LOCALIZATION OF ACETYLCHOLINE RECEPTORS BY MEANS OF HORSERADISH PEROXIDASE-α-BUNGAROTOXIN DURING FORMATION AND DEVELOPMENT OF THE NEUROMUSCULAR JUNCTION IN THE CHICK EMBRYO

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ABSTRACT

The localization of acetylcholine receptors (AChR) in the surface of developing myogenic cells of the chick embryo anterior and posterior latissimus dorsi muscles in relation to the process of innervation has been studied at the ultrastructural level utilizing a horseradish peroxidase-α-bungarotoxin conjugate. Localized concentrations of AChR were found in small regions 0.1–0.4 μm in width on the surface of myogenic cells of 10- to 14-d-old muscles. Surface specializations consisting of an external coating of extraneous material and an internal accumulation of dense material are associated with the plasma membrane in the regions of AChR concentration. As the muscle fibers are innervated, reactive surface patches are found at the region of contact of the growing nerve fiber and the surface of myotubes or their fusing myoblasts. After the establishment of contact, the patches of reaction product become more numerous and coextensive within the region of the neuromuscular junction and its immediate surroundings forming a dense continuous deposit on the postsynaptic sarcolemma. Activity becomes increasingly restricted to the site of the neuromuscular junction as the embryos approach hatching. At all stages, specializations external and internal to the plasmalemma are found at regions of high density of AChR, suggesting that they play a role in the maintenance of a higher concentration of receptors at these sites. These specializations also occur at the region of initial synaptic contact, indicating that they might be recognized by the nerve and represent preferred sites of innervation. Innervation appears to exert a stabilizing influence on the area of high AChR concentration in contact with the nerve and to induce a further increase in the AChR density of this site while the number of AChR in the remaining portions of the muscle surface declines.

KEY WORDS acetylcholine receptors · α-bungarotoxin · development · neuromuscular junction · chick embryo

The appearance and distribution of acetylcholine receptors (AChR) have been studied during myogenesis and innervation by means of microiontophoretic application of acetylcholine (ACh) and intracellular recording and by means of labeled

α-bungarotoxin (α-Btx) (5, 23, 25, 32, 40, 55, 59). These studies have shown that dividing mononucleated myoblasts lack ACh reactivity and have little or no α-Btx binding activity. However, early stages in the formation of multinucleated myotubes are characterized by a uniform distribution of AChR along their surfaces. Subsequently, the spatial distribution of AChR in developing myotubes in vitro is distinctly nonuniform with local areas of high receptor density or hot spots distributed randomly in addition to the diffuse low density labeling. Contact by nerve processes does not seem necessary for the induction of high receptor accumulations because they occur in myogenic cells that have had no previous neuronal contact (7, 48). Inhibitors of energy metabolism will cause dispersal of the AChR clusters (11).

A higher density of AChR at the region contacted by the axon has been observed consistently (3, 9, 10). The clusters of receptors have been found to be situated exactly at sites of transmitter release (20). The incoming nerve may make contact with preexisting patches of high receptor density or may induce the formation of receptors at the region of contact. In addition, after innervation there is a decrease in extrajunctional receptor density (10, 14). These changes may represent a redistribution of AChR from extrajunctional sites to the junctional region or a degradation of extrajunctional receptors. In amphibians, patches of fluorescent conjugates of α-Btx have been observed to accumulate at the synapses indicating that innervation causes a redistribution of extrajunctional receptors to sites of nerve-muscle contact (2). In the chick, the rates of turnover of junctional and extrajunctional receptors are the same, indicating that the accumulation of junctional receptors is regulated by either preferential incorporation at this region or by a restriction of receptor diffusion in the postjunctional membrane (14).

This study was undertaken to investigate changes in the distribution of AChR during these processes in developing anterior and posterior latissimus dorsi muscles (ALD and PLD, respectively) at the fine structural level. It is made possible by the use of a horseradish peroxidase-α-Btx (HRP-α-Btx) conjugate which allows for the high resolution localization of AChR (42). In particular, it is of interest to identify the regions of high receptor density and their relationship to any specializations that could be involved in preferential receptor localization and to the site of initial synaptic contact.

MATERIALS AND METHODS

Purification of α-Btx

Crude *Bungarus multicinctus* venom was obtained from the Miami Serpentarium, Miami, Fla. α-Btx was separated from the venom on a column of Whatman carboxymethylcellulose (CM-52, Whatman, Inc., Clifton, N. J.) according to the method of Clark et al. (18).

Conjugation of HRP to α-BTX

The conjugation of horseradish peroxidase (HRP) and α-BTX was based on the procedure for conjugation of HRP and proteins developed by Nakane and Kawaoi (45). The procedure and its applications are described in detail elsewhere (41, 42). While the conjugate retains specificity for AChR and is capable of competing for binding sites, it has a lower affinity for AChR than native α-Btx (42, 58). It is likely that quantities of conjugate sufficient to generate a visible reaction product bind only at sites of relatively high receptor density.

Tissue Incubation and Cytochemistry

The ALD and PLD muscles of the White Leghorn chick at several embryonic and adult stages beginning at 10 d of incubation up to 4 wk posthatching were dissected and pinned out in Petri dishes. In the earliest stages, the anterior and posterior regions of the muscle could not always be clearly distinguished. If identity is not certain, the muscle is referred to as simply LD muscle. During this procedure, the muscles were bathed in aerated (95% O_2, 5% CO_2) avian Ringer solution (29). The light fraction of the HRP-α-Btx conjugate (42) was diluted 1:10 in Ringer solution to a final concentration of 10^{-5}–10^{-6} M. The tissue was incubated in the conjugate for 2 h with gentle agitation and then rinsed with Ringer's alone for 2 h with a change of solution every 15 min. The muscles were fixed in 2% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h and rinsed with several changes of buffer for an additional hour. The tissue was then incubated for 60–90 min in the dark in 3,3′-diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, Mo.) in 0.05 M Tris buffer (pH 7.2, 50 mg/100 ml) and 0.01% H_2O_2. The oxidation of the benzidine moiety by the peroxide-peroxidase reaction produces a dense brown deposite at the site of peroxidase activity. The muscles were rinsed for three 5-min periods in buffer, trimmed into small pieces, and postfixed in 1% osmium tetroxide in cacodylate buffer (pH 7.2) for 1 h. The tissue was then processed for electron microscopy and embedded in Epon 812. Thin sections were observed unstained with a Hitachi 8B electron microscope.

Control Experiments

A variety of control experiments were performed on

the muscles. One series was preincubated in 10^{-5} M native α-Btx for 1 h. This was followed by a 2-h incubation in the HRP-α-Btx conjugate. Other muscles were preincubated in 10^{-5} M D-tubocurarine chloride (DTC) in avian Ringer for 1 h and then incubated in the conjugate plus DTC for 1 h. In other controls, tissues were incubated in HRP (10^{-5} M) or in plain avian Ringer's for 2 h. All of the tissues were then rinsed, fixed, and reacted for peroxidase activity.

