A STUDY ON ATP-ASE ACTIVITY IN DENERVATED MUSCLE

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It is well known that denervated skeletal muscle differs from the normal not only morphologically and functionally but also in its response to drugs (1, 2). Recently, our colleagues have reported that the recovery of morphologically changed muscle function in denervated muscle can be promoted by the administration with L-methionine (3), and that L-methionine has the effect of increasing the influx of potassium into an anterior tibial muscle (4).

Walker *et al.* (5) reported in 1966 that in denervated skeletal rat muscle the myofibrils were reduced in number, the nuclei and their near structures had become obscure, lamellar structures had appeared, and the basement membranes of the sarcolemmae and disappeared, and Miledi *et al.* (6), thereafter, reported in 1968 that in denervated diaphragm muscle especially the mitochondria had become smaller soon after denervation.

In the present experiment, anterior tibial muscles were denervated and their changes, especially those in ATPase activity, were examined by means of both light and electron microscopes.

METHODS

Male adult rats of the Sprague-Dowley strain weighing 200 to 250 g were used. After nembutal anesthesia in a dose of 20 to 25 mg/kg, an 1 cm sagittal skin incision was made on the dorsal side of the femoral region on one side, the sciatic nerve was taken out into the inter-muscular space and cut off in a length of 1 cm, and the skin was then sewn together with a No. 3 silk thread. This operation could be done without hemorrhage and it was done aseptically by the use of invert soap. On recovering esthesia, the operated animals could walk with the anterior legs and the one unoperated posterior leg; each animal was isolated in a clean metal cage at a temperature of 20°C to 25°C and was fed an adequate diet.

These rats were sacrificed by heart hemorrhage 3, 5, 7, 9, and 11 weeks after the denervation, and the tibial anterior muscles on both sides, this is, both the operated and unoperated ones were immediately removed. The isolated muscles were fixed in cold 4% formaldehyde for 12 hours and washed over night in cold 0.1 M cacodylate buffer in pH

7.2. Frozen sections were made at 50 microns in thickness and incubated at a temperature of 20°C for 15 minutes in the medium containing the following materials:

0.2 M tris-HCl buffer pH 9.0,	1.4 (ml)
0.15 M MgSO ₄	2.6
ATP-Na (SIGMA, 50 mg/6 ml)	2.0
0.5% lead citrate	4.0
	10.0 final pH 9.2
sucrose	0.8 g
NaCl	0.06 g
KCl	0.02 g

After incubation, the sections for light microscopy were washed in 0.1 M cacodylate buffer in pH 7.2, immersed in 1% ammonium sulfide, rinsed with distilled water, and mounted in glycerin jelly, while the sections for electron microscopy were post-osmicated after incubation with Caulfield's fixation (8) for one hour at 0° to 4°C, dehydrated in ethanol, and embeded in epon. Thin sections made with an LKB ultratome were post-stained with uranium and lead, then examined with a JEM-7 electron microscope.

RESULTS

1) *Normal anterior tibial rat muscle as to structure and ATPase activity*

Structure: Electron microscopy revealed that immediately inside the sarcolemma numerous mitochondria were observed parallel to rows of myofibrils and in their interspace as well. Alternating Z- and M-bands were seen striping each myofibril. Smooth endoplasmic reticuli were scattered in the myofibrillic interspaces. Transversally cut myofibrils consisted of thick myofilaments enveloped by numerous thin myofilaments. Both the basement membranes and sarcolemmae were in continuous contact with each other.

ATPase activity: Light microscopy revealed brown deposits of lead on sarcolemmae, capillary endothelia, and nerve fibers. The same findings are as those reported by McClurkin (9). Electron microscopy, however, revealed ATPase activity on the sarcolemmae, on the pinocytotic vesicles in the muscle cells and in the capillary endothelial cells, on the plasma membranes of the erythrocytes, and also in the spaces between the axons and Schwann's cells as shown in Figs. 1, 2 and 3.

2) *Denervated anterior tibial muscle as to structure and ATPase activity*

Structure: As seen by light microscopy three weeks after the denervation, the muscle cells had become finer than those of the controls and wavy in contour; five weeks after denervation these findings had become more pronounced. Electron microscopy revealed that, three weeks after denervation, the sarcolemmae had become deeper and more irregular in contour, and that shrunken or partially destroyed mitochondria were to be seen. The Z-bands in the degenerated myofibrils were distorted and the M-bands had become obscure. These changes, though differing in grade, were general and progressive in the all muscle cells observed as shown in Fig. 4.

FIG. 1. The ATPase activity in a normal anterior tibial rat muscle. The activity demonstrated by lead phosphate deposition is positive on the sarcolemmae ($\uparrow$), sagittal section ($\times 20,000$).

FIG. 2. A cross section of the tissue shown in Fig. 1 ($\times 30,000$).

FIG. 3. The ATPase activity in a normal anterior tibial rat muscle. It is positive on the erythrocytic membrane and on the pinocytotic vesicles in both the muscle cells and capillary endothelial cells ($\times 20,000$).

