INFLUENCE OF INNERVATION ON THE DISTRIBUTION OF ACETYLCHOLINE RECEPTORS IN SKELETAL MUSCLE: A STUDY UTILIZING FLUORESCENT STAINING

by

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ABSTRACT

α-Bungarotoxin was labelled with fluorescent dyes and used to examine the distribution of acetylcholine receptors on cultured amphibian muscle cells. Within two days in culture all non-innervated muscle cells developed discrete patches of high receptor density. Innervation caused the development of a region of densely packed receptors along the path of nerve contact. This change involved the formation of new regions of high receptor density in the subneural membrane, and occurred independently of synaptic activity. Similar changes occurred when muscle cultures were stained before adding nerve cells, indicating that innervation induced previously stained receptors to aggregate along the path of nerve contact. This process of receptor redistribution was confirmed in successive observations on identified pre-stained muscle cells. In similar experiments receptor patches on non-innervated muscle cells also formed by a process of receptor aggregation.

CONDENSE

On a utilisé une préparation d'α-bungarotoxine couplée avec des molécules fluorescentes pour étudier la distribution des récepteurs pour l'acétylcholine dans les cellules musculaires amphibiennes en culture. Toutes les cellules non-innervées développent, en moins de deux jours de culture, des régions isolées possédant une haute densité de récepteurs. L'innervation cause le développement d'une région à haute densité de récepteurs le long de la trajectoire de contact du nerf. Ce changement comporte la formation de nouvelles régions à haute densité de récepteurs dans la membrane sous-neurale, et il se produit indépendamment de l'activité synaptique. Des changements similaires se produisent quand des cultures de muscle sont exposées à l'α-bungarotoxine avant l'addition de cellules nerveuses, montrant que l'innervation induit l'aggrégation, le long de la trajectoire de contact avec le nerf, de récepteurs a été confirmé par des observations successives de cellules musculaires identifieés préalablement exposées à la préparation d'α-bungarotoxine. Dans des expériences similaires, le processus d'aggrégation des récepteurs a aussi expliqué la formation de régions à haute densité de récepteurs dans des cellules musculaires non-innervées.

PREFACE

The present thesis was prepared according to the provisions of regulation number 54h of the Faculty of Graduate Studies and Research of McGill University. This regulation allows the presentation of theses to be in the form of papers suitable for publication in a professional journal, with a supplementary introduction of the literature and concluding remarks. The main body of this thesis is thus divided into three sections (Chapters 2-4) which have either been published (Chapter 2) or submitted for publication in the Journal of Physiology (Chapters 3-4). The progression between these respective chapters will hopefully be self-evident to the reader as the methodological developments in the early chapters lead to new experimental conclusions in the latter.

The thesis is entirely original and largely the work of the author carried out in the laboratory of Dr. M.W. Cohen. Two of the experimental sub-sections, however, were carried out by other members of the group, and are included only to provide a comprehensive view of the project. Dr. E. Zorychta carried out the experiments to determine the patterns of receptor staining on identified innervated muscle cells. Drs. M.W. Cohen and E. Zorychta also carried out the radioautographic experiments, with considerable technical assistance very generously provided by I. Chow.

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TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Localization of chemosensitivity at the neuromuscular junction

During the past decade there has been an upsurge of interest in the acetylcholine (ACh) receptor of vertebrate skeletal muscle. This has come about in part because of the development of biochemical techniques for the identification and measurement of this receptor substance, but also is due to the variety of important biological processes in which it is involved.

Historically the ACh receptor was one of the first drug receptors to have been postulated, and the early studies of its pharmacological interactions were instrumental in the development of the concept of a "receptor" substance. This early progress was due in large part to the introduction into European medicine of cholinergic drugs which had long been used by tribal societies in the Americas. Thus it was observed in the nineteenth century that nicotine could mimic the effect of stimulating the motor nerves, to evoke contraction of skeletal muscle fibres (Langley & Dickenson, 1890), and that curare blocked the effect of nerve stimulation by its direct action on muscle (Bernard, 1856, 1857). However, it was not until the present century that the concept of "receptive substances" was introduced (see Langley, 1905), in part to rationalize the antagonistic effects of curare and nicotine on skeletal muscle. Soon thereafter Langley (1907) noted a dramatic variation in the sensitivity of frog muscle to small drops of nicotine he applied topically with a brush, and suggested that this variation might be explained by a localization of the "receptive substance" for nicotine on the muscle fibres at the sites of innervation. Later investigations eventually led to the discovery that ACh had

pharmacological properties which resembled those of both nictoine and muscarine (Dale, 1914), and that a humoral substance similar in action to ACh was released as result of nerve stimulation (Loewi, 1921; Loewi & Navratil, 1926).

Although Langley's suggestion, that a specific receptor substance was localized at the site of innervation, has long been accepted, it has been difficult to obtain conclusive biochemical evidence for its existence. This problem, which still exists for the majority of other drug receptors, was due to a lack of means for identifying the receptor substance, other than in a living cell. However in the case of the ACh receptor of vertebrate muscle the physiological effect is so easy to observe that considerable progress was made in characterizing it on the basis of its interactions with a host of pharmacological agents of related chemical structure. This indirect evidence led to the deduction that the nicotinic receptor has at least two sites which interact with ACh: an anionic site which undergoes a coulombic interaction with the quaternary nitrogen group of ACh, and a hydrogen-bonding site which interacts with the esteric carbonyl (see reviews, Michelson & Ziemal, 1973; Goldstein, Arqnow & Kalman, 1974). Thus many studies have indicated that the interaction of the receptor with ACh resembles in many respects that between enzyme and substrate, except that the result is not a chemical change in the transmitter, but rather a conformational change, presumably in the receptor substance, which allows a greater degree of cation permeability through the membrane.

