Neuromuscular Junctions in Flight and Tymbal Muscles of the Cicada

BY GEORGE A. EDWARDS, PH.D., HELMUT RUSKA, M.D., AND ÉTIENNE DE HARVEN, M.D.*

(From the Division of Laboratories and Research, New York State Department of Health, Albany, and the Sloan-Kettering Institute for Cancer Research, New York)

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ABSTRACT

The tymbal muscle fiber in the cicada closely resembles the indirect flight muscle fiber in its structural detail. We agree with other authors that the tymbal muscle is a modified indirect flight muscle.

The peripheral nerve branches to the tymbal and flight muscle fibers are similar to those in the wasp leg. The axon is loosely mantled by irregular turns of the mesaxon, enclosing cytoplasm. The nerve is therefore a tunicated nerve.

The neuromuscular junction in the high frequency muscle fibers shows direct apposition of plasma membranes of axon and muscle fiber, large numbers of mitochondria and synaptic vesicles in the axon, and concentrations of mitochondria, aposynaptic granules, and endoplasmic reticulum in the postsynaptic area of the muscle fiber.

Of special interest is the multitude of intracellular, opposing membranes in the postsynaptic area. They form laminated stacks and whorls, vesicles, cysternae, and tubules. They occasionally show continuity with the plasma membrane, the outer nuclear envelope, and the circumfibrillar endoplasmic reticulum. The membrane system in this area is designated "rete synapticum." It is believed to add to the electrical capacity of the neuromuscular junction, to serve in transmission of potentials, and possibly is the site of the oscillating mechanism in high-frequency muscle fibers.

INTRODUCTION

In a previous paper (Edwards, Ruska, and de Harven, 1958), the ultrastructure of peripheral nerves, muscle, and neuromuscular junctions in a low frequency femoral muscle of an insect, the wasp, was described. The present paper continues that study to clarify the nerve-muscle relationship in high frequency muscles.

The light microscopy of the indirect flight muscles of the cicada has been described by Tiegs (1955) in his thorough monograph on insect muscles. The morphology and topographic relations of the tymbal muscle of the cicada have also been well studied (Pringle, 1954 a; 1954 b) as part of the background for the physiologic analysis of song production. Essentially, it has been shown that the tymbal muscle fibers are "fibrillar," possess a large number of mitochondria and their nerve also innervates the metathoracic spiracular muscles. Physiologically, the tymbal muscle resembles the indirect flight muscles of higher insects. Its frequency of contraction may be as high as 480 per second, it shortens only 1.5 per cent, and in some species is asynchronous; i.e., above 50 stimuli per second, the muscle has a rhythmic activity greater than the frequency of the incoming nerve impulses. Pringle was led, thus, to the conclusion that the tymbal muscle is probably a modified flight muscle.

The present results indicate that although both flight and tymbal muscles are high frequency, fibrillar muscles, their fibers differ in some fine structural details. The neuromuscular junctions of both are similar, but are more complex than those of the femoral muscle of the wasp.

*Fellow of the Belgian-American Educational Foundation and of the Damon Runyon Memorial Fund. Present address: Laboratoire d'Anatomie Pathologique de l'Université Libre de Bruxelles, Brussels, Belgium.
Materials and Methods

Muscle fibers and associated peripheral nerves from the thoracic, indirect flight muscles and the tymbal muscles of the cicada (*Tibicen linnei*) were studied in thin sections with the electron microscope. The material was prefixed *in situ* for 15 minutes with 2 per cent osmium tetroxide buffered to pH 7.4, then dissected out, and pieces of less than 1 square millimeter in cross-section further fixed in cold, buffered, 1 per cent osmium tetroxide for another 45 minutes. The material was washed with veronal acetate buffer at pH 7.4, dehydrated in graded ethanols and acetone, embedded in a mixture of 80 per cent butyl-20 per cent methyl methacrylate with 3 per cent luperco initiator, and polymerized at 45°C. for 24 hours. The individual embedding capsules usually contained from 3 to 6 muscle fibers with attached nerve branches and tracheoles. Sections were cut with both glass and diamond knives (the latter manufactured at IVNIC (Venezuelan Institute for Neurology and Brain Research, Caracas), on the Porter-Blum microtome. Some of the sections were picked up in the usual manner on formvar- or carbon-coated grids. Others (cf. Figs. 10 to 12) were picked up directly on the copper grids, and then lightly covered with carbon just sufficiently to stabilize the sections in the electron beam (de Harven, 1958). Micrographs were taken in the Siemens Elmiskop I and RCA EMU electron microscopes at original magnifications of 1,800 to 40,000.

