ELECTRON-HISTOCHEMICAL INVESTIGATION OF CHOLINESTERASE IN THE NEUROMUSCULAR SYNAPSE OF RATS

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Electron-histochemical investigation of cholinesterase in the neuromuscular synapses of the rat diaphragm revealed activity of the enzyme in circular or oval structures measuring from 250 to 600 A, in contact with the synaptic folds on the side of the sarcoplasm.

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Reports of electron-histochemical investigations of cholinesterase activity in neuromuscular synapses have recently been published [1-7, 10]. Interest in this problem is understandable because knowledge of the precise localization of cholinesterase would provide a deeper understanding of the processes taking place in the synapse during function. With increasing perfection of electron-histochemical methods of cholinesterase detection, new and more precise information about its localization has been obtained. However, the results are by no means exhaustive (particularly because at times they are conflicting).

The object of the present investigation was to study cholinesterase activity in the neuromuscular synapse of the rat diaphragm.

EXPERIMENTAL METHOD

Albino rats weighing 100-120 g were used. After the animals had been anesthetized with ether the abdomen was opened and narrow strips along the branches of the phrenic nerve were quickly excised from both domes of the diaphragm. Cholinesterase activity was detected by the method of Csillik and co-workers [4]: after prefixation in a buffered formol-sucrose solution the tissue was incubated in a medium containing acetylcholine as substrate, and then placed in a solution of yellow ammonium sulfide and postfixed in buffered osmium tetroxide solution. Prefixation, incubation, and postfixation were carried out in the cold. We did not incubate frozen tissue sections (as described in the original method) but incubated pieces of tissues, and thus had to add two intermediate washings (before and after incubation) with a corresponding increase in incubation time. After dehydration in acetone the pieces of tissue were embedded in araldite. Blocks were cut on the LKB-Producter ultratome, and sections were examined in the JEM-7 electron microscope with an accelerating voltage of 80 kV. Some sections were stained with lead citrate by Reynolds' method [8].

EXPERIMENTAL RESULTS

Cholinesterase activity was judged from the presence of deposits of the end product of the reaction (lead sulfide) in the form of a fine precipitate. Enzyme activity was found in the pre- and postsynaptic membrane, including that part of it forming synaptic folds (Fig. 1, a, b). In some cases activity was also found in part of the axolemma facing the Schwann cell. Deposition of precipitate was also found in part of the synaptic space, the synaptic folds, between the axolemma and the Schwann cell, and also in the membranes of the Schwann cell. Occasionally deposition of precipitate was found in elements of the endoplasmic reticulum in the immediate vicinity of the synapse.

Cholinesterase activity was also constantly found in circular or oval structures in direct contact with the membrane of the synaptic folds on the sarcoplasm side (Fig. 1, c). These structures ranged from

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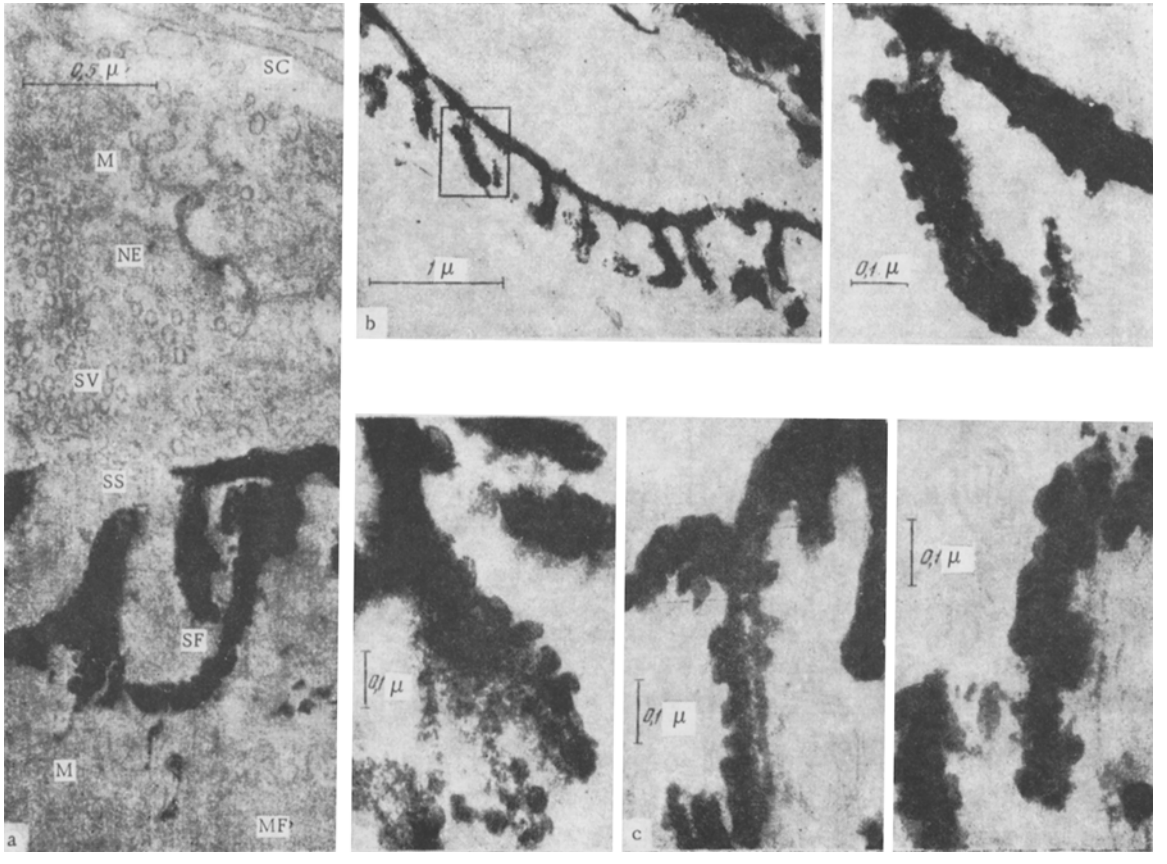