There have also been dramatic technical improvements since Langley's time in the means of examining the distribution of chemosensitivity on the surface of muscle cells. A particularly important advance was brought

about by the development of the iontophoretic technique for applying minute quantities of a drug onto a small portion of the cell surface (Nastuk , 1953; del Castillo & Katz, 1955), while recording the resulting change in membrane potential with an intracellular microelectrode. Small transient currents passed through a glass microelectrode containing ACh, or another ionic substance, lead to the ejection of amounts of drug proportional to the charge in the current pulse (see Krnjevic, Mitchell & Szerb, 1963). Thus by moving the iontophoretic electrode over the cell surface a map can be prepared of the sensitivity to the drug. Using this approach Miledi (1960a) demonstrated that sensitivity to ACh dropped one thousand-fold within 100 um of the motor nerve ending on normal rat muscle fibres. More recently the localization of the iontophoretic electrode with respect to individual branches of the nerve terminal has been enhanced by the use of Nomarski interference-contrast optics (Peper & McMahon, 1972) and the access of the electrode to the post-synaptic membrane has been improved by enzymatically removing the nerve ending (McMahon, Spitzer & Peper, 1972). Using these improvements Kuffler & Yoshikami (1975) have thus shown that ACh sensitivity falls abruptly, by a factor of fifty, within 2 um of the subsynaptic membrane.

A similar localization of chemosensitivity has also been observed for other putative transmitters such as glutamate in insect muscle (Usherwood, Machili & Leaf, 1968), for both γ-amino-butyrate and glutamate in crustacean muscle fibres (Takeuchi & Takeuchi, 1964, 1965), and for ACh on parasympathetic neurons in the frog cardiac ganglion (Harris, Kuffler & Dennis, 1971). Thus the localization of transmitter receptors to the post-synaptic membrane may be a common feature at many chemical synapses.

Interaction of snake neurotoxins with the ACh receptor

While the classical cholinergic agents have successfully been used to examine the structure-function relationships for the active site of the ACh receptor, and to map the distribution of chemosensitivity on muscle fibres, the reversibility and relatively low affinity of the drug-receptor interaction have precluded any marked success in biochemically isolating the receptor substance. This difficulty has now largely been overcome by the discovery that the venoms of several elapid snakes contain peptide toxins which act upon the ACh receptor of vertebrate skeletal muscle as specific and irreversible antagonists.

Using starch gel electrophoresis Chang & Lee (1963) separated the venom of an Asian krait, Bungarus multicinctus, into three active fractions which they named α, β, and γ-bungarotoxins. The α-bungarotoxin (αBGT) fraction was found to abolish the response of muscle fibres to ACh without altering the ACh output of the presynaptic nerve terminals and thus resembled curare in its mode of action. However, the toxin differed from curare in that its post-synaptic block was not relieved by even prolonged washing. Using intracellular and extracellular recording techniques Lee & Chang (1966) demonstrated that αBGT and a similar toxin from cobra venom produced a progressive block of the end-plate potential without affecting the resting membrane potential or terminal nerve spike. They also found that curare slowed the rate of development of the post-synaptic block produced by either toxin. The similarities between the effects of curare and the snake toxins, as well as the protection afforded by curare against the toxins, suggested that they were interacting in a competitive fashion with the ACh receptor. Later studies confirmed and extended this

idea (Miledi, Molinoff & Potter, 1971; Lester, 1972a, b). The neurotoxins were found to reduce both the amplitude of the end-plate potential and the response to iontophoretically applied ACh at the frog neuromuscular junction and Torpedo electroplax, and did so at an exponential rate proportional to the toxin concentration. Furthermore, the presence of the ACh antagonist curare, or the agonists nicotine and carbachol, slowed the rate of receptor inactivation. While the protection afforded by these agents increased with their concentration, they were ineffective in reversing the block induced by a previous exposure to the toxin. It can be concluded from these pharmacological studies that the elapid neurotoxins exert their effect by irreversibly blocking the activation of ACh receptors on muscle cells.

Chemically the post-synaptic toxins from elapid snakes are a homologous group of small basic polypeptides (see Lee, 1972) with molecular weights of approximately 8,000. Cobra neurotoxin and αBGT, for example, are composed of 71 and 74 amino acid residues respectively, with similar primary and tertiary structures. Thus, in all the toxins so far studied a similar location has been found for eight half-cysteins, and at least ten other amino acids in the sequence. A similar region in the center of each toxin molecule is thought to be the 'active site' for interaction with the ACh receptor, and contains three basic amino acids and a tyrosine which are important in conferring specificity to the interaction with the receptor. The very slow reversibility of toxin action is thought to be due to the unusually large percentage of hydrophobic amino acids in the toxin molecule.

