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Accumulation of Acetylcholine Receptors and Acetylcholinesterase at Newly Formed Nerve-Muscle Synapses

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THIS paper is a summary of some recent studies on the accumulation of acetylcholine receptors (AChR) and acetylcholinesterase (AChE) at newly formed nerve-muscle synapses in vitro. It is not an exhaustive review, but is simply intended to introduce some current questions concerning synapse formation to pharmacologists with a bent for cell biology.

I. AChRs and AChE at Adult Neuromuscular Junctions

Two important developments have provided new information about the function, the molecular structure, and the distribution of AChR within skeletal muscle membranes. First, analysis of ACh-induced membrane voltage and/or current fluctuations has shown that when receptors located in the postsynaptic membrane bind ACh, the associated ion channels open for about 1 msec (at room temperature), and, in that interval, about 50,000 ions pass through each channel (1, 44). This large flux cannot be accounted for by a carrier or shuttle mechanism: the energy of ACh binding must be translated into a conformational change that creates a transmembrane aqueous pore. Single channel events have recently been recorded directly (62). Second, the discovery of polypeptides (alpha toxins) within the venom of certain snakes (e.g., *Bungarus multicinctus, Naja nigricolis, Naja siamensis*) and of affinity alkylating reagents, i.e., 4-(N-maleimido)-benzyltrimethylammonium (MBTA), has led to the purification of receptors from electric fish and from skeletal muscle [see references in Heidmann and Changeux (40)]. Most estimates of the molecular weight of the receptor solubilized in nondenaturing detergents range between 250,000 and 300,000. The precise subunit structure of the receptor-ionophore protein complex is a matter of controversy, but it seems clear that ACh binds to a 40,000 dalton peptide. Recent reviews by Neher and Stevens (63), Heidmann and Changeux (40), and Fambrough (24) should be consulted for further detail.

AChRs are clustered immediately beneath motor nerve terminals in adult vertebrate muscles. The sensitivity of the synaptic gutter membrane to microiontophoretically applied ACh (assayed after the nerve terminals are stripped away) is more than 500-fold greater than the sensitivity of extrasynaptic membrane located a few micrometers away (48). Electronmicroscopic autoradiography has shown that (^{125}I)-α-bungarotoxin (α-BuTx) binding sites are packed at the top of the postjunctional folds at a density in excess of 20,000/μm^2 (26). This clustering is quite remarkable in light of the fact that, typically, the endplate represents less than 0.1% of the muscle surface. Indeed, synaptic receptor clusters were noted by Singer and Nicholson (81) as an

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apparent exception to their model of a fluid mosiac membrane, i.e., a uniform distribution of proteins in a sea of lipid.

A great deal has been learned about the location, molecular structure, and function of AChE from studies of adult motor endplates and eel electroplaques (10, 36, 45, 55, 56, 78, 79). AChE activity can be detected throughout the length of vertebrate muscles, but the enzyme is concentrated at neuromuscular junctions, and a high molecular weight form that is apparently unique to the endplate region of adult muscle has been identified by sucrose density centrifugation (36). This form sediments at about 16 S in the rat and 19 S in the chick. Although endplates contain approximately equal numbers of AChRs and AChE catalytic sites (5), the disposition of the two molecules is quite different. The AChR is an integral membrane protein that is concentrated at the tips of the postjunctional folds (26). AChE, on the other hand, is a peripheral membrane protein that is associated with the basal lamina throughout the primary and secondary synaptic cleft (8, 37, 57).

II. Clusters of AChRs

A. Synapse Formation in Vitro

We have examined embryonic muscle and spinal cord cells maintained in culture to determine how the clustering of AChRs comes about during the initial formation of nerve-muscle junctions. Most of our experiments are performed on chick tissue, but we have examined mammalian and amphibian cells as well. Techniques relevant to nerve and muscle cell culture and recent results have been summarized (29). Muscle cultures are prepared simply by removing a pectoral muscle or group of limb muscles from 11-day embryos, mincing them into small fragments, exposing the fragments to proteolytic enzymes, and then mechanically disrupting the digested fragments into a suspension of mononucleated cells. Soon after they settle on the culture surface, many of these cells divide, elongate, migrate over the culture surface, and fuse with one another to form multinucleated myotubes. The myotubes grow in size over the next several days and many become cross-striated and twitch spontaneously. Fibroblasts as well as myogenic cells are present in the initial cell suspension. They multiply rapidly and, if not checked, they overgrow the muscle fibers within 5 to 6 days. It is difficult to penetrate myotubes with microelectrodes in such overgrown cultures and it is impossible to visualize fine spinal cord nerve processes. The fibroblasts can be eliminated, however, by adding cytosine arabinoside (10^{-5} M), a drug that kills rapidly dividing cells, for a period of 24 to 48 hours before the fibroblasts become confluent.

Myotubes can be innervated in culture by dissociated spinal cord cells or by nerve processes that grow out of small but intact spinal cord fragments (explants). Individual uninnervated myotubes twitch on occasion. Innervated myotubes twitch more vigorously and more frequently, and several adjacent cells are usually activated in synchrony. All of the synapses that form in vitro are cholinergic: synaptic potentials are blocked by low concentrations of curare or α-BuTx, and they can be mimicked by iontophoretically applied ACh. At least some of the transmitter is released in discrete quantities (fig. 1); tetrodotoxin (TTX)-resistant miniature endplate potentials (m.e.p.p.s) can be detected in most recordings and the amplitude of stimulus-evoked endplate potentials (e.p.p.s) is adequately described by the binomial probability law. Spontaneous and stimulus-evoked synaptic potentials can be detected in myotubes adjacent to spinal cord explants within 24 hours after the explants attach. Most of this time is required for severed neurites to recover and emerge from the spinal cord fragment; some sign of synaptic transmission is usually evident within 3 hours of nerve-muscle contact. We have evoked transmitter release by stimulating stationary, but active, growth cones.

Synapses can be located precisely by focal extracellular stimulation in the presence

