Electron Microscopical Study on the Fine Structure and the Cholinesterase Activity of the Neuromuscular Junction in Progressive Muscular Dystrophy

by

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INTRODUCTION

Electron microscopical study on the neuromuscular junction of mammals has been done already by many investigators : (ROBERTSON 1953, 1954, 1956, PALADE 1954, REGER 1954, 1955, 1957, 1958, 1959, and ANDERSSON - CEDERGREN 1959). Particularly HARVEN (1959) observed the fine structure of the junction area in the human skeletal muscle. Recently, electron microscopic localization of the cholinesterase activity at the neuromuscular junction of several mammals was reported : LEHRER and ORNSTEIN (1959) examined the distribution of cholinesterase in this tissue formations by a diazo-coupling method in which α-naphtyl acetate was used as a substrate, and found that the cholinesterase activity is associated with the "subneural apparatus" as described by COUTEAUX. BIRKS and BROWN (1960), using a modification of KEOLLE's method, reported that the cholinesterase activity was detected on the junctional fold in the serratus anterior muscle of the guinea pig. BARRNETT (1962) investigated the localization of this enzyme by the thiolacetic acid method and reported that the reaction products were localized not only on the junction fold, the primary and secondary synaptic clefts but also on synaptic vesicles in the terminal axoplasm. Moreover, SABATINI, BENSCH and BARRNETT (1963) examined the acetylcholinesterase activity in the diaphragma of adult albino rats fixed with various aldehyde compounds by similar method and related that the activity was verified in the primary as well as secondary clefts and on the postsynaptic membrane. Very recently, MILEDI (1964), using KEOLLE's method, pointed out that an acetylcholinesterase activity was detected on the synaptic vesicles of the junction area in the frog skeletal muscle. He mentioned also that the distribution of the enzyme activity was considerably influenced by the incubation period.

On the other hand, there is little information, available concerning the pathological changes of the neuromuscular junction at the electron microscope level. Therefore, this study represents an attempt to observe the pathological changes of the fine structure and the subcellular localization of the cholinesterase activity at the junction area in the skeletal muscle obtained from progressive muscular dystrophy patients.

MATERIAL AND METHODS

Patients with progressive muscular dystrophy were males of fifteen and seventeen

years of age. Strips of M. triceps surae, after the application of local anaesthesia, were obtained from the transitional part of the muscle to the Achilles tendon of the patients. Specimens were fixed by the method of Holt at 4°C for 5 minutes in 4 % buffered formalin (pH 7.2) containing 45 mg/ml sucrose. Incubating mixture was prepared just before use by adding lead nitrate in the ratio of 0.5 % to thiolacetic acid (1.3%) dissolved in a veronal buffer of pH 7.2. Small tissue blocks were incubated at 1°C for 1 hour. After incubation specimens were refixed in HOLT's formalin for 1 hour and postosmicated with CAULFIELD's mixture. Tissue blocks were dehydrated in graded ethanol and embedded in Epon 812 by the method of TAKASHIO (1964).

Control specimens were treated with 0.9 % NaCl solution which contains 10^{-5}M eserine for 30 minutes and incubated as above described. For each ultrathin section an immediately adjacent section of about 1μ was taken in order to check under the light microscope whether the proper part of the specimen was sectioned.

Thin sections were stained with 1% uranyl acetate solution and examined with a Hitachi electron microscope HU-11A. Thin sections of blocks, histochemically treated, were not stained in order to avoid obscuring changes in contrast caused by the electron staining.

RESULTS AND DISCUSSION

Fig. 1 is an electron micrograph of a part of the neuromuscular junction obtained from a patient of the mild case and incubated in a substrate mixture for cholinesterase reaction. Though axolemma, being in contact with the junctional fold, was not well defined in this section, vesicles (*Syv*) of about 30 mμ, which seem to be released in the synaptic clefts, can be observed. Electron-opaque reactive products (*RP*) are deposited on clusters of these vesicles. Reaction products, however, could not be detected on the presynaptic membrane. Similar findings were also noticed by LEHRER and ORNSTEIN (1959) as well as BIRKS and BROWN (1960) in rat and guinea pig.