RESULTS

The development of the neuromuscular junction (NMJ) is divided into several stages for convenience of description; myoblast and myotube surfaces without adjacent nerve fibers, establishment of initial synaptic contacts, and development and maturation of the NMJ. The fine structural surface characteristics and the localization of the HRP-α-Btx conjugate in the ALD (slow) and PLD (fast) muscles during these stages are described below. All tissue has been viewed unstained to optimize visualization of the reaction product. About 15–25 blocks at each embryonic stage of development have been examined. Despite established differences in fast and slow muscles, including the number of synaptic contacts per fiber and degree of junctional folding, the sites and intensity of HRP-α-Btx labeling have been found to be basically the same in these muscles at every stage of development studied. Therefore, the observations on fast and slow muscle fibers are integrated in the following descriptions. It should also be noted that after establishment of initial synaptic contact, there is considerable overlap in the stages of differentiation present at each embryonic age. In later stages when some junctions are maturing, others are continuing to form and are more immature.

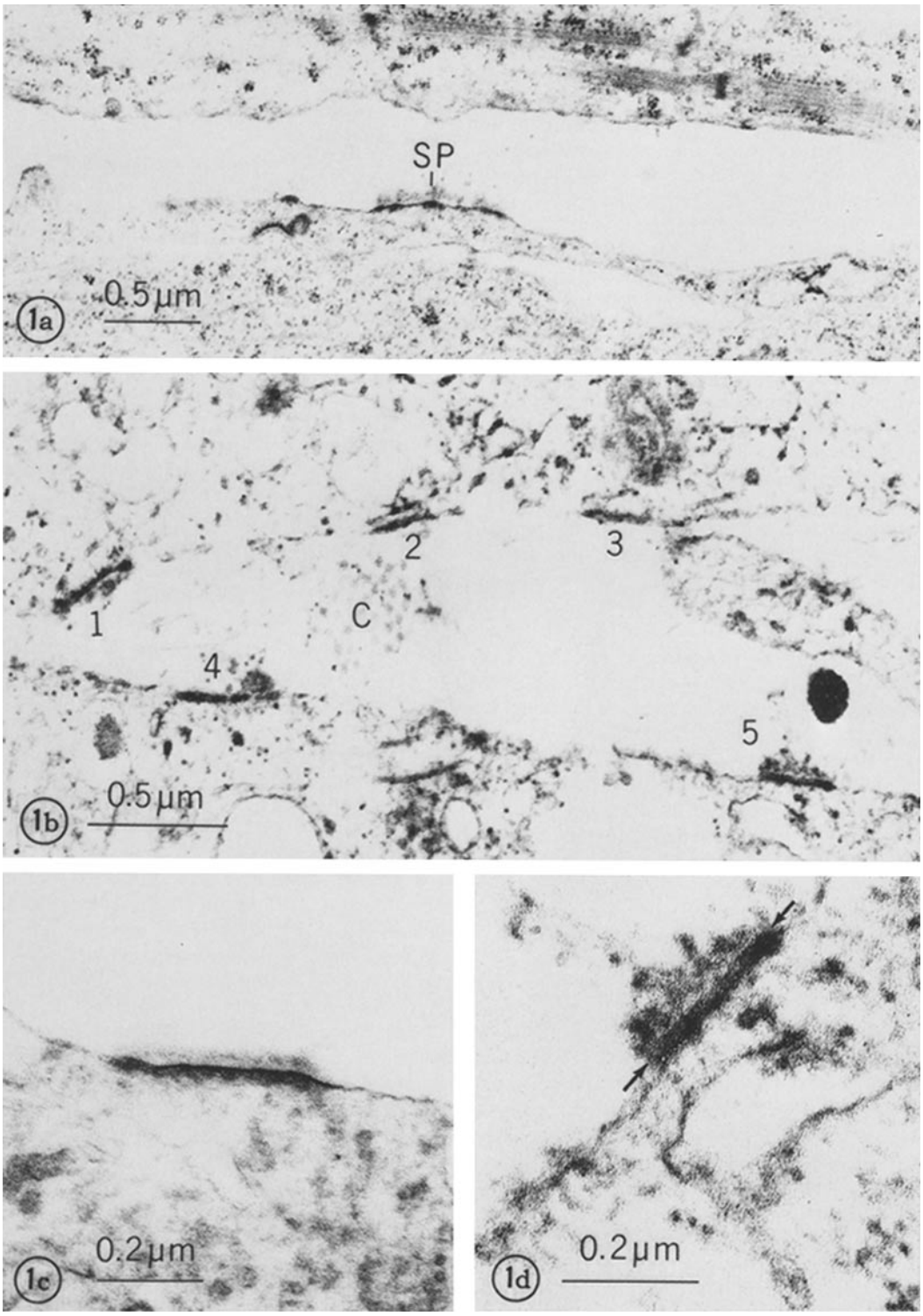
Uninnervated Surfaces of Myoblasts and Myotubes

The early chick embryo LD (10–14 d) consists of myoblasts and myotubes. The cytoplasmic constituents revealed by the plane of section make classification of the precise stage of differentiation of some cells difficult. Many of the cells appear immature but it is not always clear whether they are individual myoblasts or are in the process of fusing with adjacent myotubes. Because the identity of some cells is uncertain, they are referred to as myoblast-like cells. Other cells containing myofibrils are recognizable as myotubes. The profiles of many of the myoblast-like cells and myotubes do not have nerves associated with them and are referred to as uninnervated surfaces. However, we cannot rule out the possibility that these cells are innervated at some other site not revealed by the plane of the section.

The surface of myogenic cells is coated to a variable extent by amorphous-to-fuzzy material extending into the intercellular space (Figs. 4*a* and 8*a*). The texture and location of this material indicate that it is the precursor of the basement membrane, the glycoprotein-containing coat found over the sarcolemma of mature muscle fibers. Although previously reported to be lacking in myoblasts and to first appear on the myotube surface (26), a sparse coating can be observed on the plasmalemmas of most myoblasts and myotubes in 10-d-old LD muscles. As noted previously (43), the amorphous extraneous material occurs diffusely over the surface, forming a discontinuous poorly organized layer. At the earliest stages, it is patchy and separated by wide regions of the surface devoid of material. In maturing myotubes, it becomes more distinct and continuous.

In addition to the sparse diffuse coating, small local specializations are found on the uninnervated surfaces of myogenic cells in the early chick embryo (Figs. 1 and 8*b*). These regions which are termed surface patches are 0.1–0.4 μm in width and have an external coating of amorphous-to-fuzzy or very finely filamentous material. This material either extends directly from the sarcolemma into the intracellular space or is separated from the membrane by a thin clear space. Within the cytoplasm, a thin layer of moderately dense material occurs subjacent to the inner surface of the membrane and is coextensive with the outer surface coating. In addition, some of the patches appear as small elevations or ridges on the cell surface. The patches are distinguished from the developing basement membrane by their submembranous density and more highly developed extraneous coating.

Incubation of early chick embryo LD in HRP-α-Btx followed by the HRP reaction reveals activity associated with the surface patches of myoblast-like cells and myotubes of 10-, 12-, and 14-d-old chick embryo PLD and ALD muscles (Fig. 1). Reaction product occurs within the plasma membrane of these regions, increasing its density. The intensity of reaction, however, is not so great as in mature junctions, possibly indicating a lower den-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sity of receptors. Significantly, in some cases, reaction product occurs in a thick uniform layer which includes and overlies the plasma membrane (Fig. 1 *d*). This layer is similar to the uniform band of reaction product on the tips of the junctional folds of the mature NMJ (reference 42, see below) and on the postsynaptic surface of central synapses (41). Besides the membrane, the extraneous coating and submembranous material of these regions also appears increased in density in some cases. The sarcolemma outside the patches and the diffuse external surface layer where present in these early embryonic stages are unreactive (Fig. 1).