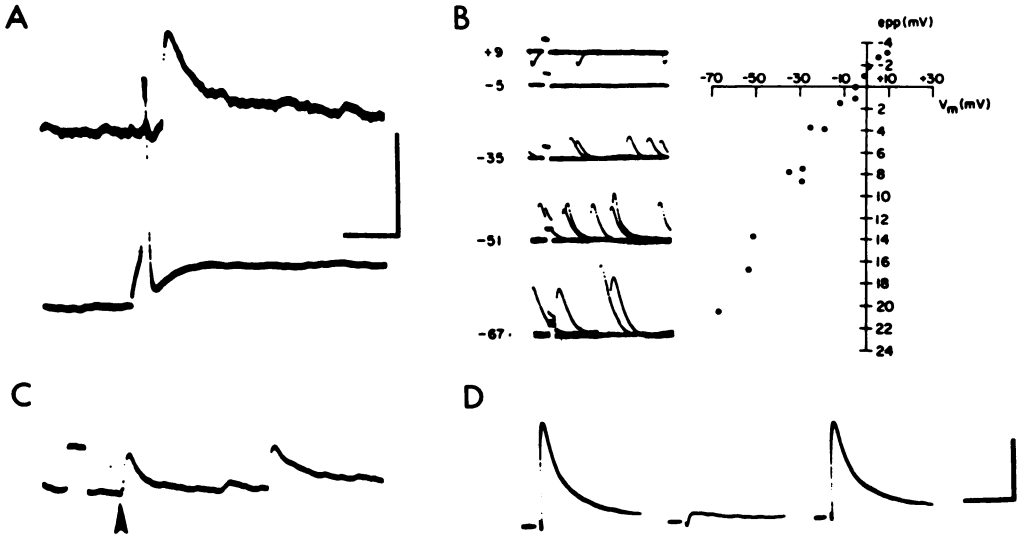

FIG. 1. Neuromuscular synaptic physiology. A. Functional contact—a depolarizing synaptic potential (upper trace) follows an action potential in a nearby neuron evoked by intracellular stimulation (current pulse not shown). Bars, 3.5 and 50 mV for muscle and neuron records, respectively, and 10 msec for both. B. Superimposed traces show the change in endplate potential (e.p.p.) amplitude as the muscle membrane potential (V_m) was altered to the values shown at the left of each record by steady currents injected through a second intracellular microelectrode. C. Acetylcholine (ACh) response (arrow) evoked by a 1-msec pulse of ACh precisely mimics a spontaneous synaptic potential recorded on the same trace; calibration, 5 mV, 10 msec. D. Reversible reduction in e.p.p. size by *d*-tubocurarine (10^{-7} g/ml); record at extreme right obtained 30 minutes after removal of the drug; bars, 15 mV, 15 msec. (From ref. 29.)

of TTX (to block propagated impulse activity) or by focal extracellular recording (20, 33, 34). Sites of transmitter release identified in this manner are not distributed all along the length of nerve-myotube contacts. Instead they are restricted to short segments along motor axons and are, in fact, rather difficult to locate. In every case examined, clusters of AChRs, evident as peaks of sensitivity to microiontophoretically applied ACh or as clusters of grains in autoradiographs of cultures exposed to (^{125}I)-α-BuTx, were found exactly at sites of transmitter release (fig. 2). A significant level of extrajunctional sensitivity persists on innervated embryonic myotubes in vitro and in vivo so the synaptic hot spots are relative peaks. This situation is quite different from that found on adult innervated fibers where the extrajunctional sensitivity is negligible.

B. Clusters on Uninnervated Myotubes

Do ingrowing motor nerves induce receptor clusters at sites of transmitter release? An interesting alternative explanation became apparent in the first experiments on uninnervated myotubes in vitro (27, 83, 84). These cells are sensitive to ACh over their entire length, but the distribution of receptors is not uniform. Sharp relative peaks of sensitivity and clusters of (^{125}I)-α-BuTx grains virtually identical to those found on innervated fibers can be located on myotubes that have never been exposed to neurons (fig. 3). Thus it seemed possible that hot spots were located at synapses because motor axons sought out and innervated preexisting clusters of receptors. The suspicion that hot spots on virgin myotubes are preassembled sites of synapse formation was based mainly on their very existence, but also on the general rule that regenerating adult motor axons grow back to the site of highest receptor density on denervated muscle—the vacated end plate (51).

The appearance of hot spots in vitro does not depend on prior exposure to motor nerves in ovo (7). They are not unique to cultured chick myotubes; they also occur

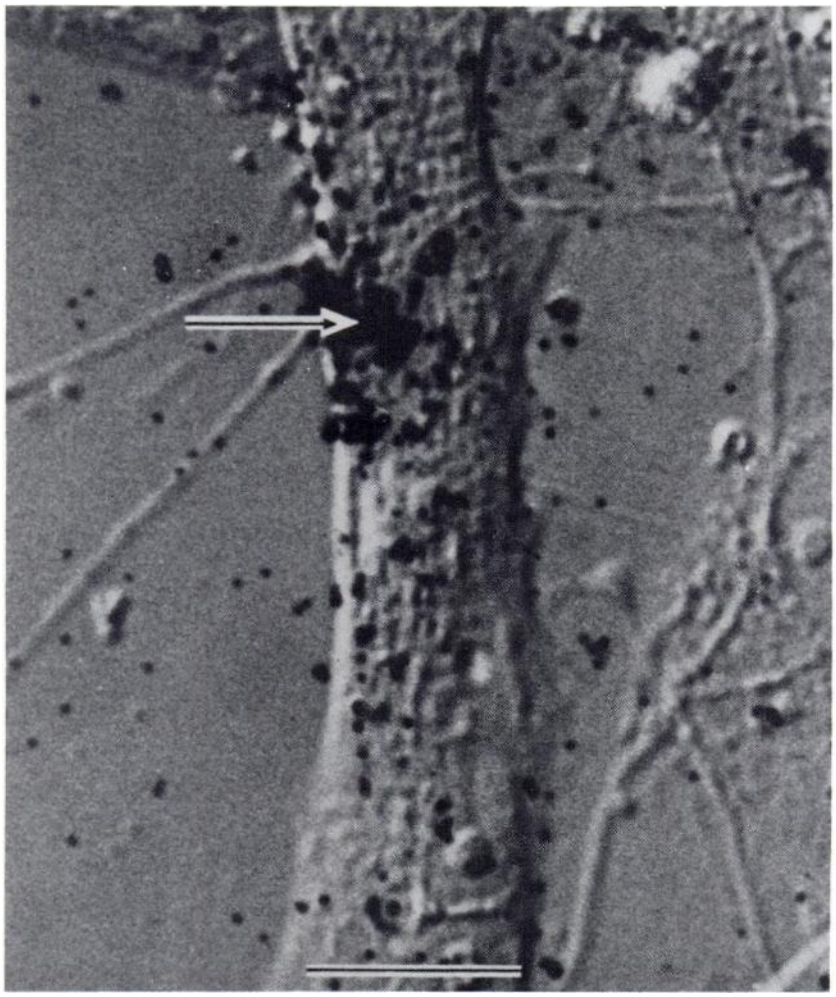

FIG. 2. Distribution of acetylcholine receptors (AChRs) on an innervated muscle fiber in vitro. Muscle cells were plated 12 days earlier; a 14-day spinal cord explant was added 4 days after plating. The synapse (arrow) was located by recording spontaneous synaptic currents with an extracellular pipette (spatial/resolution 2 to 5 μm). The culture was then incubated in 14 nM [^{125}I]-α-bungarotoxin (α-BuTx) (113 cpm/fmole) for 1 hour at 37°C, washed, fixed, coated with NTB3 emulsion, and exposed for 9 days at 4°C. AChR density (as revealed by silver grain density) is much higher in the immediate vicinity of the synapse than in extrasynaptic regions. Scale, 20 μm. (From ref. 32.)

on uninnervated rat (4, 49) and mouse (17) myotubes and on *Xenopus* monocytes (3). Myotubes derived from transformed (L6) rat myotubes are different in that the distribution of receptors is more or less uniform (49, 82). Hot spots are not unique to cultured muscle cells; they have been found recently at extrasynaptic sites on denervated adult muscle fibers (46). Thus, it may be that receptors cluster in all muscle membranes deprived of innervation. Multiple hot spots have not been found in autoradiographs of (^{125}I)-α-BuTx treated embryonic muscle fibers (9, 13). However, small clusters might be missed on the light microscopic level. Moreover, all of the fibers examined may have already been innervated.

