namba et al. 1967) and phosphomolybdic acid (Tsuji and Fournier, 1984). In the case of the secondary reactants Na sulfide/Ag nitrate the final reaction product is a dark-toning step similar to photographic development which yields an insoluble dark precipitate easily seen at reactive sites. The time required by most laboratories for conventional cytochem processing of AchE, which includes fixation of selected tissues, followed by primary and secondary incubations, is typically 8–24 h. With the advent of microwave (MW)-assisted tissue processing and MW-assisted cytochem incubations, times required for visualization of AChE can be shortened significantly.

Here is presented a MW-accelerated modified Koelle-Friedenwald–Na sulfide/Ag nitrate protocol for AChE, useful for diagnostic and investigative pathology (PATH). Guinea pig diaphragm is used as the subject tissue. The resultant histological and ultrastructural presentations of the enzyme at motor endplates are evaluated and compared with a companion study of the same AChE method using conventional procedural times. In this study, time required for the conventional procedure was 7 h. Time required for the accelerated procedure was 1.7 h.

MATERIALS AND METHODS

Since conventional methodology and procedural times for AChE visualization are well documented, this chapter presents only materials and methods for the MW-accelerated protocol of AChE localization used in this study. All tissues incubated for AChE localizations were eventually processed for routine hematoxylin and eosin histopathological study and transmission EM. The lab MW oven utilized for this study was the Pelco 3440, 800 W. The animal used was the haired guinea pig, which was euthanatized by an overdose of Na pentobarbital (1 mL/kg ip), followed by the induction of a pneumothorax. The diaphragm was removed immediately, placed in a Petri dish with saline and processed according to the following protocol.

MW Aldehyde Fixation

MATERIALS

1. Buffered fixative (1/2 Karnovsky/0.1 *M* Na cacodylate, pH 7.4, 190 mOsm).
2. Two 250-mL polypropylene beakers.
3. Temperature (temp) probe.

Process

1. Place two 250-mL beakers containing 200 mL water (23°C) in MW, as heat sinks.
2. Remove saline from Petri dish, and add 10 mL fixative.
3. Place sample in MW at a predetermined cold spot.
4. Set temp set point at 37°C, and place temp probe in Petri dish with sample.
5. Irradiate for 10 s at 100% power, 20 s at 0% power, and 10 s at 100% power.
6 Continue fixation outside of MW for 5 min.

Buffer Wash

Material required

Hank's buffer.

Process

1. Remove fixative.
2. Add buffer to Petri dish, and wash (outside MW) 3 x 10 min.

Preincubation Medium

Material

Medium (12.5 mL 0.1 *M* Na citrate, 25.0 mL Hank's buffer, 12.5 mL 0.06 *M* Cu sulfate).

Process

1. Remove buffer.
2. Incubate tissue in medium (outside MW) for 10 min.

Primary Incubation Medium

Material

Medium (12.5 mL 0.1 *M* Na citrate, 25.0 mL Hank's buffer, 12.5 mL 0.06 *M* Cu sulfate, 50 mg acetylthiocholine iodide).

Process

1. Remove preincubation medium from Petri dish, and add 10 mL primary medium.
2. Place sample in MW at a predetermined cold spot.
3. Set temp set point at 37°C, and place temp probe in Petri dish.
4. Irradiate for 30 s at 100% power.
5. Continue incubation outside of MW for 5 min.

Buffer Wash

Materials

Hank's buffer.

Process

1. Remove primary incubation medium.
2. Add buffer to Petri dish, and wash (outside MW) 3 × 5 min.

Secondary Incubation Medium

Materials

1% Na sulfide, deionized water, 0.5% Ag nitrate, 1% Na thiosulfate.

Process

1. Remove buffer, and transfer tissue to a 20-mL scintillation vial. The following steps are done outside the MW.
2. Incubate tissue in 1% Na sulfide for 1 min.
3. Remove Na sulfide.
4. Wash with deionized water 3 × 5 min.
5. Remove water.
6. Incubate in 0.5% Ag nitrate for 1 min.
7. Remove Ag nitrate.
8. Wash with deionized water 3 × 5 min.
9. Remove water.
10. Incubate in 1% Na thiosulfate for 5 min.
11. Remove Na thiosulfate.
12. Wash with deionized water 3 × 5 min, and process for light microscopy or EM.