In the Axon terminal eliptical vacuoles existed, which were surrounded by an electron dense limiting membrane of about 10 mμ. Most of these vacuoles contained material of moderate electron density (*Va*$_2$), but some of them (*Va*$_1$) revealed contents of less electron density as compared with that of the axoplasmic matrix. In these micrographs, moreover, tubular formation described by REGER (1959) or x-component of ANDERSSON-CEDERGREN (1959) (*TAR*) was found.

On the other hand, sarcolemma (*SL*) was relatively electron dense and of about 10 mμ. Numerous vesicles (*Vsc*) of about 10 mμ and large vacuoles with material of low contrast were observed in the sarcoplasm just beneath the junctional fold (Fig. 1 and 2). Small dense granules (*Gr*) of 10 to 30 mμ, which probably represent glycogen, were surrounding these large vacuoles and appeared also in the cytoplasmic matrix between the junctional fold (*Psm*) and the myofibrils (*Mf*). Mitochondria (*Mit*) observed here appeared to be swollen and showed ill-defined lamellar cristae.

Fig. 3 a illustrates the junction area obtained from a patient of a more serious case. In comparison with the findings in the mild case, it is to be noted that the postsynaptic membrane was remarkably disrupted, and the amorphous material coating the junctional

fold showed an increase in electron density. Numerous vesicles appeared just beneath the junctional fold, they seem to be engulfed in the underlying sarcoplasm. Mitochondria were so disintegrated that mitochondrial membrane and cristae were no longer sharply defined. Similar changes of mitochondria at the neuromuscular junction of the patients with progressive muscular dystrophy have been already reported by MÖLBERT (1960) and BREEMEN (1960). Slightly later OKAZAKI and OYAMADA (1963) also verified by light-microscope that the moter end-plates were disrupted and striations of myofibrils disappeared in these diseased muscles. Presumably destruction of the chondriome observed prevents the cell metabolism, especially oxidative phosphorylation at the junction area.

In regard to cholinesterase activity, final products are deposited on clusters of the synaptic vesicles (Fig. 3 b) ; however, lead precipitate (*RP*) revealed comparatively spotty localization in this material. One of the probable reasons for such a spotty localization of reaction products might be a breakdown of the surrounding membrane of synaptic vesicles and a release of internal enzymes. Concerning the functional significance of the synaptic vesicles, PALAY (1956) suggested that these vesicles could relate either to manufacture or to storage of the humoral transmitters such as acetylcholine. It seems probable that this acetylcholine-cholinesterase system, localized in the synaptic vesicles, is of prime importance in the impulse transmission at the junction area. From this point of view, it should be mentioned that the synaptic vesicles, investigated in the patients with progressive muscular dystrophy were much smaller (6-10mμ) than those in healthy human materials (30mμ) as observed by HARVEN (1959). Furthermore, in a patient of the serious case described above, it was noticeable that the synaptic membrane was destroyed, and the synaptic vesicles revealed a remarkable decrease of the cholinesterase activity.

Finally, it was verified in the control preparations which were previously treated with an inhibitor such as eserine (10^{-5}M) that the activity of this enzyme was completely inhibited, and no reaction products could be detected on the postsynaptic membrane as well as on clusters of synaptic vesicles (Fig. 4).

CONCLUSION

In two cases of progressive muscular dystrophy patients, pathological changes of the fine structure and the cholinesterase activity at the neuromuscular junction (M. triceps surae) were investigated.

1. In the patient of the mild case, presynaptic and postsynaptic membranes of the neuromuscular junction measured about 10-15 mμ. Sarcoplasm just beneath the junctional fold contained large vacuoles (about 200 mμ in diameter) and electron dense granules of about 5 mμ. Vacuoles in the junctional axoplasm were surrounded by comparatively electron dense limiting membrane. Cholinesterase activity was detected on clusters of spherical vesicles released into the synaptic clefts, while pre- and postsynaptic membranes showed no activity of this enzyme.