Hot spots on uninnervated chick myotubes are associated with clusters of intramembranous particles (21, 86, 87). Large areas of membrane are exposed when myotubes are freeze-fractured in situ on the

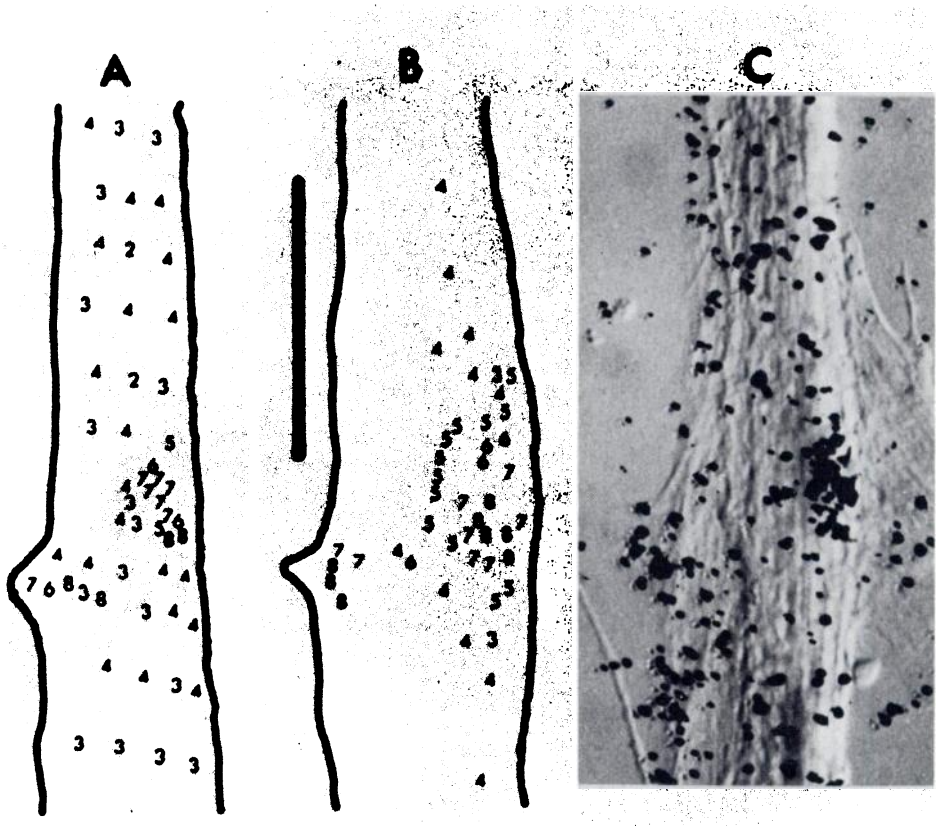

FIG. 3. A cluster of acetylcholine receptors (AChR) on an uninnervated myotube. The ACh sensitivity was mapped and each small number in A and B represents the sensitivity of the myotube to ACh at that point. The scale is: 1, 10–22 mV/nc; 2, 22–46 mV/nc; 3, 46–100 mV/nc; 4, 100–220 mV/nc; 5, 220–460 mV/nc; 6, 460–1,000 mV/nc; 7, 1,000–2,200 mV/nc; 8, 2,200–4,600 mV/nc; 9, 4,600–10,000 mV/nc. This uninnervated myotube was blocked with unlabeled/α-bungarotoxin (α-BuTx) and then remapped 24 hours later (B). After the second map was made, the culture was incubated in (^{125}I)-α-BuTx and processed for autoradiography. There is a good correspondence between ACh sensitivity and grain density. An additional finding in this experiment is that hot spots remain fixed in position even though individual receptor molecules within the cluster were rapidly metabolized. The reappearance of sensitivity after exposure to cold α-BuTx is due to the synthesis and insertion of new receptors. (From ref. 34.)

glass coverslip and it is possible to relocate the exact position on an individual cell that was previously studied electrophysiologically. Identified hot spots correspond to loose aggregates of several small islands of tightly packed intramembranous particles. The overall dimensions (ca. 10 μm across) of the aggregates are comparable to those of hot spots defined by ACh microiontophoresis or by light microscopic autoradiography. Each island measures about 0.2 to 0.5 μm across. These miniclusters may correspond to the small patches of ferritin particles found on the outer surface of freeze etched myotube membranes labeled with ferritin α-BuTx conjugates (42). This substructure is also consistent with the light microscopic appearance of hot spots on cultured *Xenopus* (3) and rat (4) myotubes labeled with fluorescent α-BuTx conjugates; many hot spots are speckled rather than uniformly labeled. Clusters of intramembranous particles have also been found in *Xenopus* myocytes (65).

The intramembranous particles resemble those found within the postsynaptic mem-

brane of adult endplates and of electric fish electroplaques. They are irregular in shape, measuring about 90 nm in longest dimension, and in some images they appear to be composed of 5 to 6 subunits. On the P-fracture face the particle density within the miniclusters is about 2000/μm^2, which is comparable to the packing density observed within adult postjunctional membranes (41, 66, 71).

We developed means for rapidly mapping the distribution of ACh sensitivity over relatively large areas of myotube membrane (32–34). At each test site the maximum ACh response was analyzed on-line with a computer and the position of the ACh electrode tip was simultaneously photographed on a frame of 16-mm movie film. With this nondestructive technique we could assay about 100 points at 10 μm intervals within 30 minutes.

Repeated maps of individual cells indicate that hot spots on uninnervated myotubes are stable with time. They can be relocated in the same position after 3 to 4 hours; several have been followed for more than 2 days (cf. fig. 3). This stable topography does not depend upon the metabolic stability of individual receptors. The great majority of receptors are degraded with a half-life of less than 30 hours (22). We found that clustered receptors disappear at the same rapid rate. Hot spots reappear in the same location when the sensitivity returns to control level some 24 hours after all receptors are blocked with α-BuTx. The reappearance of sensitivity after toxin blockade is due to the synthesis and insertion of new receptors rather than to dissociation of the toxin receptor complex. Devreotes and Fambrough's (22) compelling indirect evidence that loss of bound toxin is a reliable index of receptor degradation has been confirmed by direct measure (58). The receptor density within hot spots does not increase with time and, as expected, autoradiographic experiments showed that (^{125}I)-α-BuTx binding sites are lost from hot spots with a half-time of about 30 hours (80). Some factor(s) must maintain receptor clusters in the face of rapid synthesis and degradation.

C. Motor Nerves Induce New Receptor Clusters

To determine whether ingrowing motor axons seek out preexisting hot spots or induce new ones, we mapped the distribution of ACh sensitivity over individual myotubes before and then again after they were contacted and innervated by spinal cord explants. The answer was clear—motor nerves can induce hot spots (fig. 4). In all, we documented the formation of 22 new hot spots beneath ingrowing spinal cord

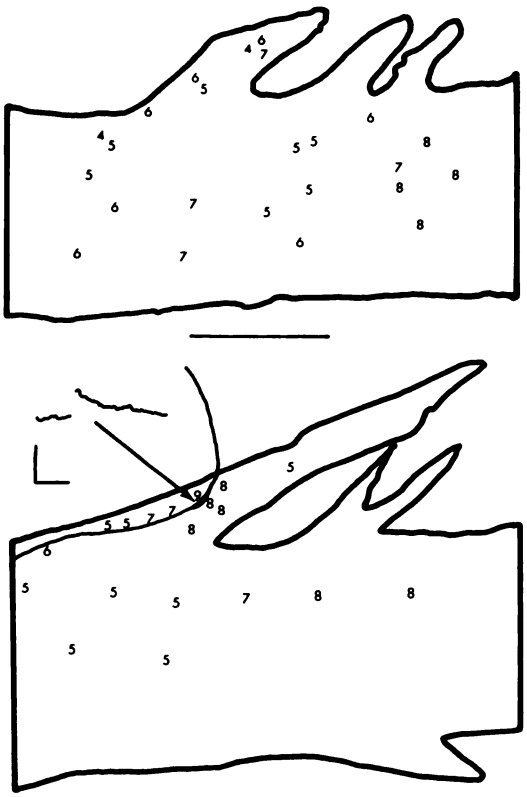

FIG. 4. A new hot spot appears during synapse formation. A growth cone palpated the upper edge of the myotube while the first map was constructed. Twelve hours later, one branch of the axon had advanced along the upper edge of the cell. Synaptic potentials were evoked (arrow) by focal depolarization in the presence of tetrodotoxin. The acetylcholine (ACh) sensitivity had increased 8- to 12-fold in the immediate vicinity of the synapse. See Figure 3 for integer scale.