Localization of ACh receptors at the neuromuscular junction

Since the initial demonstrations that αBGT is a specific antagonist which reacts irreversibly with the ACh receptors on muscle fibres, and that radioactive derivatives of the toxin not only retain its pharmacological properties, but also become localized at the neuromuscular junction (Lee & Tseng, 1966), there have been systematic attempts to develop techniques for determining the number and distribution of toxin-binding sites in cholinergic tissues such as skeletal muscle and the electroplax. In each case protein-tracing techniques which were originally developed for use with immunoglobulins have been extended to the much smaller peptide snake toxins.

The most widely used strategy has been to prepare radioactive derivatives of either cobra neurotoxin or αBGT by iodination (Miledi, Molinoff & Potter, 1971; Raftery, Schmidt, Clark, & Wolcott, 1971; Berg, Kelly, Sargent, Williamson & Hall, 1972) or acetylation with ^{3}H-labelled acetic anhydride (Barnard, Wieckowski & Chiu, 1971; Meunier, Olsen, Menez, Fromageot, Boquet & Changeux, 1972; Chang, Chen & Chuang, 1973a). The radioactive derivatives offer not only a simple means of quantitating the number of binding sites, but also permit the distribution of the bound toxin to be examined by radioautography. Using this approach the number of toxin-binding sites at vertebrate neuromuscular junctions has been estimated at between 10^{7} and 10^{8} (Miledi & Potter, 1971; Barnard, Wieckowski & Chiu, 1971; Fambrough & Hartzell, 1972; Chang, et al., 1973a, b; Porter, Chiu, Wieckowski & Barnard, 1973; Albuquerque, Barnard, Porter & Warnick, 1974). When the surface area of the post-junctional membrane is taken into account this indicates that ACh receptors are concentrated at the neuromuscular

junction with over 10^4 toxin-binding sites per um^2. Recent studies have examined the distribution of toxin-binding sites at the ultrastructural level with either radioautography (Fertuck & Salpeter, 1974, 1976), or an immunoperoxidase technique based upon the use of αBGT as an antigenic agent (Daniels & Vogel, 1975). The distribution of bound toxin was found to be limited to the post-junctional membrane and extended only about a third of the way into the complex folds at mammalian junctions. In fact the distribution of silver grains was found to be consistent with a radioactive source restricted to the region of electron-dense membrane revealed by conventional ultrastructural examination (see Fertuck & Salpeter, 1976). These findings are in agreement with the distribution of ACh sensitivity on normal muscle fibres, and indicate that chemical derivatives of the snake neurotoxins are efficient affinity labels which can be used as biochemical markers for the ACh receptor substance.

Isolation and biochemical properties of the nicotinic ACh receptor

Attempts to isolate the nicotinic ACh receptor have usually started with the electric organs of either eels or the marine electric fish (see reviews Karlin, 1974; Rang, 1975). While the snake neurotoxins abolish miniature end-plate potentials and sensitivity to iontophoretically applied ACh in both tissues without affecting the resting membrane potential (Miledi, Molinoff & Potter, 1971), the electric organs are a richer source of receptors than muscle. As described below, a variety of procedures has been used to isolate a protein fraction from the electroplax which shows binding to snake neurotoxins that is competitively inhibited by reversible cholinergic ligands. In the process several chemical properties of the ACh receptor have been demonstrated.

Early in the attempt to isolate the toxin-binding substance it became evident that it was an integral membrane protein (see Singer & Nicolson, 1972). This was demonstrated by the observation that specific toxin binding remained with tissue membrane fragments unless they were treated with detergents (Changeux, Kasai, Huchet & Meunier, 1970; Changeux, Meunier, & Huchet, 1971; Miledi, et al., 1971; Raftery, et al., 1971; Eldefrawi, Eldefrawi, Seifert & O'Brien, 1972; Franklin & Potter, 1972). In the presence of weakly ionic (Changeux et al., 1970; 1971) or non-ionic detergents (Miledi, et al., 1971; Raftery et al., 1971; Franklin & Potter, 1972) protein-detergent complexes were obtained which exhibited neurotoxin binding that was competitively inhibited by nicotinic drugs, but not by muscarine, physostigmine or choline. On the other hand, strong ionic detergents inactivated the receptor. Attempts to determine such physical properties as molecular weight and subunit composition

were however hampered by both the presence of the detergents necessary to keep the receptor in solution, and the anomalous behavior of the solublized membrane proteins (in comparison to hydrophilic protein standards) during gel-filtration and density-gradient sedimentation. Furthermore the initial efforts to fractionate receptor-containing extracts using physical techniques which separate macromolecules on the basis of their size, electrical charge or isoelectric point produced only moderate increases in specific activity as measured by neurotoxin binding. This difficulty arose from the fact that most of the proteins in the membrane extracts shared similar physical characteristics (see Raftery, Schmidt, Martinez-Carrion, Moody, Vandlen & Duguid, 1973). However, it is worth noting that the physical techniques were effective in separating the specific toxin-binding fraction from virtually all of the acetylcholinesterase activity. In fact the esterase could be removed from the membrane even by mild treatments such as salt-extraction, and therefore is not an integral membrane protein.