2. In the patient of the serious case, the fine structure of the junctional area was severely destroyed. Vesicles observed within in the sarcoplasm were much smaller (5 mμ) in size than those observed in the patient of the mild case. Mitochondria in the junctional sarcoplasm were remarkably swollen and some of them were fragmented.

3. In the progressive muscular dystrophy patients, disturbance of the impulse transmission mechanism at the junction area might be caused mainly by the destruction of an acetylcholine-cholinesterase system localized in the synaptic vesicles. In addition mitochondrial damage is likely to inhibit the cell metabolism, especially oxidative phosphorylation at the junction area.

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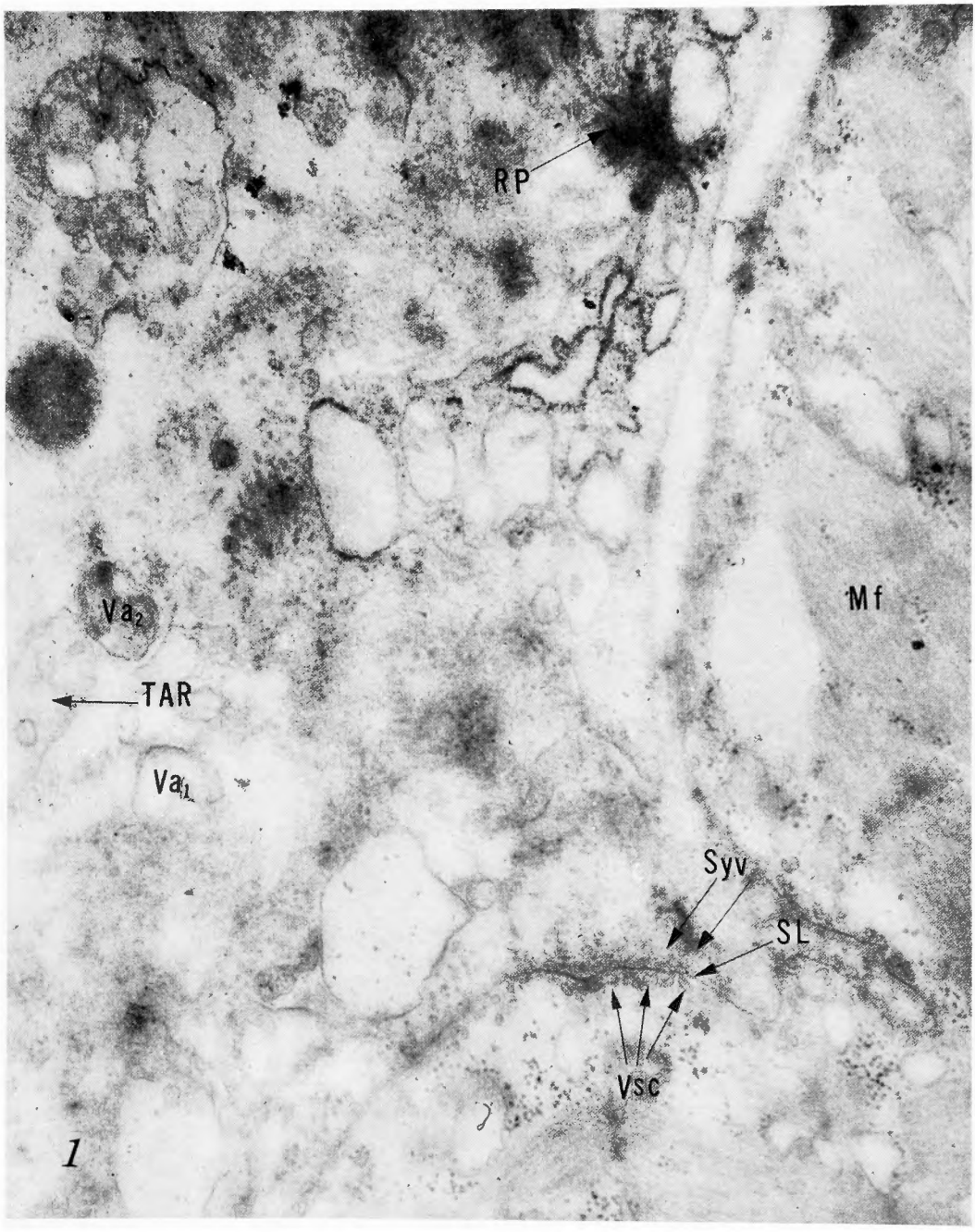