1 Insect flight is brought about principally by transmission of distortions of the thorax to the wing base through small, interposed chitinous elements. In the cicada the indirect flight muscles are concentrated in the mesothorax and comprise three major groups. The longitudinal depress, the obliques elevate, and the dorsoventral muscles elevate and extend the wings. The muscles used in the present study were from the large, heavily tracheated, median longitudinal group, originating on the phragma (internal skeleton), and inserting on the thoracic wall. The sound produced by the cicada is due to the vibration of a pair of convex tymbals on the dorsolateral surfaces of the first abdominal segment. The vibration is caused by the contractions of a large pair of tymbal muscles, originating on a ventral, V-shaped skeletal element. Each muscle inserts on a free, chitinous plate, from which a short chitinous bar inserts on one rim of the tymbal. The curvature of the tymbal is determined by small tensor muscles, which run from the posterior edge of the metathorax to the anterior edge of the tymbal. The fibers studied were from the large, tymbal muscles, believed to be modified flight muscles. (For complete details of the musculature, see Pringle, 1954 a; 1954 b; and Tiegs, 1955.)

RESULTS

A. Indirect Flight Muscle Fibers.—The indirect flight muscle of the cicada is composed of typical, high frequency, fibrillar fibers (Fig. 1). The cytolemma comprises a basement membrane of 40 mu thickness, a plasma membrane 9 mu thick, and a small interspace. The basement membrane may merge with that of lemnoblasts or tracheoblasts. The plasma membrane often shows vesiculation (pinocytosis) in regions near a neuromuscular junction. The myofibrils are generally separated by mitochondria. The fibrils average 1 mu in diameter, and are composed of myofilaments 15 mu in diameter, as seen in longitudinal section. There are indications of thin filaments between the thick ones. The endoplasmic reticulum between the fibrils is relatively scarce. Some tubules and vesicles may be seen at the level of the I region (Figs. 1 and 10). Sparse tubules may be seen near mitochondria. Between the plasma membrane and the outermost fibril, particularly near neuromuscular junctions, the tubules of the reticulum may become layered (Figs. 9 through 12). The mitochondria of the flight muscle are chunky and large (1.0 x 2.5 mu), abundant, and non-uniformly distributed between the individual myofibrils. They contain solidly packed, narrow cristae that form various alignment patterns. Often the pattern in one mitochondrion is continued in the adjacent one. "Intracellular" tracheoles are abundant and closely associated with the mitochondria. The cytoplasmic matrix is finely granular. The nuclei are numerous, generally peripheral, and large. They show the commonly described arrangement of chromatin and components of the nuclear envelope.

B. Tymbal Muscle Fibers.—The tymbal muscle fiber is an atypical, high frequency, fibrillar fiber (Fig. 3). In size and composition of fibrils, in mitochondrial size and number, in tracheolization, and in size, number, and distribution of nuclei, the tymbal muscle fiber resembles the flight muscle fiber. It differs from the flight muscle fiber in the arrangement of mitochondria and myofibrils, and in the amount and distribution of tubules and vesicles of the endoplasmic reticulum. The myofibrils (0.6 mu in diameter) occur in bundles of 2 to 5, the bundles being separated longitudinally by 1 to 3 rows of mitochondria (averaging 0.7 x 1.4 mu each). The Z line of the myofibrils is wide, the I region short (in the partially or fully contracted fibril), and the less dense H band some-
times shows a weak M line. Large quantities of tubules and vesicles of the endoplasmic reticulum surround the myofibrils, being particularly abundant at Z and M levels, and showing characteristically paired, utricular dilatations identically oriented for each sarcomere. The utricles of adjacent myofibrils lie close together halfway between the A-I and the A-H junctions.