Significant increases in purity of the receptor preparations have since been achieved by several groups using affinity-chromatography with resins or gels covalently attached to a variety of cholinergic ligands (Karlson, Heilbronn & Widlund, 1972; Olsen, Meunier & Changeux, 1972; Schmidt & Raftery, 1972, 1973; Eldefrawi & Eldefrawi, 1973; Klett, Fulpius, Cooper, Smith, Reich & Possani, 1973). However, the material isolated in each of these studies has been heterogenous on electrophoresis in the presence of the strongly ionic detergent SDS, which denatures proteins and often reveals their complex subunit composition. This complexity appears to be due to the fact that the ACh receptor exists in the membrane as a complex of several dissimilar polypeptides. While

there is still dispute over the exact subunit composition of the receptor, the most thorough analyses at the present time (Changeux et al., 1976; Raftery, Vandlen, Reed & Lee, 1976) suggest that it is composed of 2-4 proteins with molecular weights between 40- and 64-thousand daltons. These appear to be grouped in a complex composed of 6-8 subunits, forming a receptor unit with 3-4 αBGT binding sites and having a molecular weight of approximately 400,000. Each polypeptide subunit also contains about 3% by weight of carbohydrate which presumably is oriented on the outer surface of the cell membrane.

The membrane glycoprotein isolated from the electroplax has been identified as the ACh receptor on the basis of a reaction with snake neurotoxins which is competitively inhibited by nicotinic drugs. However, the ultimate proof that this substance is the ACh receptor should include the demonstration that it can also modulate cation permeability in response to cholinergic agonists. Michaelson & Raftery (1974) and Hazelbauer & Changeux (1974) have found that vesicles prepared with lipids extracted from the electroplax can be loaded with radioactive ^{22}Na, which exchanges slowly with normal ^{23}Na in the external medium. When purified 'receptor' protein is incorporated in such liposomes they respond to ACh and carbachol with a dramatic increase in the leakage rate of the ^{22}Na. While a more direct demonstration of the functioning of the isolated receptor in an artificial membrane would have been preferable, this result strongly suggests that the purified receptor is capable of functioning as an ionophore in response to cholinergic drugs.

Further evidence indicates that the receptor subunits may be arranged as a toroidal complex within the lipid bilayer, suggestive of the receptor's function as an ion channel through the membrane. This possibility has been

raised by electron microscopic studies of both negatively-stained synaptic membranes and isolated receptor extracts which reveal dense arrays of intramembranous particles that occasionally appear to have a dimple in their centre (Cartaud, Benedetti, Cohen, Meunier & Changeux, 1973; Nickel & Potter, 1973; Landowne, Potter & Terrar, 1975). Anatomical studies of the neuromuscular junction using freeze fracture techniques have also revealed a densely-packed array of intramembranous particles which is confined to the subsynaptic membrane (Peper, Dreyer, Sandri, Akert & Moor, 1974; Heuser, Reese & Landis, 1974; Rash & Ellisman, 1974) and thus coincides with the distribution of ACh receptors.

On the basis of the extensive biochemical evidence it can thus be concluded that ACh receptors are in fact complex integral membrane glycoproteins which are sufficiently large to be visualized directly in the electron microscope, and which are packed in an almost-crystalline array in the subsynaptic membrane of the neuromuscular junction.

Neural regulation of the distribution of ACh receptors in adult muscle

While it is evident that ACh receptors exist at a very high density in the post-junctional membrane, and are virtually absent from the rest of the sarcolemma of normal adult muscle fibres, it is not clear how such a non-random distribution of receptors could have developed. In fact this localized aggregation of receptors is a major anomaly in the light of current theories in which biological membranes are viewed as fluids containing a diversity of freely diffusing glycoproteins and glycolipids (see Singer & Nicholson, 1972). A central prediction of this 'fluid-mosaic' model of the membrane is that random thermal motion would lead to a uniform distribution of integral membrane glycoproteins such as the ACh receptor. If this model is correct there are likely to be specialized mechanisms which preserve the highly-ordered arrangement of receptors in the postjunctional membrane. Furthermore, since ACh receptors are concentrated only at sites of innervation the mechanism of receptor localization must depend upon positional cues provided by some form of interaction between nerve and muscle cells.

For some time it has been evident that this distribution of receptors is dependent upon normal innervation. When a motor nerve is damaged, for example by severing the axons, a number of changes occur in the denervated muscle fibres over periods from a few days to months. Within a matter of days the entire extrajunctional membrane becomes sensitive to ACh (Ginetzinsky & Shamarina, 1942; Axelsson & Thesleff, 1959; Miledi, 1960b; Albuquerque & McIsaac, 1970). Biochemical and radioautographic techniques based upon the binding of snake neurotoxins have indicated that this

extrajunctional chemosensitivity is due to the widespread appearance of ACh receptors (Lee, Tseng & Chiu, 1967; Miledi & Potter, 1971; Berg, Kelly, Sargent, Williamson & Hall, 1972; Hartzell & Fambrough, 1972; Libelius, 1975). However both the level of extrajunctional chemosensitivity and the density of toxin-binding sites remain lower than at the neuromuscular junction. In fact the density of extrajunctional toxin-binding sites only reaches levels of 10^2 - 10^3 per um^2 (Hartzell & Fambrough, 1972; Libelius, 1975), which is at least an order of magnitude less than that observed at the adult neuromuscular junction (Miledi & Potter, 1971; Barnard et al., 1971; Fambrough & Hartzell, 1972; Porter et al., 1973; Chang et al., 1973a, b; Albuquerque et al., 1974).