Fig. 1 Cholinesterase activity at the neuromuscular junction. A mild case of progressive muscular dystrophy. Vacuoles of various density (Va_1 and Va_2) exist in the axoplasm. Synaptic vesicles released into the synaptic cleft are observed and electron-opaque reactive products localized on clusters of these vesicles. ×28,000.

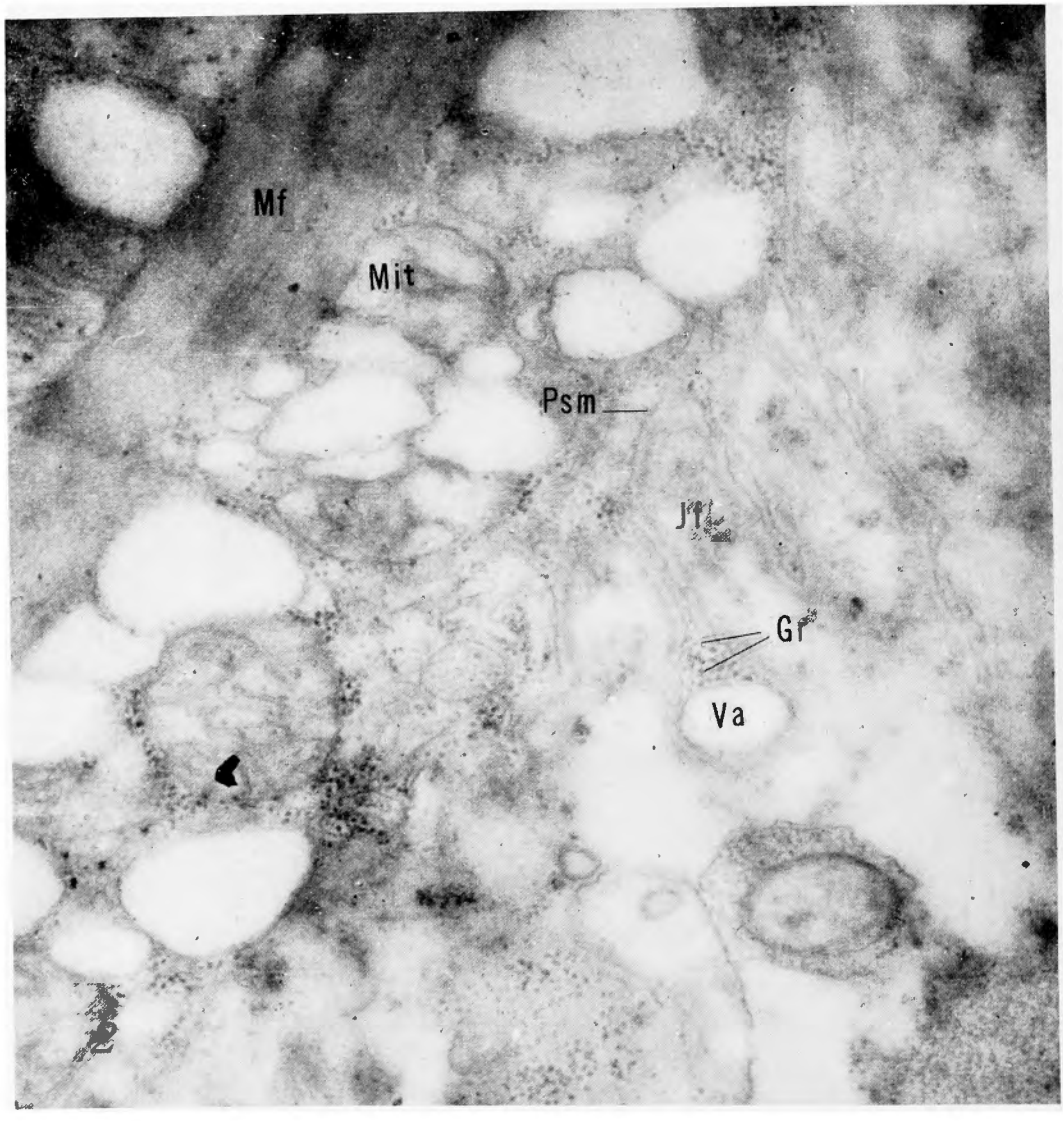

Fig. 2 Preparation for routine electron microscopy. Material was obtained from the patient as show in Fig. 1. Large vacuoles (*Va*) and numerous electron dense granules (*Gr*) are visible in the junctional sarcoplasm. Sarcolemma in disintegrated in some places. Swollen mitochondria with irregular cristae are visible. ×36,000.