Fig. 1. Localization of cholinesterase activity in neuromuscular synapse of rat diaphragm (a, b) and in synaptic folds (c). NE) Nerve endings; SV) synaptic vesicles; M) mitochondria; SS) synaptic space; SF) synaptic folds; MF) myofibril; SC) Schwann cell.

250 to 600 A in size, and the density of precipitate in them was varied. Sometimes their outer layer was dense while the part in contact with the synaptic fold was narrowed to form a neck. In some cases an active member could be seen separating these structures from the synaptic folds. The synaptic folds surrounded by these structures just described sometimes resembles a bunch of grapes.

Deposition of precipitate was variable both in different synapses and in different parts of the same synapse. In some of them deposition of precipitate was continuous in the membranes and the spaces were evenly filled with precipitate, while in others activity was interrupted in the membranes and deposition of precipitate in the synaptic spaces and folds varied in intensity and in some places was absent.

In the axon endings (presynaptic part) very tiny granules of precipitate were found as a rule on membranes of the synaptic vesicles and tubules, and sometimes inside them. The mitochondria of the ending were free from precipitate.

Several electron-histochemical methods have been suggested for detecting cholinesterase activity in synaptic structures. Those most commonly used can be divided into two groups depending on the substrate: acetylcholine [4, 5] and thioacetic acid [1, 5]. Use of acetylcholine makes the reaction more specific with respect to detection of acetylcholinesterase (true cholinesterase); the reaction using thioacetic acid is less specific, but it enables the localization of the end product of the reaction to be determined more precisely. Having chosen the method of Csillik and co-workers (using acetylthiocholine as substrate) we incubated pieces of tissue rather than sections on the assumption that this would enable the tissue structure to be preserved and that the decreased rate of substrate hydrolysis resulting from slow penetration of the substrate into the tissue would improve the picture of distribution of the precipitate. At the same time, however, the slow speed of penetration of the substrate could be one reason for the uneven activity of the enzyme, because synapses located at different depths from the surface of the fragment would be under different conditions [6]. We therefore prolonged the incubation time so that synapses with an even and clearly localized cholinesterase activity could always be found in the fragment. However, the most important reason for uneven enzyme activity in the synapse was partial elution of the precipitate during postfixation in osmium solution [4].

After contrasting of the sections, which we carried out at the beginning of the investigation, the precipitate became denser and more homogeneous. Since the possibility is not ruled out that interaction is possible between the contrast material and the precipitate of end product of the reaction, leading to partial elution or even to redistribution of the precipitate in the second, localization of cholinesterase activity was subsequently studied only in unstained sections. Furthermore, double fixation, even after embedding in araldite, enabled a sufficient degree of contrast to be obtained [9]. Staining was used only to verify integrity of the structure, and the result was always good (Fig. 1, a).

In our experiments activity of the enzyme was localized in synaptic structures, chiefly in their postsynaptic part. This agrees with data in the literature. According to the results of recent investigations, acetylcholine esterase activity is localized exclusively in the pre- and postsynaptic membrane, in the synaptic space and folds. Activity of the part of the axolemma facing the Schwann cell and in the space between them is due to nonspecific cholinesterase [6]. Using the method with thioacetic acid, Barrnett [1, 2] found cholinesterase activity in the synaptic vesicles, but the available data are insufficient to allow conclusions to be drawn regarding the presence of acetylcholinesterase activity in the vesicles.

Activity of the enzyme in elements of the endoplasmic reticulum near the synapse was discovered by Barrnett [2], also by the use of thioacetic acid as substrate. In this case, in addition to the fact that the demonstrated activity might have been due to nonspecific esterase, the possibility of diffusion of the enzyme or precipitate cannot be ruled out.

The circular structures in contact with synaptic folds in which we located cholinesterase were found frequently although not in all the synapses investigated. It is difficult to assume that they were an artefact of diffusion, because the structures had a characteristic structure which was always the same. It is possible that the age of the animals was an important factor in their discovery (we used young rats), or certain technical conditions for which no allowance was made. The final solution to this problem must await further investigation.

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