nerve processes. Synapse formation is a rare event. Most myotubes in the vicinity of the explant that are contacted by nerve processes are innervated after 1 to 2 days, but apparently only a few of the emergent neurites do the job. Stimulation of the great majority of processes fails to evoke synaptic potentials in contacted myotubes. It is remarkable, therefore, that all 12 of the neurites that induced new hot spots and that were also stimulated electrically had innervated the underlying myotube (the remaining 10 were not tested). Hot spot induction, therefore, may be a unique property of cholinergic neurons.

The ACh sensitivity was not increased all along the length of motor axon-muscle contacts. Rather, the subneural hot spots, like those on uninnervated myotubes, were discrete patches. Synaptic potentials were evoked by focal extracellular stimulation at six of eight new subneural hot spots tested in this manner. Thus, the induction of hot spots may occur only at sites of transmitter release.

We could not predict exactly where on the myotube surface a synapse would form, so relatively large areas of the muscle surface were mapped at intervals of several hours. However, when maps were repeated more frequently, it was evident that subneural clusters can appear rapidly. In one experiment, a hot spot appeared beneath a stationary (but active) growth cone within 3 hours after it contacted and paused on the myotube surface. About half of the subneural clusters appeared within 5 hours.

Although we watched several spinal cord growth cones pass within a few micrometers of and "palpate" preexisting hot spots, none formed synapses at these sites. Thus, the physiological significance of AChR clusters on uninnervated myotubes remains obscure.

Anderson et al. (3) have also concluded that spinal cord neurons induce new clusters of receptors on target muscle cells. They mapped the distribution of receptors on *Xenopus* muscle cells with fluorescent α-BuTx conjugates. Myocytes, dissociated from stage 21 somites, were innervated in culture by neurons dissociated from the neural tube. Long streaks of intense fluorescence were found along the course of neurite-muscle cell contacts, whereas only small ovoid hot spots were present on myocytes that were not contacted by neurites. The streaks strongly suggest that neurons induce a change in receptor topography. Some of the contacted *Xenopus* myocytes were innervated: they twitched when a nearby nerve cell body was stimulated. However, the same cells examined with toxin conjugates were not also assayed physiologically so it is not clear whether clustering of receptors at *Xenopus* nerve-muscle contacts occurs only at synaptic junctions. The long subneural streaks of fluorescence are quite different from the discrete hot spots found at chick nerve-muscle synapses. This disparity is consistent with the different geometries of adult motor nerve terminals in amphibia and chicks, but the relation between *Xenopus* subneural clusters and sites of transmitter release remains to be determined.

Anderson and Cohen (2) found similar streaks of fluorescence when myocyte receptors were labeled with toxin conjugates *before* addition of neural tube cells. This implies that at least some of the receptors ultimately included in subneural clusters were exposed on the cell surface before the nerve arrived. Control experiments were consistent with the possibility that they arrive by migration within the plane of the membrane. The phenomenon of lateral AChR migration is not unique to amphibian cells. Axelrod et al. (4) demonstrated with fluorescence bleaching techniques that receptors in rat myotubes are mobile. From the rate at which black (bleached) holes filled in they estimated that up to 75% of the diffusely distributed (non-hot-spot) receptors move with an effective diffusion coefficient of 7×10^{-11} cm^2/sec. It is likely, therefore, that migrating receptors contribute to new synaptic hot spots on chick myotubes, but this must be measured directly.

In addition, it must be determined whether receptors that migrate to synaptic hot spots represent a significant fraction of the total present. Receptors within innervated (and uninnervated) hot spots turn over rapidly, so they must either diffuse into and out of hot spots or they must be inserted and degraded (internalized) locally. Axelrod et al. (4) found that bleached spots within or at the edge of uninnervated hot spots do not fill in with time at least up to 10 hours. If receptors are not damaged by the photobleaching procedure, and if the same phenomenon occurs at innervated hot spots, then local synthesis and insertion of AChRs must contribute to induced subneural clusters.

Rees et al. (73) have recently described coated vesicles in the cytoplasm of cultured sympathetic ganglion neuron perikarya contacted by spinal cord neurites. It is tempting to speculate that these vesicles contribute new membrane to nascent postsynaptic sites. In some sections coated, omega-shaped flasks opened on the cell surface and the coat material appeared contiguous with a postsynaptic density. Coated vesicles are also present within cultured myotubes, and they are more numerous at physiologically identified synapses than elsewhere (S. Bursztajn and G. D. Fischbach, unpublished observations). It is commonly thought that coat material (composed primarily of the protein, clathrin) in some way provides a motive force for endocytosis or membrane retrieval. However, most coated vesicles in sympathetic neurons and myotubes do not take up the extracellular tracer horseradish peroxidase so at least some of them may be en route to the cell surface. Perhaps coated vesicles represent units of AChR rich membrane that ultimately appear as islands of intramembranous particles within hot spots. It is interesting in this regard that innervated and uninnervated hot spots are often located near myotube nuclei. Golgi complexes and clusters of coated vesicles are also located adjacent to nuclei. The receptor is a glycoprotein (11) and (^{125}I)-α-BuTx binding sites have been found within Golgi cisternae (25).

D. How do Nerves Induce Hot Spots?

The down regulation of *extra*-junctional receptors during initial synapse formation and during reinnervation of denervated adult muscles has been investigated extensively. It is due to a decrease in the rate of receptor synthesis rather than to an increase in rate of receptor degradation (15, 74). Muscle activity (electrical and/or mechanical) is an important determinant of extrajunctional receptor synthesis. Chronic stimulation of denervated adult muscle can eliminate or prevent the appearance of extrajunctional receptors (52, 53). A similar result was obtained by stimulating embryonic myotubes in vitro (19). A role for activity-independent "trophic" factors supplied by the motor nerve in the regulation of extrajunctional receptors remains controversial [see review by Fambrough (24)]. The situation is quite different regarding synaptic hot spot formation. Receptors cluster in the continued presence of α-BuTx (3) or tetrodotoxin (19); L. L. Rubin and G. D. Fischbach, in preparation). Muscle activity is not required and the search for a local influence of the presynaptic neuron is on.