FIG. 4. The ATPase activity in a denervated anterior tibial rat muscle three weeks after denervation. Myofibrils and mitochondria show some degeneration. The degeneration degree is not the same in each cell. The Z-band has been already lost on some myofibrils but not on others. The ATPase activity on the sarcolemmae is very weak ($\times 15,000$).

FIG. 5. The ATPase activity in a denervated muscle 7 weeks after denervation. It is positive on the erythrocytic membrane ($\uparrow$) and on the pinocytotic vesicles, but not on the sarcolemmae ($\times 15,000$).

FIG. 6. A higher magnification of Fig. 5 ($\times 30,000$).

FIG. 7. The ATPase activity in a denervated muscle eleven weeks after denervation. Lead phosphate deposits can be observed not on the sarcolemmae but on the degeneration products ($\times 20,000$). The arrow indicates a lamellar structure.

FIG. 8. The same preparation as in Fig. 7. The presynaptic bag of a motor end plate has been completely destroyed, but the junctional folds are relatively well preserved without ATPase activity ($\times 16,000$).

The tendency, observed by Walker *et al.* (5), for the basement membranes to disappear in the sarcolemmae after denervation, was also observed by us on our muscle preparations, made at 7 to 11 weeks after denervation (Figs. 5, 6, 7, and 8). Eleven weeks after denervation, lamellar structures, not to be seen in normal muscle, appeared inside the sarcolemmae (Fig. 7); this degeneration has also been pointed out by Walker *et al.* (5).

At the neuromuscular junctions, presynaptic membranes had vanished and postsynaptic membranes had become partially broken and discontinuous, but the sarcolemmae of the junctional folds, that is, the small bags covering the postsynaptic membranes had examined to be well preserved as shown in Fig. 7.

ATPase activity: Light microscopy revealed no activity change in the denervated muscle for the first 5 weeks, but 7 to 11 weeks after denervation, the activity appeared to be stronger than that of the controls. But electron microscopy, however, revealed that the lead precipitation on the sarcolemmae had become reduced after denervation and that it was scarcely to be seen at 7 weeks or later. Instead, the lead depositions were found only on the degeneration products which appeared between the muscle cells. Therefore, the heavier lead-staining of denervated muscle seen under the light microscope did not mean increase in ATPase activity in the sarcolemmae after denervation, but rather a nonspecific adhesion of lead phosphate to degeneration products having an affinity for lead.

In brief, the decrease in ATPase activity in the sarcolemmae of denervated muscle was seen 3 weeks after denervation, and at 7 weeks, the basement membrane of the sarcolemma had become dissociated so that ATPase activity no longer localized on the sarcolemma (Figs. 5 and 6). At 11 weeks after denervation, the sarcolemmae had partially disappeared at the neuromuscular junctions so that ATPase activity was certainly not to be found there as shown in Figs. 7 and 8.

DISCUSSION

It has been reported that fibrillation voltages occur spontaneously in denervated skeletal muscle and that denervation reduces the muscle's potassium content, even though the potassium in the circulatory blood remains unchanged. These findings were made by our colleagues in the course of their researches on inactivation atrophied muscle and its hypersensitive drug response. In the present investigation of the morphological changes in denervated muscle, gradually increasing changes in the smooth sarcoplasmic reticula were seen, lamellar structures absent in normal muscle appeared in the sarcolemmae, and the myofibrils become abnormally slender. Moreover, the changes in the mitochondria themselves were remarkable, and it has already been seen above that the localization of ATPase activity in sarcolemma was gradually reduced by degeneration.

Regarding neuromuscular junction, we saw that the ATPase activity of the postsynaptic membrane structures disappeared with their breakdown. At this place, muscle contraction is ignited, to where quantities of potassium can be mobilized, and from where the fibrillation voltages that occur after denervation can easily be led. The reduction of potassium content in a denervated muscle may be caused by some of the changes that

take place in these area, although the importance of changes that occur in other areas should not be slighted. This interpretation is permissible, if it is true that ATPase promotes the influx of potassium through membranes permitting the active transport of metabolic ions.

Under the light microscope, ATPase activity seemed to have been promoted by denervation, as the specimens were more deeply stained than the normal ones, but under the electron microscope, this activity on the sarcolemmae was seen to have actually been reduced by denervation. The electron microscope revealed that the increased precipitation of lead was actually due, not to an increase in ATPase activity, but due to an increase in lead-affine degeneration products appearing in the muscle cell interstices. This finding indicates the importance of electron microscopy in histochemical observation of ATPase activity in pathological tissue, as the observation made by light microscopy alone could be misleading.

SUMMARY

Morphological changes appearing in anterior tibial rat muscles 3, 5, 7, 9, and 11 weeks after denervation and the accompanying localization of ATPase activity were studied histochemically by means of both light and electron microscopes.

1. In denervated muscle, the number of the myofibrils had decreased. The Z-bands had become bent and the structures of the mitochondria and the sarcolemmae in the neuromuscular junctions had become changed.

2. The ATPase activity in normal muscle was definitely localized in the sarcolemmae, but in denervated muscle gradually decreased, especially at the neuromuscular junctions. This decrease in ATPase activity may be the cause of the decrease in potassium content brought on by denervation.

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