Several studies have sought to determine the source of the extrajunctional receptors in denervated muscle. Katz & Miledi (1964 b) demonstrated that nerve-free muscle segments also developed a high level of chemosensitivity, which indicated that the extrajunctional receptors must either have existed throughout the muscle fibre in an inactive form, or else been synthesized de novo after removal of the neural influence. Later studies have found that the increase in chemosensitivity following denervation is prevented by a variety of drugs which block the synthesis of either protein or RNA (Fambrough, 1970; Grampp, Harris & Thesleff, 1972; Kimura & Kimura, 1973; Chang & Tung, 1974; Sakman, 1975). More direct evidence that the extrajunctional receptors are synthesized and inserted into the membrane following denervation has recently been provided by Brockes & Hall (1975a). After incubation of denervated rat diaphragms in organ culture in the presence of ^{35}S-methionine, affinity chromatography was used to purify an extract of ACh receptors. This extract was found to

to contain ^{35}S-labelled material which could not be separated from ACh receptors by either velocity sedimentation or isoelectric focussing. Similar treatments of diaphragm which had not yet begun to develop extrajunctional receptors produced a receptor extract without the radioactive label. However, given the difficulty experienced in attempting to separate ACh receptors from other membrane proteins using such physical techniques (see Raftery, et al., 1973) it is not certain to what extent the receptor extract might have been contaminated by other radioactive membrane constituents. A somewhat different technique was used by Devreotes & Fambrough (1976) who carried out similar experiments on mouse leg muscles. In these experiments newly synthesized proteins were labelled by incubating denervated muscles in the presence of heavy deuterium-labelled amino acids. The new receptors appearing in the sarcolemma during the incubation were labelled by first blocking the pre-existing receptors with native αBGT and later exposing the muscles to ^{125}I-αBGT. Control muscles were used as a source of normal receptors and were labelled with ^{131}I-αBGT. When newly-synthesized detergent-solublized membrane proteins were partially separated from the older ones by buoyant density centrifugation, the receptors which had appeared during the period in organ culture were found to have a greater density than the receptors from control muscles. This clearly demonstrates that at least some of the extrajunctional receptors had been synthesized from heavy amino acids. On the basis of these experiments it can be concluded that denervation of muscle has dramatic effects on the metabolism of ACh receptors, and does not merely lead to the activation of previously-dormant receptors which were already present throughout the muscle cell.

The experiments described so far found no dramatic changes in the density of receptors at old end plates following denervation. Recently however, Frank, Gautvik and Sommerschild, (1975) have demonstrated that denervation also affects junctional receptors. They observed that five weeks after denervating rat soleus muscles the old end plates contained only about half as many ACh receptors as normal muscle fibres. It thus appears that the loss of normal nerve contact results in both the synthesis of ACh receptors and the development of a more random distribution of the receptor population.

It has been conventional to view the neural regulation of ACh receptor distribution in terms of a restriction on the development of extrajunctional receptors (see reviews Guth, 1968; Harris, 1975). This orientation has arisen rather naturally since in most experimental situations dramatic changes have been observed in extrajunctional chemosensitivity without correspondingly obvious changes at the neuromuscular junction. An important focus of interest has therefore been the mechanism by which the neural influence is transmitted to the muscle cell.

There are several forms of message which motor neurons might conceivably employ in order to influence the muscle fibres with which they are in contact. These can be grouped into two general categories on the basis of their involvement in the sequence of events which leads to muscle contraction. Thus, in priniciple, the interaction of ACh with the post-synaptic membrane, depolarization of the sarcolemma, or the contraction of the muscle cell could each be important for the regulation of ACh receptors. On the other hand, neural influence might be expressed by some mechanism independent of activity, such as the release of a 'trophic' substance or the actual

physical contact between the two cell surfaces. While these latter possibilities remain somewhat speculative due to a lack of well-documented biological precedents, they are likely to reflect the means of 'inductive' influence between nonexcitable cells. Furthermore, since there is no reason to presume that only a single regulatory factor is involved in the interaction between nerve and muscle, a combination of the above alternatives may be required in any of the several possible permutations. It is also conceivable that the nature of the neural influence may not remain constant throughout the life of an organism. In particular, somewhat different mechanisms might operate in embryonic tissue while the synapse is forming, compared to the adult where only maintenance of structure is required.

It is also worth noting that innervation is important in the regulation of several characteristics of normal muscle fibres in addition to the distribution of ACh receptors. This has been demonstrated by experiments in which the resting membrane potential (Albuquerque, Shuh, & Kaufman, 1971) membrane resistance (Albuquerque & McIsaac, 1970) and sensitivity of action potential generation to tetrodotoxin (Redfern & Thesleff, 1971) have all been found to change after denervation. It is also clear that the high level of junctional acetylcholinesterase (Filogamo & Gabella, 1966; Sonesson & Thesleff, 1968) and the complex folding of the post-junctional membrane (Reger, 1959; Birks, Katz & Miledi, 1960; Bauer, Blumberg & Zachs, 1962; Nickel & Waser, 1968; Miledi & Slater, 1968) likewise depend upon innervation. It is therefore likely that mechanisms which mediate the neural influence on the distribution of ACh receptors also affect other substances involved in synaptic transmission.