Establishment of Initial Synaptic Contact

The LD nerve of the superior brachial plexus emerges from the spinal cord at the level of the last three cervical segments to innervate the anterior and posterior branches of the LD muscle (38). Aggregates of naked or loosely and incompletely Schwann cell-enwrapped processes are observed between myogenic cells of 10-d and older chick embryos. The most characteristic feature of the immature nerve fibers is their dark appearance due apparently to the density of the axoplasm (Fig. 3). This density, which is of unknown nature and may be a fixation artifact, tends to obscure the organelles or cause them to appear in negative contrast. However, most fibers contain a few microtubules, elements of the smooth endoplasmic reticulum, mitochondria, and electron-lucent vesicles. In addition, many fibers contain a dilated protuberance at their periphery filled with membranous sacs and large vesicles (Fig. 3 *a*). These structures have been referred to as mound areas and occur in elongating axons and growth cones (13).

Some of the small immature nerve fibers or growth cones come into close contact with the surface of the muscle cells. The fibers may appear in section as a single small oval or round process (Fig. 3 *b*), a more elongated irregular profile extending along the muscle surface (Fig. 3 *a*), or enveloped by a Schwann cell (Fig. 2). It has been reported that in developing PLD, individual axons are completely ensheathed by Schwann cells, whereas in ALD the axons are naked initially (4). The processes are separated from the muscle surface by a regular cleft 700–800 Å in width.

The sarcolemma in the region of nerve-muscle contact is characterized by the presence of surface patches (Figs. 2 and 3). These structures are invariably present at or nearby the point of contact. After the establishment of contact, the patches become more numerous and coextensive within the region of the synapse and its immediate surroundings, forming a basement membrane layer (Figs. 3 and 4). While innervation is occurring, the extraneous layer is most prominent at the vicinity of contact and is poorly developed in other regions such as the opposite surface of the muscle fiber (Figs. 3 and 4 *b*).

After incubation in the HRP-α-Btx conjugate, activity is found at the region of contact in the plasma membrane of the surface patches (Fig. 2). Activity is more prominent at those junctions where the patches are more numerous (Fig. 3). In some regions, reaction product occurs in the characteristic thick band which includes the sarcolemma and thin overlying layer (Fig. 3, *inset*). After innervation, reactive surface patches become less numerous and disappear from extrajunctional regions of the cell surface. In addition to postsynaptic activity, there appears to be some activity

FIGURE 1 Reactive surface patches on chick embryo LD cells after HRP-α-Btx incubation and HRP reaction. (*a*) Surface patch (*SP*) on a 10-d LD myotube. Amorphous-to-fine textured material covers the plasma membrane of the patch and extends into the intercellular space. Dense material is applied to the inner surface of the membrane. Elsewhere, the cell surfaces are unspecialized and the basement membrane is absent or poorly developed. Activity is present in the plasma membrane of the patch and extends into the underlying material. (*b*) A series of reactive patches (*1–5*) on the surfaces of two myotubes in 12-d-old PLD muscle. The inner and outer surfaces of the patches are lined to a variable extent by fuzzy material. Activity occurs in the plasma membrane of the patches. *C*, collagen. (*c*) Reactive surface patch on a myogenic cell of 14-d-old PLD. The external surface of the densely reacted region of sarcolemma is coated by fuzzy material. An accumulation of material occurs on the cytoplasmic surface of the membrane as well. Reaction product is localized to the plasma membrane of the patch. The material on the inner surface of the membrane also is increased in density. Adjacent uncoated plasma membrane is much less dense. (*d*) Higher magnification of patch No. 5 in *b*. Reaction product is present as a thick uniform layer (between arrows) overlying the portion of plasmalemma coated by the extraneous material. The latter is enhanced in density also. (*a*) × 29,000; (*b*) × 42,000; (*c*) × 80,000; (*d*) × 102,000.

FIGURE 2 Early stage of innervation of 10-d-old chick embryo LD muscle after HRP-α-Btx incubation and HRP reaction. A small immature nerve fiber (*NF*) completely enveloped by a Schwann cell (*SC*) comes into close proximity to the surface of a myoblast. The axoplasm of the fiber is dense. Small reactive regions (arrows) occur on the surface of the myoblast at the region of contact of the nerve-Schwann cell complex with the cell surface. Reactive patches are absent on other regions of the myoblast surface. The myoblast is undifferentiated and contains large numbers of free ribosomes. It has begun to fuse with an adjacent myotube containing myofibrils (*Mf*) below. *N*, nucleus. × 15,000.

detectable in the presynaptic axonal membrane at an early state (Fig. 3). This activity occurs in small localized regions of the axolemma where the axon is closest to the muscle surface.

Development of the NMJ

As the NMJ develops, the zone of synaptic contact lengthens (Figs. 5 and 6). Schwann cells are more consistently and closely associated with the nerve terminals and cover their outer surfaces. The terminal is increased in size and its axoplasm becomes electron-lucent. The 500 Å synaptic vesicles increase greatly in number as the organelles of the immature stages are replaced by structures characteristic of the mature NMJ. Both ALD and PLD muscles may have multiple axon profiles on their developing surfaces (4).

Nerve terminals are separated from the muscle surface by a relatively constant synaptic cleft of 700–800 Å. A well developed layer of filamentous material occupies the synaptic cleft and is continuous laterally with the basement membrane of the muscle fiber. The latter becomes more extensive and covers the entire muscle surface. At the NMJ, the contour of the sarcolemma becomes broadly undulated and the small ridges of the surface patches disappear (Fig. 5). Junctional folds occur largely after hatching, but the secondary synaptic clefts formed as a result are shallow and few in number (Fig. 7).

Activity after incubation in the conjugate progressively increases in intensity beyond the low levels present at the earlier developmental stages (Figs. 5 and 6). Activity which was confined earlier to patches becomes coextensive to form a continuous reactive band on the postsynaptic surface. At first, the zone of activity extends well beyond the immediate region of nerve-muscle contact (Fig. 4). At later stages, reaction product does not extend as far beyond the zone of contact which itself is enlarged (Fig. 6), and in the adult chicken it is largely restricted to the immediate synaptic surface of the NMJ. When junctional folds are forming, activity occurs on the upper portions of the folds and is absent in membranes lining the bases of the secondary synaptic clefts (Fig. 7). Postsynaptic reaction product occurs in a uniform thickened layer which includes the plasma membrane. In addition, densely packed granular deposits of reaction product occupy the synaptic cleft in intensely reacted specimens (Figs. 6 and 7). The origin of this activity is unknown, but diffusion of reaction product from the postsynaptic surface or trapping by the fibrous intercellular material has not been ruled out (42).