C. Peripheral Nerves.—The peripheral nerves innervating the flight and tymbal muscle fibers observed in the present study show similar structure; hence will be described as one general type. The large nerves, occurring free in the interstitium, are “tunicated” nerves, containing a large, central, heavily mantled axon (Fig. 4), and several smaller, peripheral, less mantled axons. The nerve lemnoblast characteristically has a thick basement membrane, which may vary considerably, however. The average thickness of the basement membrane of the interstitial nerve was 200 μm, which is 10 or more times that found in vertebrates. Between the basement membrane and plasma membrane is a small interspace. The plasma membrane, averaging 8 μm, invaginates (Fig. 5) to form the mesaxon, whose membranes occasionally diverge to form lacunae of differing sizes. The mesaxon finally winds loosely around the axon in 4 to 9 turns. The membranes of the mesaxonal mantle enclose variable amounts of cytoplasmic matrix, and even mitochondria. The nucleus of the lemnoblast is ovoid and situated to one side of the cell. The cytoplasm of the lemnoblast contains numerous, small mitochondria (averaging 190 x 330 μm) with few cristae, membrane-bound profiles, and a finely granular matrix. The cytoplasm of the axons of nerves passing through the interstitium contains a few mitochondria, membrane-bound profiles of varying sizes, and neurofilaments.

The smaller nerve branches (Fig. 2) to the individual muscle fibers generally contain one large central axon mantled by turns of the mesaxonal membrane. The nucleus and cytoplasm of the lemnoblast of the smaller branches are similar to those of the larger ones. The more distal axons, e.g. those of a nerve in contact with the muscle fiber, show fewer neurofilaments and more mitochondria, concomitant with the appearance of axonal synaptic vesicles. The nerve is usually accompanied by tracheoblasts. The basement membranes of nerve lemnoblasts, tracheoblast, and muscle fiber merge at the region of nerve-muscle contact (Fig. 5).

D. Neuromuscular Junction.—As in the femoral muscle of the wasp, the small nerve branch in the cicada courses longitudinally along the muscle fiber in a groove (Fig. 6). In the light microscope the nerve appears “inside” the muscle fiber. In the electron microscope it can be seen that the axon makes a longitudinal synapse with the muscle cell. The outer portion of the axon is capped by the lemnoblast and tracheoblast. The synapsing inner portion of the axon is free of its lemnoblast sheath. The axon contains many mitochondria and synaptic vesicles (Figs. 7 and 8). The plasma membrane of the axon is intimately apposed to that of the muscle fiber, with only a small interspace intervening.

The postsynaptic area of the muscle fiber (Figs. 6 and 7) is large, occupying the muscle fiber periphery to a depth of 4 microns and extending laterally from the axon as much as 6 microns. The entire area contains nuclei, mitochondria, membranes, and granules. Considerable distance and material, therefore, intervene between the axonal synapse and the most peripheral contractile material of the muscle fiber. Immediately beneath the synapsing plasma membrane of the muscle fiber, the sarcoplasm is filled with large numbers of aposynaptic granules (Figs. 6 through 8). These are dense, osmiophilic, homogeneous granules of 5 to 15 μm diameter. They are aggregated most closely in the region of the synapse and become more dispersed with increasing distance from the plasma membrane. Even more prominent than the sarcoplasmic, aposynaptic granules are the ramifying and layered membranes of the “rete synapticum” (Figs. 6, 7, and 9 through 12). These occur as large and small vesicles, meandering membrane-bound profiles, (Figs. 6 and 7), parallel layers of 4 to 8 membranes (Figs. 6, 9 through 12), and concentrically wound membranes (Fig. 6), enclosing cytoplasm. The plasma membrane in the postsynaptic area may show considerable vesiculation and occasional deep infolding. The deep folds may form spirals of several turns, or break up into numerous vesicles, or be continuous with the layered membranes (Figs. 6 and 9). In some regions, one or more small vesicular profiles are seen within a larger profile (Fig. 7). Near the most peripheral myofibril of the region, and near nuclear borders the membranes form long (4 to 6 microns) parallel layers (Figs. 9 to 11). The more internal membranes appear continuous with the endoplasmic reticulum surrounding the myofibrils (Figs. 9 and 10). Occasionally a con-
nection is noted between the layered profiles and the outer nuclear membrane. The "rete synapticum" could constitute a special arrangement of the endoplasmic reticulum involved in the electrophysiologic activity specific to the synaptic region of the high frequency muscle fiber.

