The Structure of the Ileofibularis Muscle in the Turtle *Trionyx sinensis*

P. GOPALAKRISHNAKONE

Department of Anatomy, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore

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Summary. The ileofibularis muscle of the turtle *Trionyx sinensis* was examined by light and electron microscopy. Acetylcholinesterase staining showed an “en grappe” type of terminal. The muscle showed succinic dehydrogenase activity with three different types of fibers. The fibers appeared fibrillar type under the electron microscope. Neuromuscular junctions showed the presynaptic portion containing clear vesicles and mitochondria, whereas the postjunctional portion showed only few junctional folds.

The correlative studies on the structure and function of reptilian muscles, and their classification based on those studies have brought about much confusion (see review by Morgan and Proske, 1984). Guthe (1981) reported in detail the difficulty in classifying amphibian, reptilian and avian muscle using cytochemical, electron microscopic and physiological criteria. Among reptiles, the turtles, terrapins and tortoises belong to a special group. The slowness of tortoise muscle contraction has attracted the attention of many physiologists since it has been observed that the movement of the skeletal muscles in the tortoise is about 5-50 times slower than that of the frog (Page, 1968). It is of still more interest to study turtle muscles which act slowly on land but swiftly in water. Since the iliofibularis muscle of the tortoise has been studied both physiologically and electron microscopically (Page, 1968), the same muscle of the turtle was selected for this study.

MATERIALS AND METHODS

Adult fresh water terrapins and young hatchlings of *Trionyx sinensis* were obtained from commercial farms in Singapore. For light microscopic studies the turtles were anesthetized with thiopentone sodium or hypothermia or a combination of both. The ventral plates of the carapace were removed using bone forceps and the animal was perfused through the ventricle with 10% formal saline. The iliofibularis muscle was isolated by dissecting the hind limb and then processed for hematoxylin and eosin staining.

For an acetylcholinesterase reaction and detection of the succinic dehydrogenase enzyme, the iliofibularis muscle was removed under anesthesia and frozen. The tissues were cut into transverse sections, about 24 μm in thickness, in the cryostat.
The sections were stained for cholinesterase (KOELLE and FRIDENWALD, 1949); every other section of the series was stained for the succinic dehydrogenase enzyme (DUBOWITZ and BROOKE, 1973).

For transmission electron microscopy, the fixative used was mixed aldehyde solution made up of 2% paraformaldehyde and 3% glutaraldehyde in 0.1M cacodylate.

Fig. 1-4. Legends on the opposite page.
buffer pH 7.3. The ileofibularis muscle was dissected out and trimmed into small pieces about 1 mm in length which were further fixed in a freshly mixed aldehyde solution for 3 hrs at 4°C. After overnight rinsing in sucrose buffer, the tissues were postfixed in 1% osmium tetroxide containing 1.5% potassium ferrocyanide at 4°C for 2 hrs. The tissues were then dehydrated in an ascending series of ethanol and embedded in Araldite. Semithin sections were cut with a Porter Blum MT2 ultramicrotome and stained with 1% methylene blue in 1% borax. Selected areas of blocks were trimmed for ultrathin sections which were stained with uranyl acetate and lead citrate and examined in a Philips 400T electron microscope.

**OBSERVATIONS**

**Light microscopy**

The muscle showed an outer covering of perimysium which sent septa divide the muscle in fascicles. The muscle fibers were rounded or hexagonal in transverse sections and showed centrally located nuclei. Blood vessels, mainly capillaries and nerve bundles, were seen between the muscle fibers. In longitudinal sections, the fibers showed centrally located nuclei along their lengths: some nuclei were also seen closer to the sarcolemma. The nuclei also showed prominent nucleoli and peripheral condensed chromatin. There were fibroblasts and pigment cells (GOPALAKRISHNAKONE, 1986) between the muscle fibers. The latter cells, which were distributed also close to blood vessels, could be observed even in unstained sections. The cells were dark in color and possessed many cytoplasmic processes (Fig. 1, 2).

**Acetylcholinesterase staining**

The presence of pigment cells made the confirmation of the acetylcholinesterase reaction difficult, since both appeared as semilunar in shape. Post junctional folds were not stained. There was no branching of the synaptic gutters. The appearance was that of an "en grappe" type of terminal without extensive infolding of the postsynaptic membrane of the sarcolemma (Fig. 1, 2).