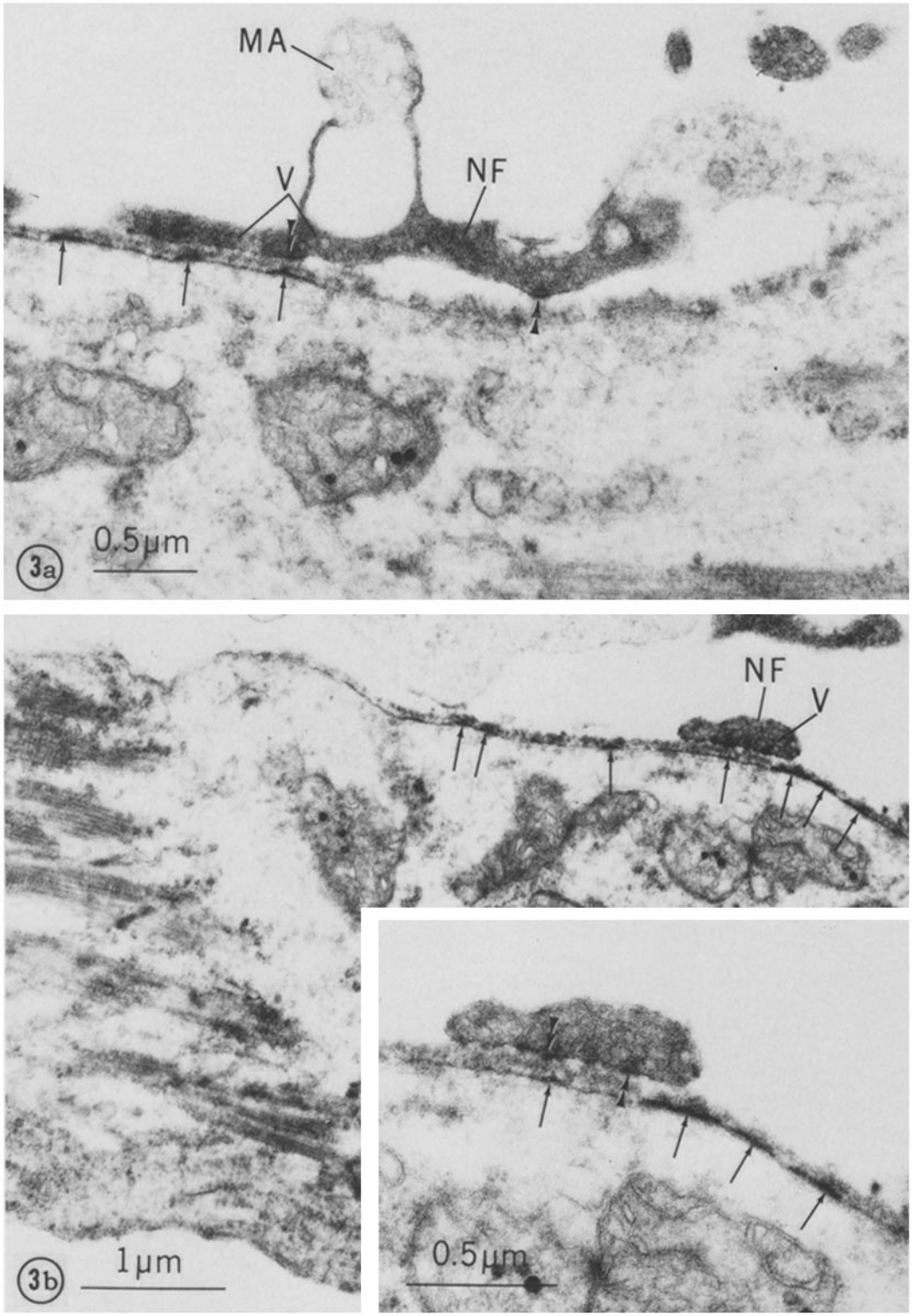
Activity also occurs in the presynaptic axonal membrane (Figs. 5–7). It is more prominent on the surface of the terminal facing the muscle fiber. The lateral and outer terminal surfaces usually are less reactive, although activity can be detected in them. Reaction product occurs in patches separated by unreactive membrane at earlier stages (Fig. 5) and over wider areas of the presynaptic surface at later stages (Figs. 6 and 7). The label is confined to the axolemma and does not form a thick layer as on the postsynaptic surface. Schwann cell membranes including those immediately adjacent to reactive pre- or postsynaptic surfaces are unreactive (Figs. 6 and 7).

Control Reactions

After incubation in avian Ringer's and reaction for HRP, the surfaces of myogenic cells including patches (Fig. 8 *a* and *b*) and the pre- and postsynaptic surfaces of the NMJ are unreactive, demonstrating the absence of endogenous peroxidase activity at these sites. Some intracellular lysosomes are reactive due to their endogenous peroxidase activity. In some preparations, mitochondria, nuclear chromatin, and as noted previously early nerve terminals are enhanced in density after the DAB reaction and osmium tetroxide fixation. The density of surface patches and synaptic surfaces is not increased by incubation of muscles in HRP, indicating that HRP does not bind nonspecifically to these regions. Tissues preincubated in native α-Btx to block nicotinic AChR before exposure to the conjugate are unreactive (Fig. 8 *c*). Preincubation in DTC to competitively inhibit HRP-α-Btx binding is found to result in an absence or a decrease in activity (Fig. 8 *d*). In all control preparations, surface patches and junctional folds have some inherent density due mainly to the submembranous cytoplasmic material. However, the density of these regions is enhanced after incubation in the conjugate and reaction for HRP.

DISCUSSION

The localization and distribution of AChR in the developing ALD and PLD muscles of the chick embryo have been studied by HRP-α-Btx labeling (Fig. 9). Initially, AChR are found to be present in small regions or patches of plasmalemma unrelated to nerve fibers in myoblast-like cells and myotubes of 10, 12, and 14-d-old chick embryos. No activity is detected with this procedure on the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remaining surface of the myogenic cells. It is not known with certainty whether these cells are uninnervated, as there could be contact with nerve in a plane not revealed by the section. Localized surface specializations are associated with the AChR accumulations. Elsewhere, only the less well organized basement membrane is present in various stages of formation on the outer surface of the myogenic cell plasmalemma. Other studies utilizing markers attached to α-Btx have not shown structural differences between regions of high and low receptor density in myotubes (35, 40, 57).

Clusters of AChR or "hot spots" are present on the surface of noninnervated myotubes in primary culture (5, 9, 22, 25, 40, 48, 55). The patches of high receptor density observed in the present study are considerably smaller than those detected by microiontophoretic techniques or labeled α-Btx at the light microscope level. Hot spots have been reported to be ~5–60 μm in diameter (3, 5, 9, 40, 48). This difference could be accounted for by the observation that the hot spots are actually compound structures composed of many smaller lines or spots (3). Thus, it is possible that groups of the small patches observed in the present study (Fig. 1 *b*) might represent the larger hot spots detected at the light microscope level. Alternatively, differences in the size of AChR clusters could be a reflection of differences in in vivo and in vitro conditions.

In the present study, reactive patches were observed on relatively undifferentiated myoblast-like cells in addition to myotubes. Areas of high AChR density have not been observed on myoblast surfaces in other investigations. In fact, little or no labeling is seen on the surface of myoblasts after application of a variety of techniques: fluorescently-conjugated α-Btx (5), radiolabeled α-Btx (40, 48, 55, 56), and electrophysiological determinations of ACh sensitivity (23, 40). However, some internal labeling has been observed (56). It is possible that the immature myoblast-like cell profiles observed in this study to have small patches of high AChR concentration are cells in the process of fusing with adjacent myotubes not visible in the plane of section. Alternatively, the patches of AChR may appear on the surfaces of myoblasts but are too small or widely separated to be detected by the other techniques.

In the present study, localized surface specializations are consistently observed in regions of the plasmalemma containing a high density of AChR. An extraneous coating is present on the outer surface of the plasma membrane and a thin layer of dense material is applied to the inner surface. Similarly, in the mature NMJ, a well developed basement membrane occupies the synaptic cleft and a dense matrix on the inner surface of the sarcolemma of the tips of the junctional folds is coextensive with the highest concentration of AChR (24). The presence of specializations associated with the membrane at regions of high receptor density suggests that they may be related to the mobility or positioning of receptors.