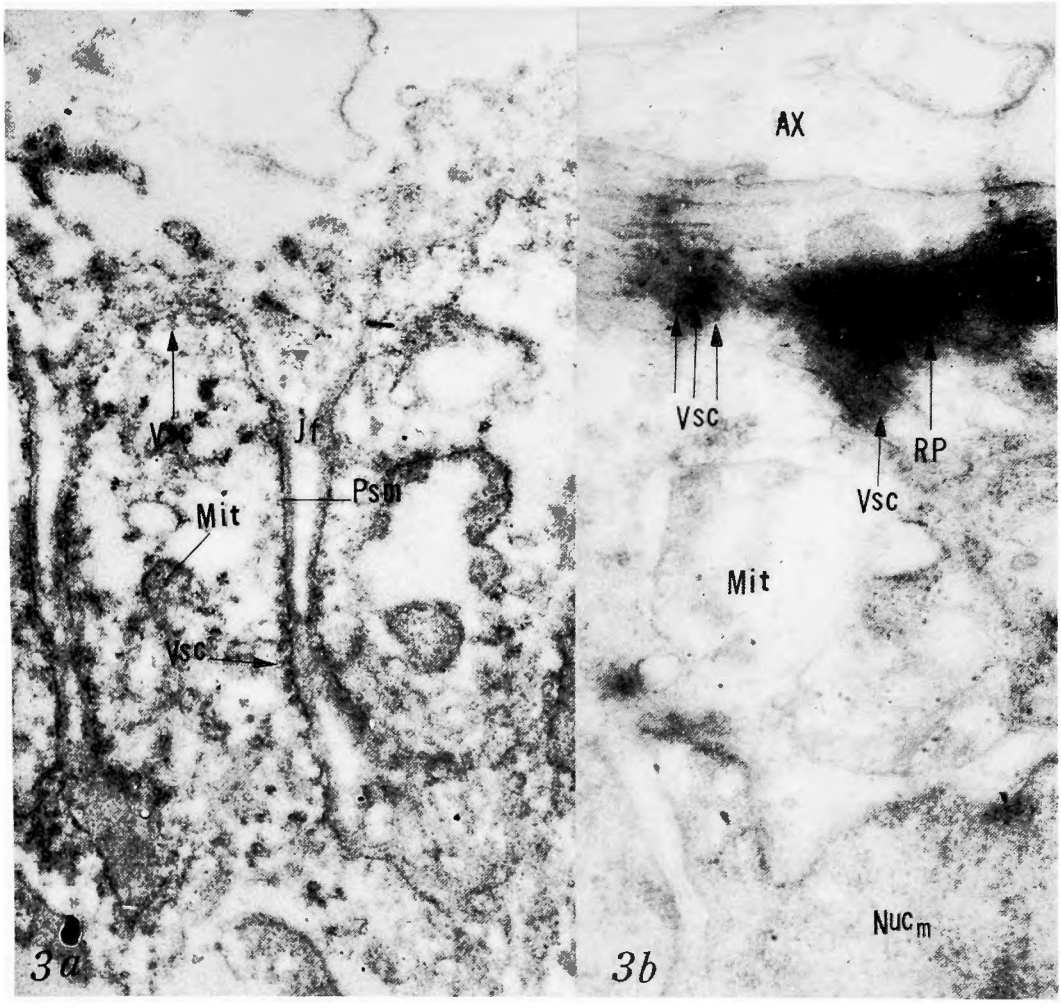

Fig. 3a Junction area obtained from a patient of the serious case. Sarcoplasm just beneath the junctional fold shows poor preservation of fine structure, and vesicles (about 5 mμ in diameter), engulfed into sarcoplasm, are abundantly observed. Mitochondria are swollen, and some of them are fragmented.

Fig. 3b Cholinesterase reaction of the same specimen as shown in Fig. 3a. Reactive products show spotty localization, however, they are observed on the cluster of the spherical vesicles in the synaptic clefts. ×35,000.

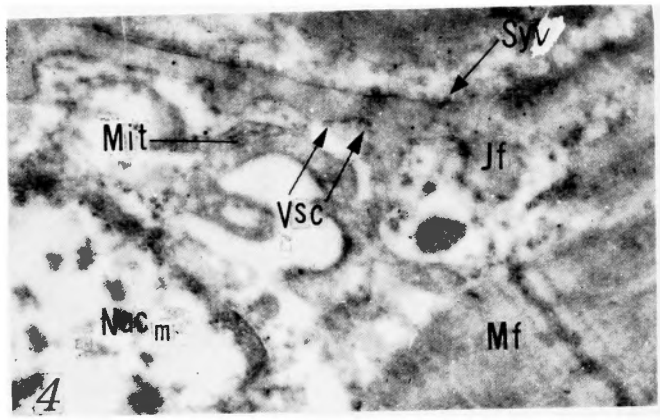

Fig. 4 Control preparation of cholinesterasc-test. Specimen was obtained from a patient of the mild case, described above and previously treated with eserine (10^{-5}M). No reaction products can be detected in this section. ×13,000.