**Succinic dehydrogenase enzyme activity**

The muscle fibers stained darkly for this mitochondria bound enzyme. The smaller diameter fibers stained darkly and the larger diameter fibers stained lightly, with intermediate fibers staining at varying intensities. At higher magnifications, small

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**Fig. 1.** Light micrograph of the acetylcholinesterase reaction of the ileofibularis muscle. Note the semicircular areas of reaction products and also a diffusion of the staining. The dark cells are pigment cells (arrow) which cause confusion with the AcchE reaction products. The background was stained with 1% eosin which shows the hexagonal muscle fibers and central nuclei. ×52

**Fig. 2.** Higher magnification of a similar area as in Figure 1; showing the diffusion pattern of the acetyl cholinesterase reaction (arrows) and the pigment cells. The shape of the muscle fibers and the central nuclei are clearly seen. ×380

**Fig. 3.** Light micrograph of the succinic dehydrogenase (SDH) of the turtle muscle showing darkly staining, lightly staining and intermediate staining fibers. Note the lack of a regular pattern of distribution for the fibers. ×9

**Fig. 4.** Higher magnification of an area similar to Figure 3, showing darkly staining fibers (D), lightly staining fibers (L) and intermediate staining fibers (I). A pigment cell (P) can also be seen. ×380
Fig. 5. Electron micrograph of a longitudinal section of muscle fiber showing the dark A band, light I band and the Z disc. Between the myofibrils, the triads (arrows) with a central transverse tubule and two terminal cisternae and mitochondria are seen. ×16,000

Fig. 6. Higher magnification of a similar area as that in Figure 5 distinctly showing the M bands (M) and the H line (arrow). Note also the distribution of glycogen granules (G) in between myofibrils. ×36,000
diformazan granules were seen distributed evenly throughout the muscle fibers (Fig. 3, 4).

**Electron microscopy**

The A and I bands and the Z lines were clearly seen. The H zone showed a well defined M line in the center. The presence of two dark lines within the I band, one on either side of the Z line, known as the N lines, could be recognized only in certain regions (Fig. 5). The sarcomere was about 1.7 μm in length and the A band was about 1.4 μm, while the Z disc was about 0.9 μm in width. Glycogen granules were richly scattered among the myofibrils. Mitochondria with irregular cristae were seen in longitudinal rows among the myofibrils (Fig. 6).

The nuclei, longitudinally elongated, were seen in the central region of the muscle fibers. The sarcolemma showed a large number of vesicular invaginations (Fig. 7).

The triad consisting of a transverse tubule in the center and a the cistern of the sarcoplasmic reticulum on either side, was mostly observed at the junction of the A and I bands (Fig. 6, 8).
Satellite cells were seen scattered among the fibers. These were situated outside the sarcolemmal membrane but inside the basal lamina without disturbing the conformity of the fiber. These cells showed a prominent nucleus surrounded by a rim of cytoplasm containing only a few organelles, such as mitochondria. The cells were about 9–9 μm in length (Fig. 8).

Blood capillaries uniformly distributed between the muscle fibers comprised the endothelium of the continuous type and occasional pericytes. The pigment cell ultrastructure has been described elsewhere (GOPALAKRISHNAKONE, 1986).

**Neuromuscular junctions**

The presynaptic portion of the neuromuscular junction appeared as a spindle-shaped structure in both longitudinal and transverse sections. Two types of vesicles were distinguished: one was clear and the other was dense core type (Fig. 9–13). The former were scattered throughout the presynaptic portion and were pleomorphic in shape and size; on the average they were round in shape and measured about 35 nm in diameter. The dense core vesicles were fewer in number than the clear vesicles.

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**Fig. 8.** Transverse section of muscle fiber showing a satellite cell (S). Note the large nucleus in comparison to the minimal cytoplasm; also the cell situated outside the sarcolemmal membrane but inside the basement membrane. ×28,000

**Fig. 9.** Longitudinal section showing the neuromuscular junction. The presynaptic region contains small translucent vesicles and few large dense core vesicles (arrows). Note the absence of postsynaptic folds. ×28,000

**Fig. 10.** Transverse section showing the neuromuscular junction. The presynaptic portion shows pleomorphic translucent vesicles and a large mitochondrion. ×36,000

**Fig. 11.** Transverse section of a neuromuscular junction showing the accumulation of glycogen in the presynaptic portion. The presynaptic membrane appears irregular and associated with vesicles in the post synaptic membrane. ×28,000
They were round in shape and about 120 nm in diameter. Some terminals showed an accumulation of glycogen (Fig. 11). Only a few mitochondria were seen scattered among the vesicles. The mitochondria were variable in size and shape in addition to their irregularly branching cristae. Occasionally neurotubules and neurofilaments

Fig. 9-11. Legends on the opposite page.
Fig. 12. Neuromuscular junction very distinctly showing the translucent vesicles and the dense core vesicles; also note that there are two postsynaptic folds (arrows) in the membrane.  \( \times 28,000 \)

Fig. 13. Three terminals (1, 2, 3) could be seen in relation to one muscle fiber.  \( \times 28,000 \)
were seen in the terminal portion. Multiple terminals were also seen in some fibers (Fig. 12, 13).