Two populations of AChR characterized by different diffusion rates are found to exist (5). AChR in areas of low density, diffuse distribution move relatively freely in the membrane while high density patches of AChR are predominantly immobile (5). In addition, the receptors at the postsynaptic surfaces of mature synapses are stable (15, 22, 28).

Considering the generalized cell surface, receptors are thought to be integral glycoproteins which

FIGURE 3 Immature neuromuscular contacts along the surface of 10-d-old embryo LD myotubes incubated in HRP-α-Btx and reacted for HRP. (*a* and *b*) Nerve fibers (*NF*) are situated along the surface of myotubes. The axoplasm of these immature fibers is dense, obscuring the organelles; however, synaptic vesicles are present in negative contrast. The upper process (3 *a*) bears a mound area (*MA*) identifying it as a growth cone. These fibers are not ensheathed by Schwann cells. The fibers are separated from the muscle surface by a cleft 700 Å in width. A layer of fibrillar material occupies the cleft. It is best developed in the region of contact and extends beyond it somewhat. It is less prominent at greater distances from the contact and is largely absent on the opposite surface of the myotube (3 *b*). Reaction product (arrows) occurs in patches over the sarcolemma. The reactive patches are present in the immediate area of contact and extend beyond it but become less common at increasing distances. The patches are identical to those seen on uninnervated surfaces of myogenic cells. In some places, the reaction product forms a uniform layer of greater thickness than the membrane (*inset*). There is also increased density presynaptically in small patches of the axolemma facing the postsynaptic surface (double arrowheads). Fig. 3 *b* inset is a higher magnification of the nerve terminal in Fig. 3 *b*. (*a*) × 32,000; (*b*) × 22,000; (*inset*) × 46,000.

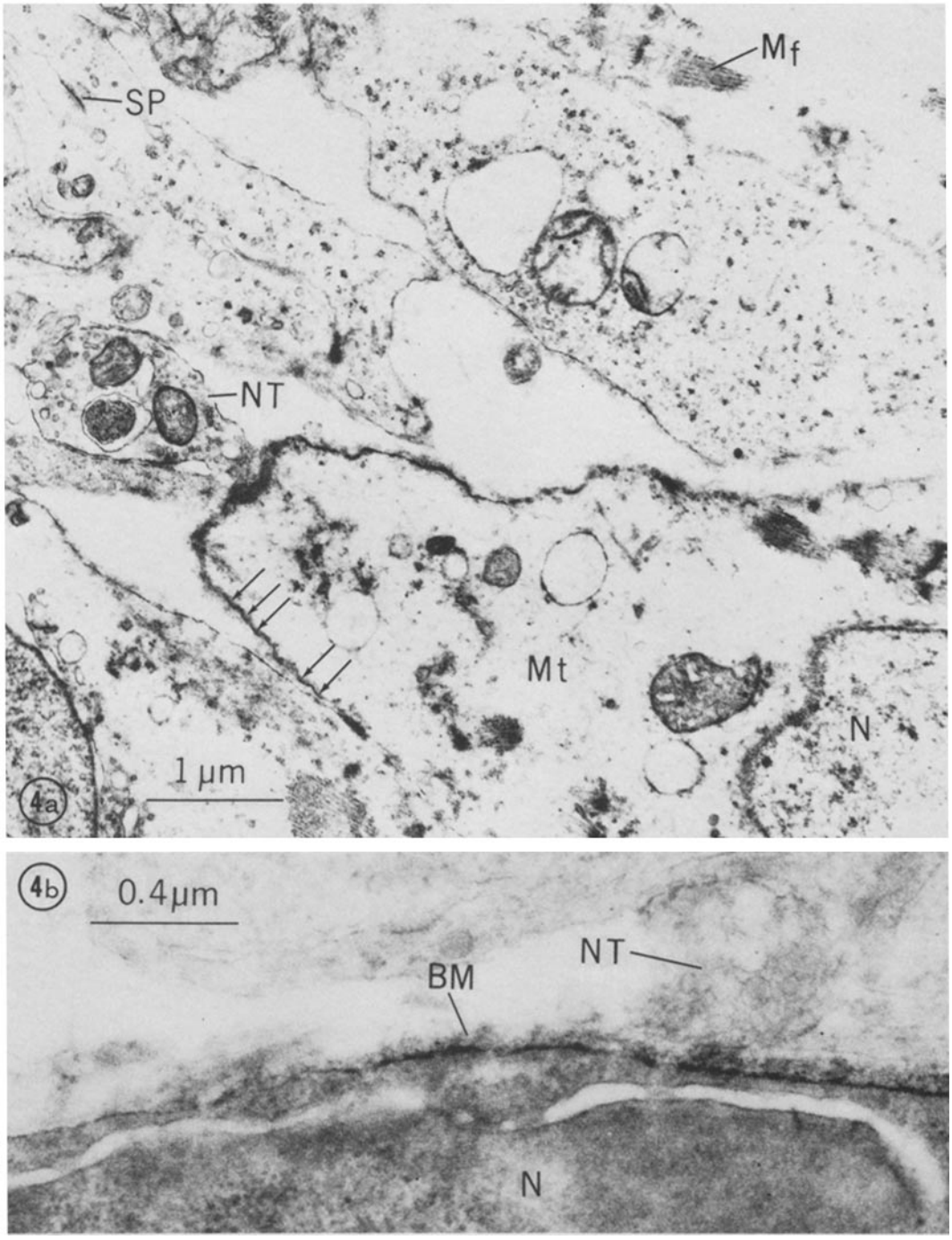


FIGURE 4 (*a*) Maturing nerve fiber in contact with the surface of a 12-d-old chick embryo PLD myotube reacted with HRP-α-Btx. The nerve terminal (*NT*) containing a few synaptic vesicles closely approximates the end of a myotube (*Mt*) with myofibrils (*Mf*). The surface of the contacted myotube is reactive. The zone of activity extends considerably beyond the point of contact and includes the entire pole of the cell. In one region, portions of the reactive membrane are elevated and appear as small ridges along the cell surface (arrows). A coating of material occurs on the outer surface of the membrane in the reactive region. On other myotubes and myogenic cells in the field, the degree of development of the basement membrane is variable, present in some areas and sparse or discontinuous in others. A surface patch (*SP*) is present on a myogenic cell in the upper left. *N*, nucleus. (*b*) Nerve terminal (*NT*) containing synaptic vesicles in contact with the surface of a 17-d-old embryo ALD myotube. Incubated in HRP-α-Btx and reacted for peroxidase activity. Reaction product forms a continuous band along the postsynaptic surface and extends beyond the immediate point of contact. The basement membrane (*BM*) is more uniform and regular over the reactive region. Where activity is absent to the left, the basement membrane is less well developed or absent. *N*, nucleus. (*a*) × 20,000; (*b*) × 54,000.