To determine whether the restriction of extrajunctional chemosensitivity is dependent upon synaptic activity the most common approach has been to remove some component of physiological activity while leaving synaptic structure intact. It has remained difficult however to demonstrate that pharmacological agents which are effective for this approach do not also disrupt some other biological process in addition to their conspicuous effect on synaptic transmission. Despite this drawback the chronic effects of several neuromuscular blocking agents have been examined to determine whether they might mimic denervation. Chronic blockade of synaptic activity by either botulinum toxin (Thesleff, 1960; Tonge, 1974; Bray & Harris, 1975), β-bungarotoxin (Hofmann & Thesleff, 1972), αBGT (Chang, Chang & Huang, 1975) or curare (Berg & Hall, 1975a) leads to a development of extrajunctional ACh receptors analogous to that produced by denervation. Similar results were also obtained by Lomo & Rosenthal (1972) who used chronic topical application of a local anaesthetic onto the sciatic nerve to block impulse conduction, while allowing sub-threshold spontaneous activity. The practical difficulties inherent in this approach were demonstrated by the later discovery that local anaesthetics also block axoplasmic transport from the soma to the nerve endings and would be expected to interfere with other processes dependent upon supplies from the cell body (see Anderson & Edstrom, 1973).

More convincing evidence that synaptic activity has long-term effects on the distribution of ACh receptors has come from experiments in which chronic electrical stimulation of a denervated muscle has been shown to produce a reduction in the extrajunctional chemosensitivity (Jones & Vrbova, 1970; Lomo & Rosenthal, 1972; Drachman & Witzke, 1972; Purves & Sackman, 1974; Lomo & Westgaard, 1975). These studies have not only demonstrated that artificially -induced contractile activity can reduce

the chemosensitivity of denervated muscle fibres, but that appropriate patterns of stimulation restore the level of sensitivity to the range found in normal innervated fibres (Lomo & Westgaard, 1975). However, in muscle fibres where extrajunctional chemosensitivity had been reduced to normal levels by electrical stimulation, no changes were observed in the level of sensitivity at the region of the old end plate. These studies therefore indicate that extrajunctional receptor density is profoundly affected by the level of muscle activity, and suggest that the mechanism which maintains the high density of ACh receptors at the neuromuscular junction may either operate independently of activity or be too slow a process to have been detected in these experiments.

There is, however, also evidence that factors independent of activity have an effect on extrajunctional chemosensitivity. For example, blocking axoplasmic transport by the application of colchicine or vinblastine to motor nerve trunks, either by injection into the nerve sheath or implanting silastic cuffs containing the drug around the nerve, results in the development of extrajunctional chemosensitivity in innervated muscle (Hofmann & Thesleff, 1972; Albuquerque, Warnick, Tasse, & Sansone, 1972; Pilar & Landmessar, 1972). This increased sensitivity was observed in muscle fibres which had miniature end plate potentials and could be activated by electrical stimulation distal to the region treated with the drug. However these studies did not exclude either a direct effect of the drugs on the muscle itself (see Lomo, 1974) or on the pattern of muscle usage. They nevertheless provide suggestive evidence that another neural mechanism dependent upon axonal transport may be important in restricting extrajunctional chemosensitivity.

There is also more convincing evidence that nerve contacts have effects on the distribution of ACh receptors that are independent of muscle activity. Miledi (1960a) partially denervated multiply-innervated frog muscle fibres, and subsequently found an increased ACh sensitivity in the vicinity of denervated end plates but not around functional end plates on the same muscle fibres. Since muscle contractile activity would have been similar throughout an individual fibre under these conditions, some other effect of innervation must have been withdrawn. Another line of evidence has shown that the time before the onset of denervation changes, such as the development of extrajunctional ACh receptors, increases with the length of the stump between the point of nerve section and the muscle (Luco & Eyzaguirre, 1955; Emmelin & Malm, 1965; Salafsky, Bell & Prewitt, 1968; Harris & Thesleff, 1972). Since muscle activity stops at the time of denervation, some other neural factor, presumably delivered to the nerve ending by axoplasmic flow, must retard the development of extrajunctional chemosensitivity. Furthermore when motor nerves regenerate either after denervation (Miledi, 1960c; Bennett, Pettigrew & Taylor, 1973) or botulinus intoxication (Bray & Harris, 1975) the level of extrajunctional sensitivity falls before the regenerating nerve is able to elicit muscle contraction. Recently both Lavoie, Collier & Tenenhouse (1976) and Pestronk, Drachman & Griffin (1976) have examined the effects of both muscle inactivity and surgical denervation on the development of extrajunctional receptors, measured as ^{125}I-αBGT binding sites, in mouse leg muscles. Inactivity, brought about by placing silastic cuffs containing tetrodotoxin onto the sciatic nerve, or by its injection into the nerve sheath, resulted in the development of extrajunctional receptors. However, the rate of appearance of new receptors, and their final

level after seven days was considerably greater in the denervated muscles than those with the nerve-conduction block.

From this evidence it therefore appears that motor nerves restrict the development of extrajunctional receptors both by evoking muscle contraction, and also by a still-obscure mechanism which is independent of synaptic activity.

While studies of the changes in ACh receptor distribution produced by denervation and other means in adult muscle have produced many important insights, they have not revealed any physiological role or purpose for extrajunctional receptors. Likewise they provide almost no information regarding the means whereby unusually high densities of junctional receptors develop in the sarcolemma at sites of innervation.