The subneural clustering of receptors might be due to physical contact between nerve and muscle. That hot spots are restricted to short segments (5–10 μm) along the length of motor axons that may course over myotubes for more than 300 μm indicates that simple proximity between a competent nerve process and a receptive muscle fiber is not sufficient. However, "proximity" is a vague term and it is likely that contacts at newly formed synapses are specialized in some way. Our recent scanning (fig. 5) and thin section electronmicroscopic results indicate that synaptic contacts are specialized at an early stage (32–34). It may be that any firmly attached process is capable of altering the distribution of receptors in the subjacent membrane. Clusters of receptors have been described on L6 myotubes near

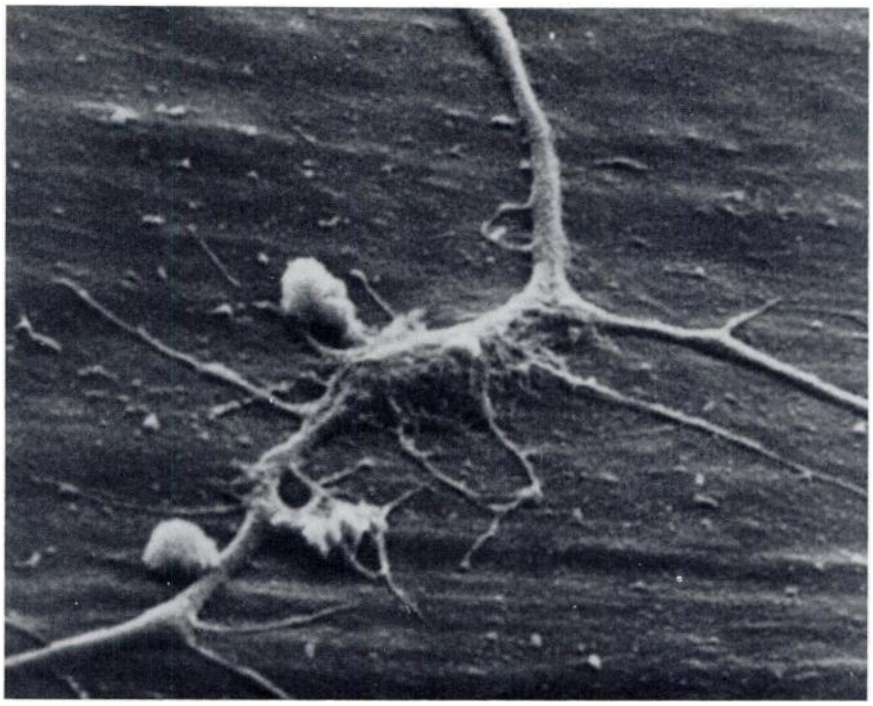

FIG. 5. Scanning electron micrograph of a functionally identified nerve-muscle synapse. Synaptic potentials were evoked at the swelling in the nerve. Two characteristic features are apparent: the spongy surface texture of the synaptic varicosity and the long filopodia projecting from it over the surface of the myotube. Neither of these specializations was seen at nonsynaptic varicosities. Magnification, 8500 ×.

neuroblastoma nerve processes (38). These neurons do not innervate L6 myotubes, but the processes are tightly adherent.

Alternatively, or in addition, the formation of subneural clusters might depend upon the release of a chemical factor at newly formed synapses. In our first study of myotubes innervated by intact spinal cord explants we noted that the overall (non-hot-spot) ACh sensitivity and the density of (^{125}I)-α-BuTx binding sites of myotubes immediately adjacent to the explants were increased compared to myotubes located farther away on the same coverslip or to myotubes on sister coverslips that did not contain an explant (20). It was suggested that the increase in receptor number might be mediated by a soluble factor released from the spinal cord. Evidence for such a factor has been obtained. Saline extracts of embryonic rat brain increase the number of (^{125}I)-α-BuTx binding sites on rat L6 myotubes and induce receptor clusters (69). The density of binding sites is ordinarily quite low in these cells compared to primary rat myotubes and the distribution of sites is more or less uniform (49, 82). Extracts of a neuronal cell line increase the number of receptor clusters but apparently not the total number of toxin sites on mouse myotubes (17).

Saline extracts of chick spinal cord or brain produce a 4- to 9-fold increase in receptor number (fig. 6) and a 40-fold increase in number of receptor aggregates (fig. 7) (43). The effect is dose dependent between 10 and 300 μg of extract protein (added each day). The increase occurs over the course of 3 to 4 days and it persists for at least 2 to 3 days after the CNS supplements are stopped. Extracts prepared from embryonic cerebellar cortices produced smaller increases in receptor number; retina, liver, heart, and fibroblast extracts were without effect. Brain extracts do not decrease the turnover of receptors so the

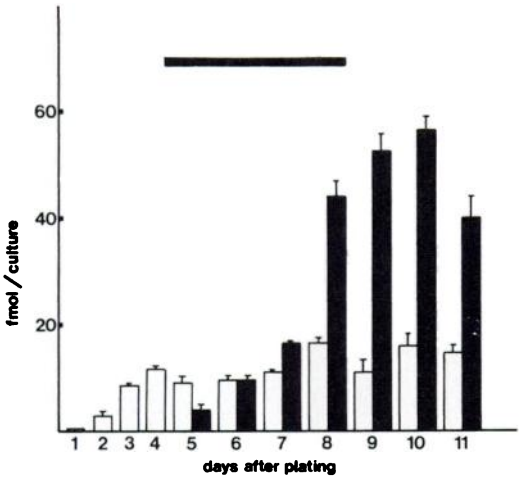

FIG. 6. Brain extract-induced increase in acetylcholine receptor (AChR) number in cultured myotubes. The mononucleated cells were plated at a density of 6×10^4 cells per 18 mm of collagen-coated Falcon well in 400 μl of medium. Brain extract was prepared by homogenizing 14-day embryonic chick brains with a Dounce homogenizer in 3 volumes of Earle's balanced salt solution (BSS) at 4°C. The homogenate was centrifuged at $1500 \times g$ for 10 minutes and the supernatant was centrifuged again at 20,000 $\times g$ for 60 minutes. Extract was added at 280 μg of brain protein every 24 hours from day 4 through day 8 in a volume of 5 to 50 μl. Control cultures were fed with the same volume of BSS. Receptor number was estimated by measuring the specific binding of (^{125}I)-α-bungarotoxin (α-BuTx). Open columns, control; filled columns, extract-treated. (From ref. 43a.)

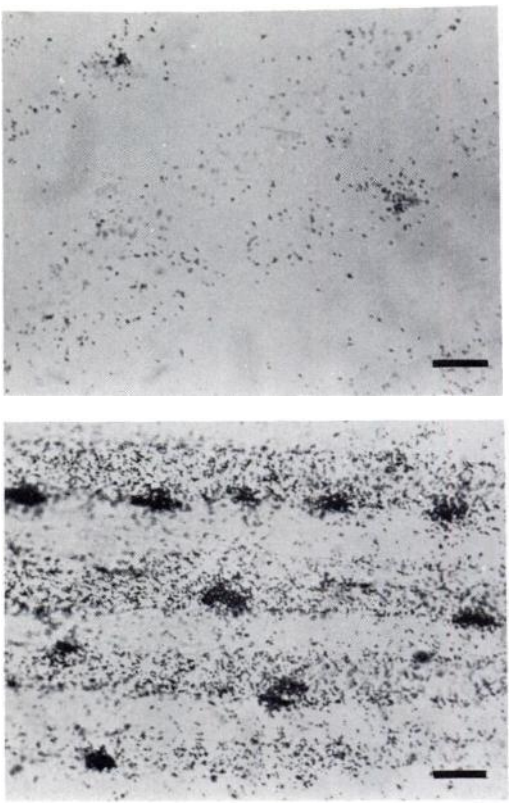

FIG. 7. (^{125}I)-α-bungarotoxin (α-BuTx) autoradiography of control and brain extract (780 μg/day for 4 days)-treated cultures. Upper panel, control 8-day myotubes; two grain clusters are present on the four myotubes in this bright-field micrograph. Lower panel, brain extract-treated 8-day myotubes. The overall grain density is increased and 11 grain clusters are evident on the four myotubes. Bars = 20 μm.

increase in receptor number must reflect a stimulation of receptor synthesis.