**DISCUSSION**

The present investigation supports Pringle's conclusion (1954b) that the tymbal muscle in the cicada is a modified flight muscle. His evidence was that the chitinous V for the basal attachment of the tymbal muscles is a posteriorly directed projection of the metathoracic sternum, that cutting the metathoracic and abdominal nerves did not interfere with flight; and principally, that the tymbal muscle of some cicada species has a rhythmic mechanism similar to that of dipteran (Pringle, 1949) and hymenopteran (Roeder, 1951) indirect flight muscles. Not all cicadas possess an asynchronous tymbal muscle, however. Hagiwara (cited in Pringle, 1954b) has shown that certain Japanese cicadas show a one to one relationship between nerve impulses and sound pulses. Morphologically, the tymbal muscle of the common cicada, Tibicen linnei, is more similar to the indirect flight muscles of other higher insects (cf. Chapman, 1954; Hodge, Huxley, and Spiro, 1954; Hodge, 1955; Edwards and Ruska, 1955; Edwards, Ruska, de Souza Santos, and Vallejo-Freire, 1956) than to the flight muscle of lower insects or to abdominal and leg muscles (cf. Edwards and Ruska, 1955; Edwards, Ruska, de Souza Santos, and Vallejo-Freire, 1956). It is intermediate between leg and flight muscle in that its myofibrils are arranged in bundles, and it possesses a well organized endoplasmic reticulum with characteristic utricles. The paired utricular dilatations, identically situated between adjacent sarcomeres, probably correspond with the "terminal cisternae" of the triads described by Porter and Palade (1957) in other muscles. The general distribution of the tubules, vesicles, and utricles of the endoplasmic reticulum of the tymbal muscle of the cicada are not exactly similar to any of the arrangements figured in their study, nor to those found in flight or leg muscle of the insect. The quantity and distribution of endoplasmic reticulum have been linked to the transmission and distribution of endopotentials (Porter and Palade, 1957; Ruska, Edwards, and Caesar, 1958). Hence, as the tymbal muscle in various species is variable in its myogenicity, it is not too surprising that some variation in the endoplasmic reticulum also occurs.

In a previous communication (Edwards, Ruska, and de Harven, 1958) it was shown that the peripheral nerve in the wasp leg is tunicated, i.e., the axon is surrounded by irregular, cytoplasm-enclosing, turns of the mesaxon. Similarly, in the cicada peripheral nerve branches to the tymbal and flight muscles, the axons are loosely mantled by three to nine windings of the mesaxon. Between the irregular turns of the mesaxon there are cytoplasmic areas containing a granular matrix and even mitochondria. Thus again, we observe that the peripheral nerve branches in this insect are tunicated, rather than myelinated. We consider that in its evolution the axon-sheath cell relationship is equivalent to that seen in the chick embryo (Geren and Schmitt, 1955). Myelination would be a more advanced stage in which multiple lipoprotein layers result from the tightest possible packing of the spiralling, infolded plasma membrane of the lemnoblast.

Of particular interest in the present study are the neuromuscular junction and postsynaptic area of the muscle fiber. The axon-muscle relationship is similar to that in insect leg muscle (Edwards, Ruska, and de Harven, 1958), but different from those in vertebrate striated muscle (compare Robertson, 1956; Reger, 1957). The area of the muscle fiber between axon and peripheral contractile material is strikingly different from previously observed junctional areas. It is large, contains nuclei, mitochondria, and aposynaptic granules. Principally, it is the region where membranes take forms reminiscent of the sacculi in dictyosomes. The important problem is the meaning of the alignment of the membranes into layers and their connections with plasma membrane, outer nuclear membrane, and circumfibrillar membranes. Morphologically, the system can probably be described best as a "rete synapticum." Such a continuous system, specific to the synaptic region in the high frequency muscle fiber, should have a specific function. The physiologic role can only be surmised. The topography and morphology suggest that the membranes of the "rete synapticum" may carry membrane potentials, may add to the electrical capacity of the neuromuscular junction, and act in the transmission of the impulse within the fiber. Pringle postulated (1954a) that the mechanism for the myogenic rhythmic activity in these muscles is contained in the im-
dependent myofibrils. We would submit that the rhythmic mechanism could just as well be a function of the evolved postsynaptic area as of the myofibril. It is more justified, then, to conceive of the oscillation mechanism as being in the membrane layers in the postsynaptic area.