RESULTS

Histopathological examination of MW-processed diaphragm revealed AChE reaction products strongly specific for motor endplates to the exclusion of other muscle sites (Fig. 1). Except for focal areas of rarefaction of some muscle fibers, the structural presentation of the diaphragm and localization of the enzyme were unchanged from that seen with conventional fixation and incubations (Fig. 2). At the ultrastructural level, with the exception of occasional dilatation of mitochondria and some minor presynaptic swelling surrounding presynaptic vesicles, the subcellular details of the muscle fibers at motor endplates were mostly unchanged from that seen with conventional processing. Reaction products for AChE were specific for the primary cleft and junctional folds of motor endplates (Fig. 3), and were mostly replicate, compared with conventional processing (Fig. 4).

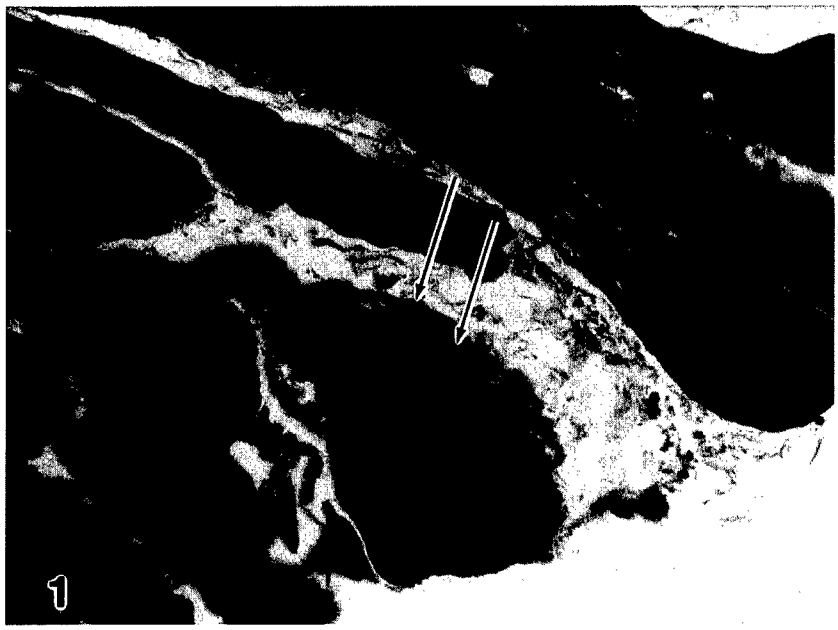

Fig. 1. Histological section of MW-processed diaphragm, showing AChE reaction product (arrows) at a motor endplate.

Fig. 2. Histological section of conventionally processed diaphragm, showing AChE reaction product (arrows) at a motor endplate.