Activity is destroyed by trypsin and by boiling, so the factor is probably a protein. It is stable in 1 N HCl and gel chromatography indicates that it is a small peptide less than 2000 daltons in molecular weight. A reasonable correlation between receptor-inducing activity and levels of choline acetyltransferase was found in different regions of the brain and in the periphery so the peptide may be concentrated in cholinergic neurons. A pure population of cholinergic neurons dissociated from chick ciliary ganglia increased the number of receptors on cocultured myotubes 2- to 3-fold, whereas neurons dissociated from sensory (noncholinergic) ganglia had no effect. Studies of the cellular localization of the factor and of its action on target tissues must await further purification.

E. Subsequent Modification of AChRs

There are a number of differences between synaptic receptors at adult endplates and extrasynaptic receptors that appear following denervation. Of relevance here are the facts that junctional receptors are: 1) packed at a higher density (39, 59); 2) metabolized less rapidly (6); 3) gated open by ACh for a shorter time (44, 61); and 4) more negatively charged (11). These differences may point to changes that occur during synapse formation de novo. In a holistic scheme, they would all be related (cf. 16).

For example, a signal, perhaps from the nerve, might induce a covalent modification such as phosphorylation in subjacent receptors that causes them to stick together, resist degradation, and alter their channel kinetics. However, our data indicate that these phenomena are not related in a simple way.

Receptors within embryonic synaptic clusters in vitro (80; see above) and in vivo (9, 13, 14) are degraded with a half-life of about 30 hours. In contrast, the half-life of adult endplate receptors is at least 5 days. They may be even more stable, but the α-BuTx dissociation rate is on the order of 5 days, so longer times cannot be assayed. Judging from light microscopic autoradiography and ACh iontophoresis, the receptor densities at embryonic and adult synapses are comparable so metabolic stability is probably not linked to receptor density or to the fact of innervation. Small, but perhaps crucial, differences in receptor density or microdistribution would not be detected on the light microscopic level or by physiological assay. A more definite conclusion must await ultrastructural studies.

We determined the mean channel open time, τ_0, of receptors in small patches of membrane by recording small current fluctuations with a focal extracellular electrode filled with 40 μM ACh (80). The increase in baseline noise recorded when such an electrode is pressed against a myotube surface reflects the random opening and closing of individual ACh channels (fig. 8). The noise is blocked by curare and is absent when the electrode is pressed against relatively insensitive muscle membrane or against membranes that contain no AChRs at all. Our first studies focused on chick muscle fibers. All estimates of τ_0, based on the computed spectral density function, were about 4 msec at 22°C–24°C, regardless of whether the receptors tested were clustered at a synapse, clustered at an uninnervated hot spot, or diffusely distributed at lower density (fig. 9). Thus τ_0 is also independent of receptor density and of the fact of innervation.

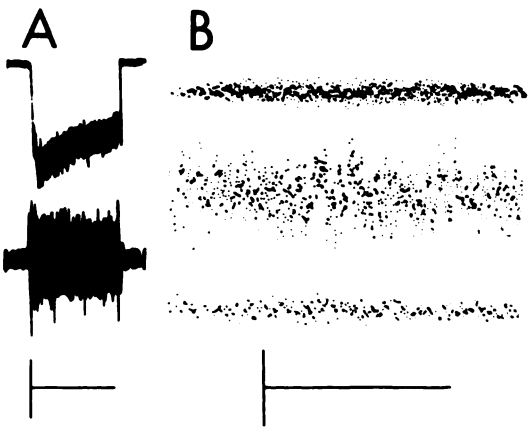

FIG. 8. A. Acetylcholine (ACh) noise recorded with an extracellular electrode filled with 40 μM ACh placed on the surface of an uninnervated myotube. Upper, dc-coupled; Lower, band pass-filtered (0.5–1100 Hz). Vertical bar = 200 μV (upper) or 100 μV (lower). Horizontal bar = 10 seconds. B. Digitized records of ACh noise. Top, background (control) noise of the recording system obtained with the electrode positioned far above the cell surface. Middle, ACh noise recorded after placing the electrode on the myotube surface. Bottom, attenuated noise recorded from the same membrane patch after addition of *d*-tubocurarine (0.1 mM) to the bath. Calibration bars = 100 μV, 0.5 second. (From ref. 80.)

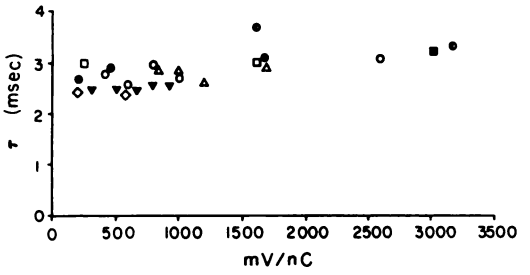

FIG. 9. Mean channel open time as a function of acetylcholine (ACh) sensitivity (mV depolarization of muscle per nc changed passed through the iontophoretic electrode). Each of the eight symbols represents a different, uninnervated myotube. Temperature = 26–29°C. (From ref. 80.)

When posthatched chicks were examined, we found, to our surpise, that the gating time of synaptic channels did not decrease to 1 msec as expected from work in other species. Estimates of 3 to 4 msec were obtained from fluctuation spectra and also from the time constant of synaptic current decay (cf. 54) at motor endplates as

late as 18 weeks after hatching. Nevertheless, the initial conclusion, that τ_0 is not altered by receptor clustering or by innervation, remains valid. The turnover rate of chick endplate receptors decreases markedly during the second week after hatching (14) so, in this species, τ_0 is also independent of metabolic stability.

We also examined mean channel open time at developing rat neuromuscular junctions. Synaptic potentials can be recorded intracellularly in many cultured rat myotubes that are contacted by spinal cord nerve processes, but it was difficult to locate synapses by focal extracellular recording. Histochemical staining for AChE indicated that many of the synapses were located on the undersurface of the myotubes, and this probably accounts for our poor success in recording synaptic currents. Because of this problem, we turned to intact muscles in embryonic and neonatal rats. Synaptic current decay rates and ACh spectra showed that τ_0 was prolonged (3–4 msec at 22°C–24°C) at all stages of embryonic life and it was also prolonged until 4 to 5 days after birth (fig. 10). The relatively slow synaptic currents in neonates are not due to lack of AChE. The enzyme can be demonstrated histochemically at neonatal endplates, and

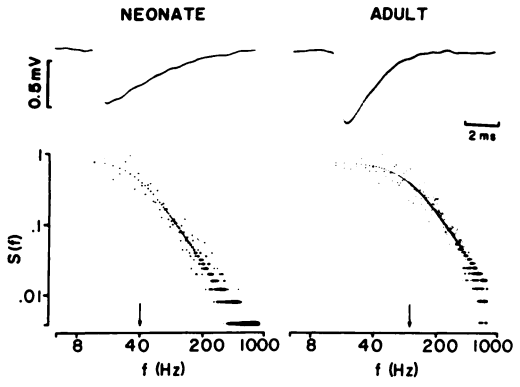

FIG. 10. Synaptic currents and acetylcholine (ACh) power spectra at neonatal and adult rat endplates. The estimates of channel open time at the neonatal junction are prolonged (τ_0 = 3.9 msec; τ_{syn} = 3.7 msec) compared to estimates at the adult junction (τ_0 = 1.06 msec; τ_{syn} = 1.6 msec). All recordings were performed at 23°C.

inhibitors of AChE further prolong the already slow synaptic currents. Moreover, synaptic current decay rates agree closely with spectral estimates of channel open time. Synaptic potentials and receptor clusters are present in 15-day rat embryos so, as in the chick, the kinetics of rat synaptic channels are independent of receptor density and of innervation. Berg and Hall (6) presented evidence that AChRs at rat endplates are metabolically stable at birth. Thus, as in the chick, the gating time at rat junctions is apparently independent of changes that decrease the rate of receptor degradation.