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**FIG. 1.** Longitudinal section through an *indirect flight muscle fiber*. Visible in the micrograph are parts of three myofibrils (*Mf*), extending over approximately $2\frac{1}{2}$ sarcomeres. The fibrils are contracted, hence only the Z line is visible. Very little endoplasmic reticulum surrounds the muscle fibrils, appearing most noticeably as paired utricles (*Er*) between adjacent myofibrils at the level of the A region. The fibrils are separated by large, chunky mitochondria (*Mi*), with the closely packed internal membranes characteristic of high-frequency muscle. The cytolemma consists of a thin plasma membrane (*PM*), a small interspace, and a basement membrane (*BM*). In this micrograph, the basement membrane of the muscle fiber is merged with that of the accompanying tracheoblast, whose nucleus (*Nu*) is at the left of the picture. $\times$ 55,000.
Fig. 2. Cross-section through a small nerve branch between two indirect flight muscle fibers (M, M). The nerve is accompanied by a tuft of tracheoles (Tr) enclosed within the cytoplasm and membranes of the tracheoblast. The basement membrane of the tracheoblast is merged with that of the upper muscle fiber, and with that of the nerve lemnoblast (at BM₁). The nerve branch consists of a single, central axon (A) suspended within the mesaxon. The mesaxon is formed by invagination of the plasma membrane of the lemnoblast, and courses irregularly through the lemnoblast, at times diverging to form lacunae. The cytoplasm of the lemnoblast (L) contains small mitochondria and granules. The basement membrane of the lemnoblast is in contact with the basement membrane of the lower muscle fiber (at BM₂). The sarcoplasm is filled with mitochondria (Mi) characteristic of areas close to nerve-muscle contact. × 35,000.

Fig. 3. Longitudinal section through the interior of a tymbal muscle fiber. Two myofibrils are surrounded by rows of mitochondria (Mi). The individual myofibrils are in turn surrounded by tubules and vesicles of the endoplasmic reticulum (Er₁ and Er₂). Characteristic of the tymbal muscle fiber are the paired utricles (Ut) of the reticulum at the A level of the sarcomere halves. In the fully contracted fiber the only line visible is the Z in the extremely shortened I region. The Z band comprises continuous filaments, a Z substance, and some profiles of the endoplasmic reticulum. × 50,000.
(Edwards et al.: Neuromuscular junctions)
Fig. 4. Cross-section through part of a large, *interstitial nerve branch*. The lemnoblast of the nerve shows the typical, thick basement membrane (*BM*). The plasma membrane (*PM*) of the lemnoblast exhibits pinocytosis. The mesaxon (*MA*) courses in various directions through the lemnoblast cytoplasm, finally making several turns around the axon (*A*) and enclosing cytoplasmic areas between its membranes. The lemnoblast cytoplasm contains numerous small mitochondria (*Mi*) and granules, in addition to small membrane-bound profiles. Large lacunae (*La*) are formed by divergence of the mesaxon membranes. The axon of an interstitial nerve branch generally contains mitochondria (*Mi*) and neurofilaments (*nf*). Vesicles (*V*) are variably present in the axons of proximal nerve branches. × 50,000.
Fig. 5. Cross-section through interstitial nerve branch. The thick basement membrane of the nerve lemnoblast is merged with an accompanying tracheoblast at BM₁ and again at BM₂ with a tracheoblast containing a small tracheole. The plasma membrane of the lemnoblast invaginates to form the mesaxon (MA at single arrow), which makes complex turns throughout the lemnoblast. The cytoplasm of the lemnoblast (L) shows mitochondria (Mi₁) containing few cristae, and granules of varying size and density. The mesaxon (ending opposite double arrows) makes several turns around the axon (A). The axon contains mitochondria (Mi₂) and neurofilaments (nf). × 50,000.
(Edwards et al.: Neuromuscular junctions)
Fig. 6. Cross-section through a neuromuscular junction and postsynaptic area in an indirect flight muscle fiber. The axon (A) occupies a groove in the muscle fiber surface. The outer portion of the axon is capped by the merged basement membranes (BM) of the lemnoblast and muscle fiber. The axon contains many small mitochondria (Mi), and a few synaptic vesicles, the latter occurring close to the synapsing plasma membrane (arrows). The receptive (postsynaptic) area of the muscle fiber is that large area between the synapsing plasma membranes and the first myofibril of the muscle fiber. It contains nuclei (not shown), mitochondria (Mi), aposynaptic granules (G), and a complex system of membranes of the endoplasmic reticulum (Er). The granules are most closely aggregated near the plasma membrane, but may be scattered. The membranes occur in stacks (Er1), or whorls (Er2), or as isolated vesicles and tubules (Er3). They may be continuous with the plasma membrane (upper right), and with the outer nuclear membrane and the circumfibrillar reticulum. The total membrane system of the postsynaptic area is designated “rete synapticum.” In upper right hand corner of the micrograph is an extension of a tracheoblast, containing a small tracheole (Tr). The basement membrane of the tracheoblast is merged with that of the muscle fiber. In general, tufts of tracheoles are found in and around the receptive area. X 60,000.
(Edwards et al.: Neuromuscular junctions)
FIG. 7. Section through a neuromuscular junction and postsynaptic area in a flight muscle. The axon (A) at left contains synaptic vesicles (v), aggregated near the synapse (arrows). The apposing portions of the plasma membranes of the axon and muscle fiber are thicker and denser than in non-synaptic areas. Aposynaptic granules (G) are found throughout the sarcoplasm of the receptive area, but are closely packed near the synapse. At right is part of a nucleus (Nu). A large part of the area between the axon and nucleus is occupied by the “rete synapticum” (Er). Its walls appear as long parallel membrane profiles near the nucleus, whorls of membranes in lower center, profiles of vesicles within vesicles, and non-ordered tubules and vesicles. X 35,000.