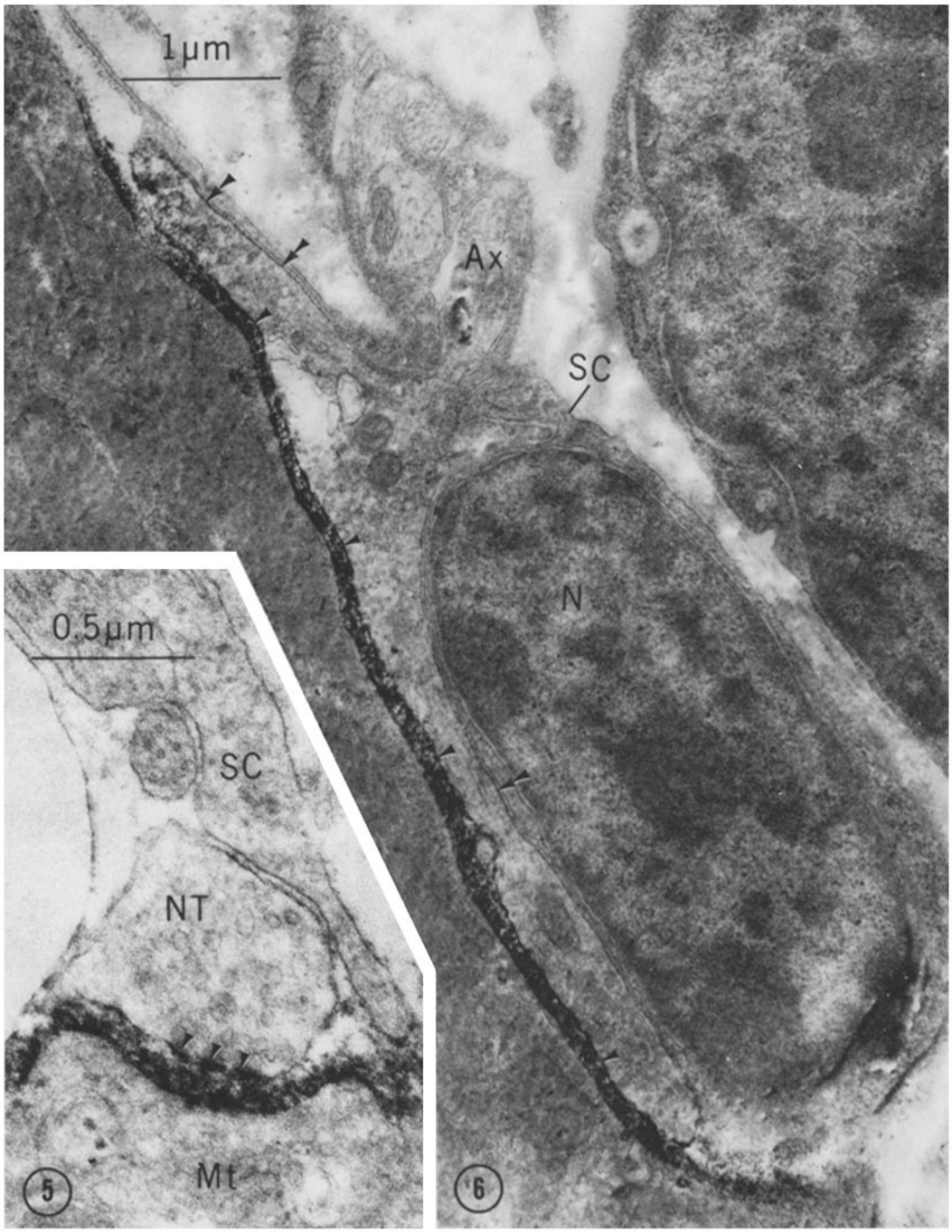


FIGURE 5 Nerve terminal (*NT*) in contact with a 16-d-old chick embryo ALD myotube (*Mt*) after HRP-α-Btx incubation and HRP reaction. The axoplasm is low in density and synaptic vesicles are apparent. The nerves are partially enveloped by Schwann cells (*SC*). Postsynaptically, reaction product forms a continuous band along the sarcolemma. Granular reaction product is present in the synaptic cleft. The postsynaptic surface is thrown up into broad folds. Activity also occurs on the presynaptic surface in small dense patches (arrowheads). × 47,000.

FIGURE 6 NMJ of 19-d-old chick embryo ALD muscle incubated in HRP-α-Btx and reacted for peroxidase activity. An axon (*Ax*) forms a broad, vesicle-filled terminal expansion on the myofiber surface. A Schwann cell (*SC*) completely covers the outer surface of the terminal. Heavy accumulations of reaction product occur at the neuromuscular contact. The postsynaptic membrane is labeled and dense granular deposits occur in the synaptic cleft. Postsynaptic activity extends a short distance beyond the immediate zone of contact. Activity also occurs presynaptically in the axolemma. It is most intense in the portion