和 文 抄 録

進行性筋ジストロフィーの運動神経終板の微細構造と Cholinesterase 活性の電子鏡的観察

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進行性ジストロフィー患者の下腿三頭筋で，運動神経終板の構造と cholinesterase 活性を観察した．その方法はチォール酢酸法に従つた．

軽症例の運動神経終板の連接前膜(presynaptic membrane) の厚さは約 15mμ，連接後膜は厚さ約 11mμで，ここでは連接隙 (synaptic cleft) が甚だ狭い．褶入 (fold)の間に進入した筋形質には，円形の約 5 mμの小胞の他に，大きな液胞も認める．連接後膜の直下にある小胞は約 6 mμ で甚だ小さい．

連接後膜は厚さ 7～10mμ で所々断裂している．連接隙に放出された 3～6 mμ の円形，楕円形の小胞の集団に cholinesterase 活性を認めた．

重症例では，連接突起が強く破壊され，褶入間の筋形質に大きな液胞が貯つている．連接後膜の肉形質側には 2～5 mμ の小胞が並び，この小胞は軽症例のそれより小型である．糸粒体は著しく破壊され，内部構造が不明瞭となつている．

連接隙には，軽症例の小胞より小型で，均質で，電子密の円形の小胞が認められる．この小胞の密集した部分には，反応生成物なる硫化鉛の沈着が見られ，cholinesterase 活性を証明する．しかし重症例の方が電子疎の小胞が多く，軽症例に比べて活性度が低い．

すなわちこの研究から，進行性筋ジストロフィーは，筋神経連接部における連接小胞のもつ cholinesterase 活性低下の他に，糸粒体の破壊によるエネルギー代謝障害と連接部の細胞膜の損傷による膜内外の電気的バランスの破れが加わり，運動神経終板における神経刺激伝導機構の障害が充分考えられる．