Studies in adult muscle have however shown that conditions which lead to the development of extrajunctional chemosensitivity also cause muscle fibres to become receptive to further innervation. This correlation has led to the suggestion that a high level of chemosensitivity may be a requirement for the formation of synapses between muscle and nerve (Katz & Miledi, 1964; Fex, Sonesson, Thesleff & Zelena, 1966). Of great significance also has been the discovery by Diamond & Mildei (1962) that foetal and neonatal muscle fibres in the rat diaphragm have a widespread sensitivity to ACh which resembles the distribution in denervated muscle. This suggested that the effect of denervation was to produce a reversal of the normal developmental sequence, and was not a mere experimental or pathological curiosity. It also revealed that the remarkable localization of ACh receptors at sites of innervation was the result of a somewhat gradual process related to the differentiation of the muscle cell. Innervation can

in fact be seen as inducing a final step in muscle differentiation whereby ACh receptors change from the more uniform distribution characteristic of most integral membrane glycoproteins to the non-random arrangement found on normal adult fibres. The localization of receptors at the site of innervation is therefore a developmental process, and the question of mechanism should most appropriately be examined in developing muscle. As is described in the following sections experimental difficulties involved in working with normally developing embryos have led to a reliance on cell culture techniques, and evidence concerning developmental changes in the distribution of ACh receptors remains quite indirect.

Acquisition of ACh receptors during the normal development of the neuromuscular junction

Anatomical changes occurring during the normal development of nerve-muscle junctions have been examined at the ultrastructural level in a variety of vertebrates such as amphibians (Lentz, 1969) the chick (Hirano, 1967; Atsumi, 1971), rat (Teravainen, 1968a, b; Kelly & Zachs, 1969) and human (Juntunen & Teravainen, 1972). Several common events have emerged from these studies. The earliest evidence of structural specialization along the intramuscular branches of growing motor nerves was the development of 'synaptic' vesicles at otherwise undifferentiated regions of nerve-muscle contact (Hirano, 1967; Teravainen, 1968b; Lentz, 1969; Kelly & Zachs, 1969). At a presumably later stage of development an increased density of heavy-metal staining was found in the sarcolemma opposite nerve terminals containing vesicles (Hirano, 1967; Teravainen, 1968b; Kelly & Zachs, 1969; Lentz, 1969). Somewhat later still the development of basement membrane in the synaptic cleft and post-junctional folds was observed (Hirano, 1967; Lentz, 1969; Teravainen, 1968b). Histochemically detectable acetylcholinesterase also appeared to develop late, after the onset of physiological activity (Teravainen, 1968a) and at the time post-junctional folds had begun to form (Lentz, 1969). A significant complication for studies of the developmental changes in muscle associated with innervation was also illustrated in these experiments. Even within a single muscle the junctions did not develop synchronously, and considerable variation was found in synaptic morphology at most developmental stages (Teravainen, 1968b; Kelly & Zachs, 1969). In the light of recent evidence that the distribution of ACh receptors at adult mammalian end plates may be co-extensive with the region of post-synatpic 'thickening'

observed in conventional ultrastructural studies (see Fertuck & Salpeter, 1974, 1976), these results suggest that the development of a local region of high receptor density associated with the site of nerve-muscle contact may be one of the earliest signs of post-synaptic specialization. However there is no direct evidence that such heavy-metal staining is invariably associated with a high receptor density (see Vogel & Daniels, 1976).

It has not been determined whether ACh receptors are present on developing muscle fibres prior to nerve contact during normal development, but several lines of evidence suggest this is the case. Giacobini, Filogamo, Weber, Boquet & Changeux (1973) examined the development of receptors in the chick limb bud. They found that binding of ^{3}H-labelled cobra neurotoxin became detectable during the developmental stage in which the limb bud was first invaded by growing nerve fibres. Since synapse formation as revealed by neurogenic motor behavior began at the same stage, it is difficult to estimate the sequence of events on individual cells. The experimental situation has been found to be somewhat simpler in the case of myotomal muscle cells in amphibian embryos which undergo a more synchronous sequence of developmental changes than is found in the limb bud. It has thus been determined that myotomal muscle cells depolarize to bath applied ACh about 1-2 hours before nerve processes grow out of the developing neural tube (Kullberg, 1974; Lentz, T., unpublished observations). Specific binding of ^{125}I-labelled αBGT can also be detected at the same stage, before the outgrowth of nerve fibres (I. Chow, unpublished observations).

Thus the study of receptor production and incorporation into the sarcolemma has proved difficult in normally developing embryos of the higher vertebrates, and many studies have resorted to the use of cell culture.