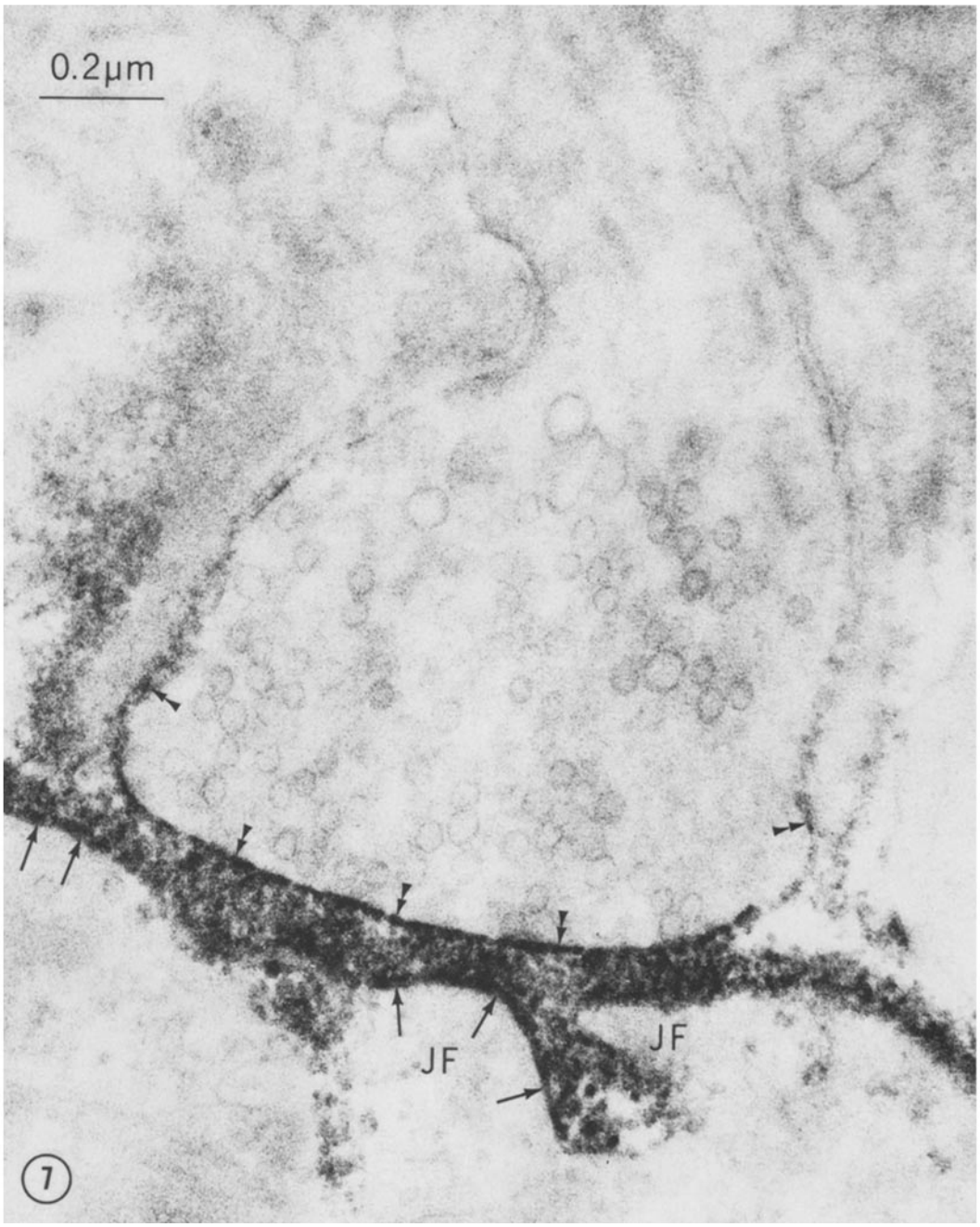


FIGURE 7 HRP-α-Btx reacted NMJ from 3-wk-old chicken ALD muscle. A few junctional folds (*JF*) are present in the mature junction. A thick layer of reaction product occurs on the upper and lateral surfaces of the folds (arrows). Granular reaction product deposits are present in the synaptic cleft. Reaction product also occurs on the axolemma (double arrowheads) facing the muscle surface and less intensely on the lateral surface of the terminal. × 87,000.

span the entire lipid bilayer matrix (21, 46). This establishes the potential for interactions at both the inner and outer leaflets of the membrane to influence the mobility and topographic distribution of the surface receptor (8, 21, 46, 51, 60). These interactions could take place with adjacent submembranous filamentous and tubular structures such as microfilaments or microtubules and/or the extraneous filamentous coating. The possibility of such mechanisms for controlling receptor mobility and distribution is supported by investigations of the ligand-induced formation of patches and caps on the surface of lymphocytes (21, 60), ovarian granulosa cells (1), and polymorphonuclear leukocytes (34). It is possible, therefore, that interaction of diffusely localized mobile AChR with specializations of the surface patches could be responsible for the localization of a higher concentration of receptors at these sites by anchoring receptors and restricting lateral diffusion.

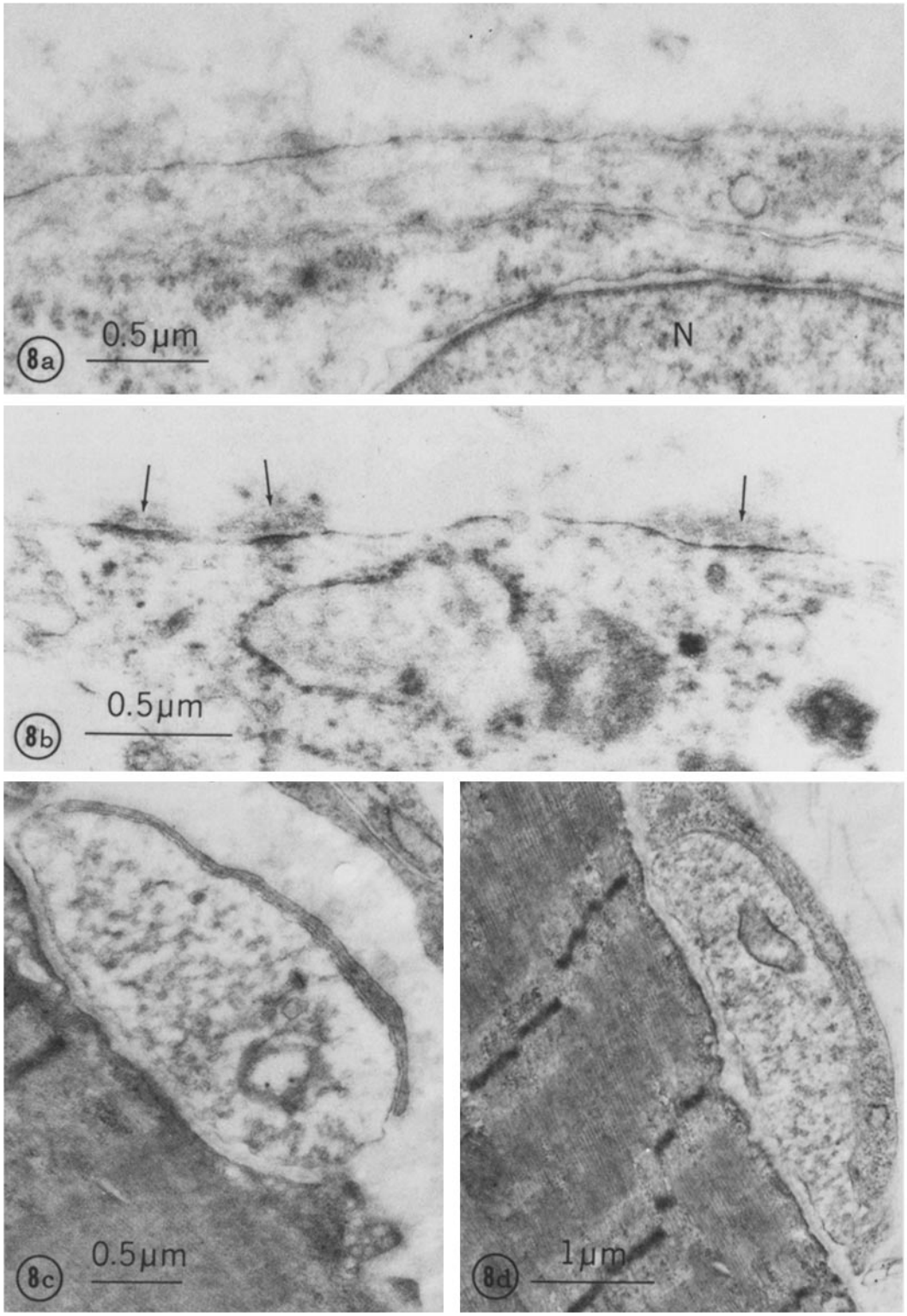
The extent and distribution of AChR on the surface of developing myogenic cells is influenced subsequently by the presence of nerves. Innervation results in an accumulation of AChR in the region of nerve contact (2, 3, 9, 10). In the present study, reactive surface patches were more numerous in the region of the innervating nerve. Subsequent formation of a continuous band of activity at and beyond the region of nerve-muscle contact was paralleled by the coalescence and enlargement of the morphologically specialized regions. At the same time, reactive patches disappeared elsewhere on the surface. Burden (14) has also observed the establishment of a spot of high density AChR in chick embryo PLD myotubes. That the nerve plays a role in the establishment and stability of these regions of high receptor density is further evidenced by the disappearance of clusters of receptors in myotubes maintained for long periods of time in culture in the absence of innervation (48). Subsequent development in the chick consists of a restriction of the reactive band to the immediate region of nerve-muscle contact, increased intensity of reaction, and, with the appearance of junctional folds after hatching, further restriction of sarcolemmal activity to the upper portions of the junctional folds. At all stages of development, regions of high density of AChR are characterized by the specializations external and internal to the membrane (Fig. 9), suggesting that they play a role in the binding and stabilization of AChR. If the membrane specializations function in this manner, it seems likely that they could bind both newly synthesized receptors and mobile receptors diffusing from extrajunctional regions.