By 12 days after birth all endplates contain predominantly fast channels (about 1.0 msec at 22°C–24°C). Between 4 and 12 days, estimates of mean channel open time vary widely among individual fibers within a given muscle. Some endplates are as "slow" as neonatal endplates and others in the same muscle are as fast as adult endplates. Intermediate estimates are obtained at some junctions during this critical interval. However, ACh spectra at "intermediate" junctions did not decay with 1/(frequency)^2 as expected from the usual two-state (closed-open) model of channel function, and synaptic currents did not decay as single exponentials. One interpretation of complex spectra and synaptic current decays is that two (or more) populations of receptors, one fast and one slow, are present within the same endplate. However, other interpretations are possible (cf. 77) and more data are needed. It will be of interest to determine whether this change in gating time is associated with the appearance of new receptors and whether the change resides in the receptors themselves or in the state of the surrounding lipid microenvironment.

The period between 1 and 2 weeks after birth may be an important developmental "window" at the rat neuromuscular junction. In adult animals, each endplate is innervated by one motor axon, but at birth the endplates are multiply innervated; some receive more than 10 motor-nerve

fibers. Nearly all of the extra motor nerve terminals are lost during the second week after birth (12, 72). Perhaps more relevant to a change in receptor gating time is the fact that secondary postsynaptic folds form and elongate during the first and second week after birth. Finally, a dramatic change in the action of curare during the second week after birth has recently been described. Curare is a competitive antagonist of ACh at adult endplates. It does not exert agonist action on its own. However, in embryos and neonatal animals, curare increases the conductance and depolarizes the endplate membrane (88). The change in curare efficacy occurs at about the same time as the change in mean channel open time.

III. Accumulation of AChE

Little information is available concerning the first appearance of AChE during the process of nerve-muscle synapse formation. Evidence from embryonic and regenerating adult junctions suggests that the enzyme does not accumulate at newly formed synapses until several days after the onset of synaptic transmission. In the chick embryo, for example, focal "spots" of AChE reaction product first appear in thigh muscles on the 11th day of incubation (30, 35, 85), whereas synaptic transmission within the same muscle mass can be demonstrated on the 4th day (50). Neuromuscular junctions continue to form in vivo over a period of several days, so this interval must be considered an upper limit.

It should be possible to determine more precisely when synaptic AChE first appears in spinal cord/muscle co-cultures. AChE is present in uninnervated myotubes (23, 31, 70). At least some of the enzyme is particulate, but it is diffusely distributed throughout the cell and it is not clear whether any is exposed on the surface membrane (28). Early histochemical studies in which myotubes (or strips of adult muscle) were cocultured with embryonic spinal cord explants (intact fragments) emphasized that foci of AChE first appeared at nerve-muscle contacts after 3 to 5 weeks in vitro (60, 64, 67, 68). Recent data suggest an earlier appearance in vitro. Koenig and Vigny (47) demonstrated the presence of the endplate-specific (16 S) form of AChE in monolayer cultures of dissociated rat spinal cord and muscle cells 5 days after plating. Frank and Fischbach (33, 34) described a synapse in a chick spinal cord explant/muscle culture that stained for AChE 3 days after the onset of synaptic transmission. Electron microscopy showed that reaction product was located in the synaptic cleft and in some of the large vacuoles immediately beneath the muscle cell surface. These vacuoles accumulate horseradish peroxidase and ferritin so they must communicate with the extracellular space. The cleft and vacuole AChE reaction product was associated with a distinct basement membrane.

We examined the early appearance of AChE in more detail by correlating a physiological assay for the enzyme—the rate of decay of synaptic currents—with a histochemical assay (75). When AChE is inhibited at adult endplates, the decay of synaptic current is markedly prolonged, probably because the liberated ACh binds sequentially to several receptors as it diffuses out of the synaptic cleft (45). Thus in principle the rate of synaptic current decay, compared to the rate at which individual ACh channels close, is a sensitive index of synaptic AChE activity.

There is marked variation in the time constant of synaptic current decay (τ_{syn}: determined by computer fit to a simple exponential) between synapses in young cultures (fig. 11). At 30°C the shortest are ca. 1.3 msec and the longest are 8 to 9 msec. The fastest synaptic current decays are comparable to the mean channel open time, τ_0 estimated from spectra obtained at the same synapses at 30°C. At 22°C to 24°C, τ_0 and the shortest values of τ_{syn} are ca. 4 msec. At each synapse histograms of τ_{syn} were constructed from about 50 potentials and the means were arbitrarily divided into three categories: fast ($\tau_{syn} < 1.8$ msec), intermediate ($\tau_{syn} = 1.8$–2.6 msec), and slow

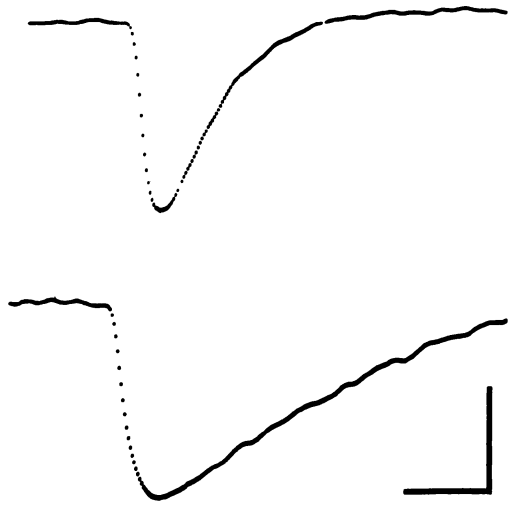

FIG. 11. Variation in rate of synaptic current decay. The two extracellularly recorded synaptic potentials were recorded at different synapses in the same culture. Upper trace, τ_{syn} = 1.30 msec; lower trace τ_{syn} = 4.02 msec. Bars = 200 μV and 2 msec. τ = 30°C. (From ref. 75.)

(τ_{syn} > 2.6 msec). Of the fast synapses 74% stained for AChE, whereas none of the 14 slow synapses were stained (fig. 12). Reaction product was evident at 64% of the intermediate synapses, but the intensity of the stain at these junctions was less than at the fast synapses. The possibility that slow rates of synaptic current decay reflect similarly slow rates of ACh channel closing was shown not to be the case by analysis of ACh current fluctuations. Estimates of mean channel open time at 30°C were identical at slow (τ_0 = 1.34 msec ± 0.34, N = 5) and fast (τ_0 = 1.34 ± 0.16, N = 4) synapses. Thus, the physiological and histochemical measures agree; AChE appears early during synapse formation and when present it serves to terminate transmitter action.