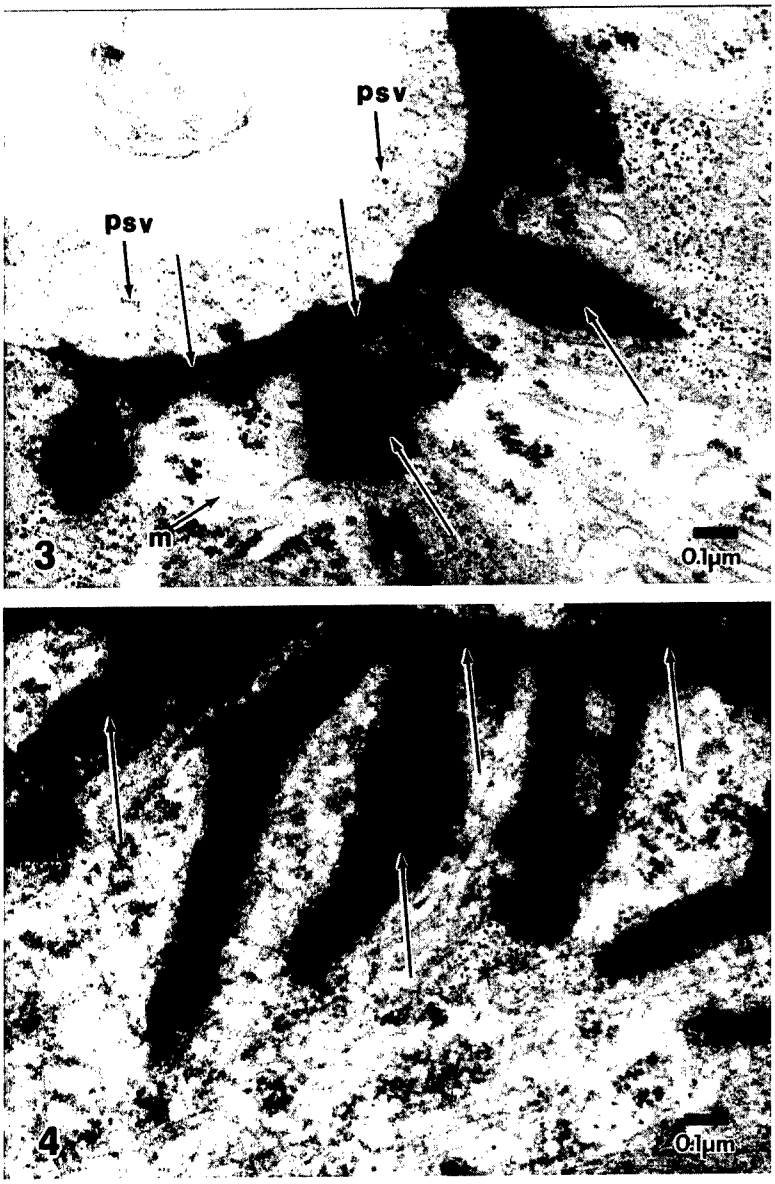

Fig. 3. Ultrastructural section of MW-processed diaphragm, showing AChE reaction product (arrows) in the primary cleft and junctional folds of a motor endplate. m, dilated mitochondrium; psv, presynaptic vesicles.

Fig. 4. Ultrastructural section of conventionally processed diaphragm, showing AChE reaction product (arrows) in the primary cleft and junctional folds of a motor endplate.

DISCUSSION

Results of this study add to the growing value of the lab MW oven in diagnostic and investigative PATH. The 79% decrease in time required, to achieve results that closely approach or are equal to those of conventional methods for the characteristic localization of AChE, makes MW processing for this enzyme now rapid and reliable. Although requiring standardized time for hematoxylin and eosin paraffin processing, light microscopy evaluations in this study were especially useful for quick diagnostic evaluations of large expanses of diaphragm for the presence or inhibition of the enzyme at motor endplates. The ultrastructural portion of this study attests to the ability of MW processing to shorten response times, when addressing the investigative subcellular disposition of the enzyme. The authors' lab, which now embraces the lab MW oven as an on-line integral part of tissue processing to include special staining and embedding, has now significantly reduced the extended times needed by most EM labs to determine ultrastructural localizations of AChE.

However, still lacking are standardized, uniform lab MW setups for any given procedure. MW literature is resplendent with personalized strategies regarding, among others, cold spots, hot spots, specimen size, temps, sensor-probe placements, fixing solutions, position of heat sinks, power applications, and so on. This ambiguity is reminiscent of the halcyon days of the EM sciences, when all was in formative stages. When MW processing does become standardized, as it must, then it might be suggested, at that juncture, that the lab MW oven might well prove to be the one instrument that finally places the EM sciences in the mainstream of diagnostic and investigative PATH.

REFERENCES

Karnovsky MJ, Roots L (1964) Direct-coloring thiocholine method for cholinesterase. *J Histochem Cytochem* **12:**219–221.

Koelle GB, Friedenwald JS (1949) Histochemical method for localizing cholinesterase activity. *Proc Soc Exp Biol Med* **70:**617–622.

Namba T, Nakamura T, Grob D (1967) Staining for nerve fiber and cholinesterase activity in fresh frozen sections. *Am J Clin Pathol* **47:**74–77.

Tsuji S, Fournier M (1984) Ultrastructural localization of acetylcholinesterase activity by means of the electron dense precipitate derived from Koelle's cuprous thiocholine iodide by treatment with phosphomolybdic acid and osmium tetroxide. *Histochemistry* **80:**19–21.