The manner in which the nerve induces muscle to synthesize additional receptors or the specializations which may affect receptor localization and stability is unknown. Recently, soluble extracts of nerve tissue have been found to influence the number and distribution of AChR on myotubes (17, 37, 47). Thus, it is possible that these processes are influenced by a soluble trophic factor released by nerves.

A final question to be discussed concerns the relationship of the regions of high receptor density to the site of innervation. The presence of external specializations at these regions and the consistent localization of the patches at sites of nerve-muscle contact make it attractive to hypothesize that these regions are somehow recognized by the nerve and represent preferential sites of synapse formation. We cannot rule out, however, the alternative possibility that the patches in the region of nerve-muscle contact are induced by the presence of nerve or appear subsequent to contact. It is clear that if the nerve makes contact with a preexisting patch, it induces others to form since large groups of patches like those at sites of nerve-muscle contact were not seen on uninnervated surfaces.

In general, carbohydrate moieties covalently linked to either protein or lipid on the cell surface could function as unique and recognizable entities in cellular interactions (6, 31, 53). In a number of cell types, specific surface glycoproteins have been observed to function as mediators of cell recognition and selective adhesiveness (6, 33, 61) or fusion (6, 21, 27). Glycoproteins have been identified in the postsynaptic membrane (39) and density (31) of central synapses. External plasma membrane glycosyltransferases may also function in specific cell interactions by binding to their naturally occurring substrate (acceptor) on a neighboring cell surface (52). A number of glycoprotein:glycosyltransferases have been identified as components of synaptosomal plasma membrane fractions isolated from the central nervous system and have been proposed to function in cell recognition and selective adhesion during synaptogenesis (12, 30).

The fact that the AChR is a glycoprotein (15, 50) and that patches of high AChR concentration are consistently observed in the present work to be localized at or near the region of contact suggest that these AChR accumulations may play an essential role in initiating or guiding synapse formation by the growing motor axon. On the other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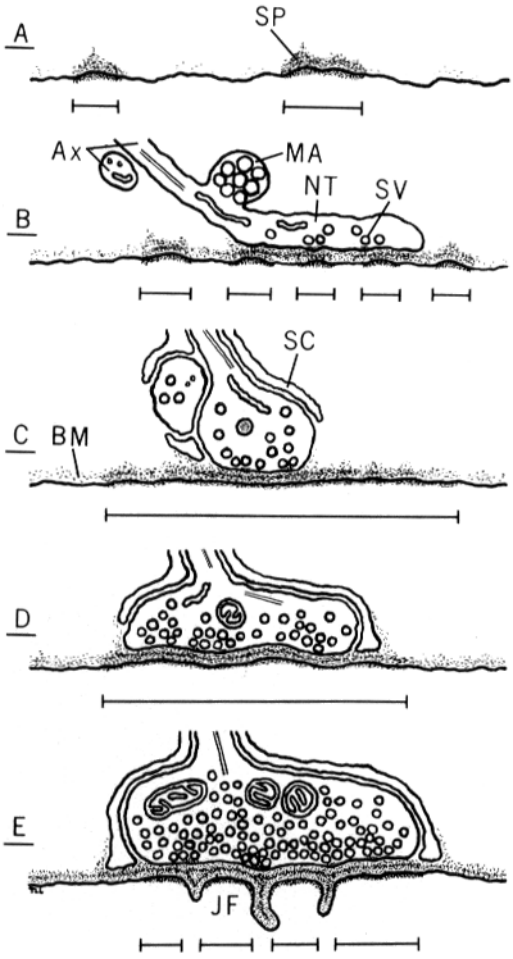

hand, functional synaptic connections have been reported to form in the absence of dense AChR accumulations as on the surface of myotubes possessing a uniform AChR distribution (40). Furthermore, the presence of physiologically active AChR is not required for the establishment of nerve muscle contact because it occurs as rapidly and extensively as normal in the presence of suf-

FIGURE 9 Diagram summarizing changes in distribution of AChR during development of the NMJ in the chick embryo as demonstrated by HRP-α-Btx staining which reveals high density accumulations of receptors. The regions occupied by AChR as demonstrated with this procedure are indicated by bars. Initially, AChR occur on the myogenic cell surface in widely distributed, small patches (*A*). The patches are morphologically specialized and consist of a slight surface elevation or ridge, external amorphous material, and submembranous dense material. Growth cones and developing nerve terminals make contact with the myotube surface (*B*). Patches containing AChR are present at these sites and are more numerous in the vicinity of contact while decreasing elsewhere on the cell surface. Coalescense of reactive regions produces a broad zone of AChR localization extending beyond the point of nerve-muscle contact (*C*). As the junction differentiates, the zone of AChR is increasingly restricted to the zone of contact (*D*). In the mature junction, AChR are localized largely to the immediate region of contact and occur on the upper portions of junctional folds (*E*). *Ax*, axon; *BM*, basement membrane; *JF*, junctional fold; *MA*, mound area; *NT*, nerve terminal; *SC*, Schwann cell; *SP*, surface patch; *SV*, synaptic vesicle.

FIGURE 8 Control preparations. (*a*) Surface of a 10-d chick embryo LD myogenic cell incubated in avian Ringer's alone and reacted for HRP activity. The surface of the cell is covered by an irregular matting of amorphous material extending for variable distances into the intercellular space. No reaction product is present. *N*, nucleus. (*b*) Surface patches on a 12-d-old embryo PLD myogenic cell incubated in avian Ringer's alone and reacted for HRP. Extraneous material (arrows) occurs in localized patches along the cell surface. A much sparser coating occurs elsewhere. In addition, moderately dense material is applied to the inner aspect of the plasma membrane of the patches. The membrane of these regions appears somewhat more dense than that of the adjacent uncoated surface. The areas associated with extraneous material are elevated and appear as small ridges on the cell surface. Accumulations of reaction product like those after incubation in the conjugate (Fig. 1) are absent. (*c*) NMJ of 3-d-old chicken ALD muscle preincubated for 1 h in native α-Btx followed by 2 h incubation in HRP-α-Btx and reaction for HRP. Prior incubation in native α-Btx results in the absence of reaction product deposits on the nerve and muscle plasma membranes in the region of neuromuscular contact. (*d*) NMJ of 3-d-old chicken ALD muscle preincubated in DTC for 1 h, incubated in the conjugate and DTC for 1 h, and reacted for HRP. Activity on the pre- and postsynaptic surfaces is blocked. (*a*) × 37,000; (*b*) × 45,000; (*c*) × 28,000; (*d*) × 19,000.

ficient DTC (19, 36), α-Btx (36), or α-neurotoxin (54) concentrations to block all functional AChR activity. However, the binding of toxin does not block all AChR carbohydrate moieties as concanavalin A and toxin binding occur simultaneously with no competition between them (50).

It is possible that some other specialization associated with the region of high AChR concentration on the muscle surface might function in establishing this area as a preferential zone of nerve contact (54). Some constituent of the external coating of the surface patches such as a glycoprotein would seem to be the most likely candidate for this function. A recent study has revealed that regenerating axons reinnervate disrupted phagocytized muscle fibers precisely at the portion of the still intact basement membrane formerly present in the synaptic cleft of the original NMJ (44). If the fibrous patches represent the sites of recognition on myotubes, it is to be noted that initially they are widely distributed over the cell surface and would allow for multiple contacts of nerve and muscle. Subsequently, one of these sites is stabilized while others regress and are terminated (see references 16, 49).

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