The Schwann cell of the terminal showed a prominent nucleus with a well developed Golgi apparatus. The presynaptic membrane and the synaptic gap appeared regular in most places, but in some regions Ω-shaped indentations were seen in the presynaptic portion. The synaptic gap appeared uniform throughout its length, being about 70 nm in width. In most terminals the postsynaptic membrane did not show any clear infoldings; only very few terminals showed two or three short junctional folds without any branching (Fig. 12).

**DISCUSSION**

The turtle ileofibularis muscle showed many similarities to those reptilian muscles such as in the reticulate python, *Python reticulatus* (GOPALAKRISHNAKONE, 1983) and *Cobra naja naja* (GOPALAKRISHNAKONE and JAYATILAKA, 1978), also to fish (NAKAJIMA, 1969) and bird muscles (GOPALAKRISHNAKONE, 1985). Although the basic myofibrillar organization of the turtle muscle was similar to that of tortoise muscle (PAGE, 1968) there was one feature which was present in tortoise muscle and absent in the turtle muscle, namely the N line. The N line (Nebenscheiben) was observed as a densely staining lining within the light I band of the tortoise muscle. PAGE (1968) could not postulate any possible function for the presence of N line in tortoise muscle. At any rate, the absence of the N line in the turtle muscle in the present study indicates that there are structural variations even among closely related genera. The features of vertebrate slow muscles have been reviewed (HESS, 1970).

Based on such ultrastructural data as fiber diameter, mitochondrial content, width of the Z disc and features of the sarcoplasmic reticulum, mammalian skeletal muscle fibers have been classified into red, white and intermediate fibers (PADYKULA and GAUTHIER, 1970). The fibers observed in the present study are judged to be of the red and intermediate types: they were of larger diameter with fewer mitochondria, abundant glycogen, and possessing few fibers around the peripheral region of the muscle.

Using the histochemical criteria such as succinic dehydrogenase (SDH) activity, the majority of the turtle muscle fibers were lightly stained; around the peripheral region of the muscle belly there were intermediate and darkly staining fibres, but the distinction between the intermediate and dark fibers was not marked, similar to avian muscle (GOPALAKRISHNAKONE, 1985). Another study on the histochemical composition of the locomotory muscles in a lizard showed three types of fibers namely fast twitch glycolytic, fast twitch oxidative glycolytic, and tonic (PUTNAM, GLEESON and BENNETT, 1980). As printed out by GUTHE (1981) the present study confirmed the difficulty in classifying reptilian muscles on the basis of cytochemical, electron microscopic and physiological criteria. Based on the ultrastructural data, BOWMAN and MARSHALL (1971) categorized avian muscles into the fibrillar (Fibrillensstruktur) type and (Felderstruktur) type. According to this classification, the iliolobularis muscle of the turtle could be referred to as a fibrillar type in which the myofibrils are polygonal in cross section, separated from each other by the sarcoplasmic reticulum, clearly visible, and the H zone and M band are distinct.

The acetylcholinesterase reaction and the interpretation of the results were complicated by the presence pigment cells. However, the stained areas of semilunar shape could be clearly recognized, showing no branching of the synaptic gutters. This
type of ending has been observed in the dorsal fin muscle of the sea horse (*Hippocampus hudsonius*; BERGMAN, 1967), in the garter snake (HESS, 1965) and in snake fish (*Ophiophagus argus*) muscle (NAKAJIMA, 1969), in the myotomal muscles of the dog fish (*Scyhydrhines canicula*; BONE, 1972) where the neuromuscular junctions showed postsynaptic folds, and also in some other striated muscles of vertebrates (COLE, 1955). The functional significance of the post synaptic folds is not known. It is also open to criticism whether they are present only in highly evolved animals such as mammals, because of the presence of post junctional folds in some fish (BONE, 1972). The two types, i.e., those with and without post junctional folds have been observed in lizard muscle (PROSKE and VAUGHAN, 1968).

The type of neuromuscular junction observed in the present study appears to differ in ultrastructural features from the mammalian neuromuscular junction because of the absence of postsynaptic folds. The criteria used by PADYKULA and GAUTHIER (1970) could not be applied for the purpose of classification. The description of 'en gruppe' and 'en plaque' types of neuromuscular junctions has enabled the present neuromuscular junctions to be described as an 'engrappe' type. The presence of two types of vesicles, namely the small clear vesicles and the large dense ones, suggests that there might be two different transmitters. From the above observations it can be concluded that the structure and classification of reptilian muscles are still not conclusive and can not be generalized.

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Dr. P. GOPALAKRISHNAKONE
Department of Anatomy
Faculty of Medicine
National University of Singapore
Kent Ridge
Singapore 0511