Four days after addition of spinal cord explants, about 25 patches of AChE reaction product were evident beneath neurite contacts around each explant. A few sub-

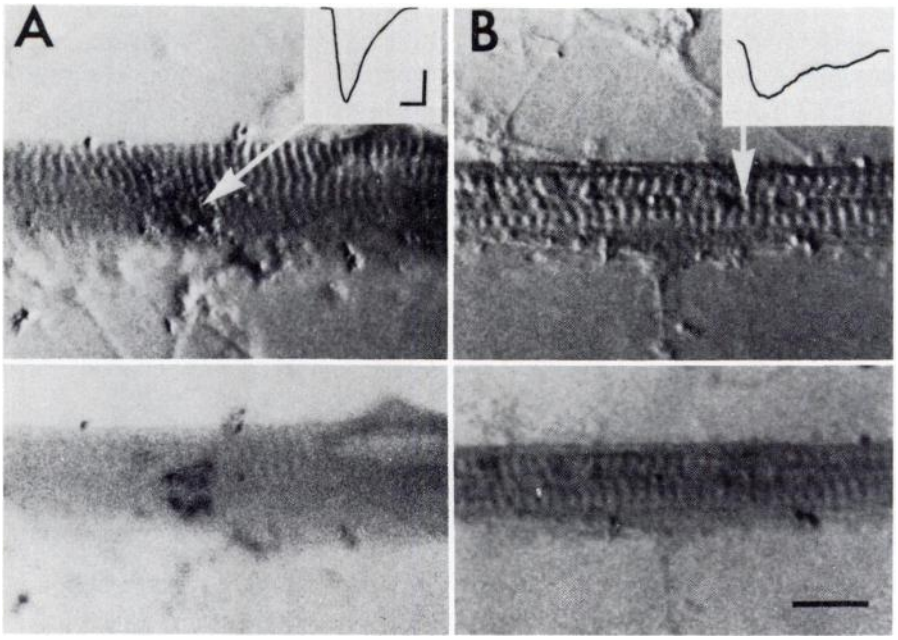

FIG. 12. Correlation between τ_{syn} and acetylcholinesterase (AChE) stain. The two synapses shown in A and B were on noncontiguous segments of the same myotube. Upper, interference contrast; lower, bright field. Bar = 10 μm. Synaptic currents recorded at each site are shown in the insets. Calibration bars = 1 msec, 200 mV; τ = 37°C. The rapidly decaying current is associated with AChE stain (A), whereas the slowly decaying current is not (B). (From ref. 75.)

neural AChE patches were present 36 hours after the explants attached. If 12 hours are allowed for the first neurites to emerge from the spinal cord explants, this implies that AChE can appear within 24 hours after nerve-muscle contact.

We assayed for the large, endplate-specific form of AChE (19 S in chick muscle) by velocity sedimentation through 5 to 20% sucrose gradients (76). The 19 S form was present in muscle cultures seeded with a large number of dissociated spinal cord cells (ca. 2×10^6 cells/60 mm dish), but not in muscle cultures or spinal cord cultures grown alone, confirming the report of Koenig and Vigny (47).

Synapses form and AChRs cluster at functional contacts in the absence of synaptic transmission (2, 18). In contrast, we found that the appearance of AChE at synapses does depend on synaptic transmission. When spinal cord explant/muscle cultures were grown in TTX, curare, or α-BuTx, the mean rates of synaptic current decay were prolonged. The percentage of fast synapses decreased by a factor of 5, and only 2% of the synapses stained for AChE (fig. 13). The total AChE activity was unchanged in spinal cord/muscle cell cultures grown in curare, but the 19 S form was not detectable. One consequence of synaptic transmission is that myotubes contract and our results suggest that such electrical and/or mechanical activity is necessary for accumulation of AChE at synapses (76). Myotubes innervated by spinal cord explants in the presence of curare were stimulated directly via an extracellular electrode in the continued presence of curare for 8 to 12 hours at a rate of 2.5/sec. The mean τ_{syn} at 7 previously identified synapses decreased from 2.76 to 1.74 msec. This change, in which each synapse served as its own control, is highly significant.

Factors other than muscle activity are probably also important for accumulation of synaptic AChE. The fact that the enzyme appears to be restricted to sites of transmitter release argues for a local influence of the motor axon. Moreover, in a few cases, AChE positive and AChE negative synapses were located on the same myotube within a few hundred micrometers of each other (cf. fig. 12). If both contacts were exposed to the same level of muscle activity then something else must be required. The same CNS extracts that increase the number of AChRs also produce a 2- to 3-fold increase in AChE in uninnervated myotubes (R. Siegel, T. Jessell, and G. D. Fischbach, unpublished observations). It will be important to determine whether the

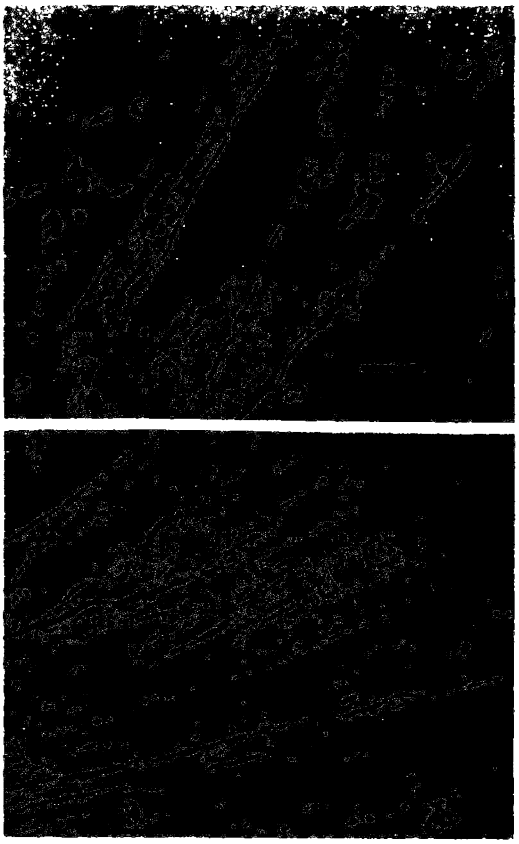

FIG. 13. Bright field view of control (upper) and 50 μM curare-treated (lower) myotubes stained for acetylcholinesterase (AChE) according to the method of Karnovsky and Roots (17) 4 days after addition of spinal cord explants. Both fields were near (within 300 μm) the explants. Numerous patches of reaction product near fine nerve processes (not visible under bright field illumination) are evident on control myotubes. Few, if any, patches are present on curare-treated myotubes. Bar = 100 μm.

same factor is responsible for the increases in receptor number and AChE activity.

We have begun to investigate factors that may mediate the effect of muscle activity and the local influence of the nerve. Dibutyryl cyclic GMP can reverse the suppression of AChE caused by inactivity. The majority of synaptic currents decay rapidly, 70% of the synapses stain for AChE, and 19 S AChE is present in cultures grown continuously in 50 μM curare and 50 to 100 μM dibutyryl cyclic GMP.

IV. Summary

Nerve-muscle synapses form and the postsynaptic membrane begins to differentiate rapidly in vitro. New clusters of AChRs have been located at transmitting synapses within 3 hours after a competent neurite contacted a receptive myotube. Receptors within new subneural clusters are not identical with receptors at adult endplates: they are metabolized rapidly and their mean channel open time is prolonged. In this sense synapse formation must be considered a drawn out affair. AChE also accumulates at synapses soon after functional contacts are established. The cleft enzyme is apparently regulated differently than receptors in the postsynaptic membrane. Muscle activity is necessary for the early accumulation of AChE, but not for the clustering of receptors. Further studies in vitro should be useful for analysis of the role of muscle activity, of the local neural influence on the postsynaptic membrane, and of the mechanism of the muscle response.

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