FIG. 8. Detail of a synapse in a tymbal muscle fiber. The synaptic apposition of plasma membranes of axon and muscle is the same in tymbal, flight, and leg muscles of insects. The synapsing axon (A) contains mitochondria (Mi) and synaptic vesicles (V). The sarcoplasm of the muscle fiber contains aposynaptic granules (G) close to the plasma membrane. The apposition of plasma membranes (arrows) is more like that in vertebrate smooth, than vertebrate striated muscle. X 110,000.

FIG. 9. Detail of part of the “rete synapticum” (Er) near a peripheral myofibril (Mf). Of the four branches of the “rete” (Er), the outermost courses through the receptive area in continuity with non-ordered tubules. The innermost is continuous with the circumfibrillar endoplasmic reticulum (Er), particularly at the Z line level. The section was stabilized by lightly covering with carbon. X 50,000.
(Edwards et al.: Neuromuscular junctions)
Fig. 10. Section through part of a postsynaptic area in an indirect flight muscle fiber. All the figures (Figs. 10 to 12) in this plate are from unsupported sections that were stabilized by lightly covering with carbon (de Harven, 1958). Visible in the micrograph are portions of a nucleus (Nu), myofibril (Mf) with surrounding endoplasmic reticulum (Er) and Z line, aposynaptic granules (G), and layered membranes of the "rete synapticum" (Er). The phase-separating membranes of the "rete synapticum" may be continuous with the perinuclear and circumfibrillar endoplasmic reticulum. They occur in that part of the receptive area near the peripheral myofibril and the nucleus. × 48,000.

Fig. 11. Detail from a postsynaptic area in an indirect flight muscle fiber, showing the layered membranes of the "rete synapticum" (Er) near the myofibril (Mf). External to the layered membranes are scattered aposynaptic granules and discontinuous vesicular profiles. An "intracellular" tracheole (Tr) at left of micrograph. × 60,000.

Fig. 12. Occasionally the layered membranes (Er) may occur close to the plasma membrane of the muscle fiber in the postsynaptic area as shown in this detail. In lower center, an interstitial tracheole (Tr) in a tracheoblast extension contacts the basement membrane of the muscle fiber. Aposynaptic granules are scattered throughout the sarcoplasm. × 55,000.
(Edwards et al.: Neuromuscular junctions)