Development of ACh receptors in cultured embryonic muscle

Primary cultures of chick, rat and mouse myogenic cells have been used in numerous studies of muscle development (see reviews, Murray, 1972; Nelson, 1975). In these preparations mononucleated cells are obtained by disrupting embryonic muscle tissues with trypsin and mechanical agitation, and are then sprinkled into culture chambers which have a collagen-coated substrate. The mononucleated cells which survive this treatment are composed of a mixture of fibroblasts and myoblasts that are indistinguishable morphologically. After a period of cell proliferation, which can be controlled to some extent by the culture conditions, the migratory myoblasts fuse to form compound multinucleated myotubes. These are stationary cells that continue to fuse with myoblasts and other myotubes to produce elongated cells reminiscent of muscle fibres in vivo. They usually develop striations and, when older, often undergo spontaneous contraction. However, the fibroblasts also continue to multiply and may obscure the developing myotubes unless the cultures are treated with drugs which are toxic to dividing cells. After such treatment virtually pure cultures of long striated myotubes are obtained (Fischbach, 1972). These cultures allow ready access to individual muscle cells and can reproducibly be obtained in substantial quantities. They have thus provided a considerable amount of information regarding the synthesis, insertion into the sarcolemma and eventual degradation of ACh receptors on non-innervated embryonic muscle cells.

Fambrough & Rash (1971) examined cell cultures of rat myoblasts and myotubes to determine at which stage sensitivity to iontophoretically applied

ACh could first be detected. While they observed only a small population of precocious mononucleated cells sensitive to ACh, all of the myotubes showed depolarizing responses to the drug. In another study using a cloned cell line of rat myoblasts Patrick, Heinemann, Lindstrom, Schubert & Steinbach (1972) found that specific binding of ^{125}I-labelled cobra neurotoxin also began at about the time of myoblast fusion. Hartzell & Fambrough (1973) examined the kinetics of the incorporation of ACh receptors, measured as ^{125}I- αBGT binding sites, into the sarcolemma of growing chick and rat myotubes in cell culture. They exploited the irreversible binding of native toxin to mask 'old' receptors and then measured the appearance of new sites with radioactive toxin. These results were compared with both iontophoretic mapping of ACh sensitivity and radioautography, which revealed the distribution of both the total and new receptor populations. They found an approximately steady rate of incorporation which amounted to a 4-5% increase in the number of receptors per hour. This increase was greater than the change in surface area over the same time interval and thus reflected an increase in the density of receptors in the surface membrane. The increase in the number of binding sites was the result of active processes within the cells and could be prevented by cold, metabolic inhibitors and cycloheximide, but not by αBGT or actinomycin-D. This suggested that the increase in number of toxin-binding sites was dependent upon the synthesis of new protein but not new messenger RNA. The synthesis of new receptors has been implicated more directly by Devreotes & Fambrough (1976) who demonstrated the incorporation of heavy deuterium-labelled amino acids into new toxin-binding material on rat and chick myotubes. The increase in the density of toxin binding sites observed by Hartzell &

Fambrough (1973) was paralleled by an equivalent increase in ACh sensitivity. In addition, the distribution of binding sites and chemosensitivity was uniform over the cell surface for both the entire population of receptors and those which appeared after an initial block with αBGT.

Devreotes & Fambrough (1975, 1976) have extended these studies to examine the pathway of ACh receptor metabolism in cultured chick and rat myotubes. They observed, in addition to the population of receptors in the sarcolemma which could be pulse-labelled with extracellular αBGT, two additional membrane-bound 'pools' of specific toxin-binding sites which could be labelled in detergent extracts of the cells. One of these, which was equivalent in number to 30% of the surface sites, could be saturated by overnight exposure to extracellular αBGT. This pool was not sensitive to treatment of the cells with protein synthesis inhibitors, and probably corresponds to surface receptors in the process of degradation. In other experiments the kinetics of receptor turnover was examined after pulse-labelling the surface receptors with ^{125}I-αBGT. Radioactivity was released from the labelled muscle cells with a half-time of 22-24 hours, and could be accounted for quantitatively by the appearance of ^{125}I-tyrosine in the culture medium. In the presence of protein synthesis inhibitors which block the appearance of new receptors, the release of radioactivity after pulse-labelling paralleled the decline in the number of receptors in the membranes of control myotubes. These results indicate that surface-bound toxin was taken up into the muscle cells in association with ACh receptors and degraded by proteolytic enzymes. Electron microscope radioautography further indicated that uptake of the labelled toxin had occurred into secondary lysosomes within the muscle cells, which suggested

that they were probably the sites responsible for the degradation of the toxin-receptor complexes.

The second pool of cytoplasmic receptor sites, corresponding to about 10% of the number of surface receptors, could not however be labelled even by overnight exposure to extracellular αBGT. Also, during treatment with the protein synthesis inhibitor puromycin, the number of binding sites in this pool declined to zero by the time new receptors had stopped appearing in the sarcolemma. It is therefore likely that this pool represents newly synthesized receptors awaiting insertion into the surface membrane.

It would seem on the basis of these observations that extrajunctional ACh receptors are first synthesized from amino acids into a precursor pool of receptors that is associated with cytoplasmic membranes. After a period of time, which may be required for glycosylation, these newly-synthesized receptors are inserted into the sarcolemma. Subsequently the receptors are lost from the surface in a random first-order process and become incorporated in a pool associated with lysosomal granules where they are presumably degraded back to their constituent amino acids. Essentially similar patterns of receptor metabolism have been observed in denervated adult muscle where extrajunctional receptors are also synthesized de novo from amino acids (Brockes & Hall, 1975a; Devreotes & Fambrough, 1976) and later removed from the sarcolemma for proteolytic degradation (Berg & Hall, 1974, 1975b). In each case the half-life of the extrajunctional receptors on the surface of avian and mammalian muscle cells was about one day.

In the above experiments, carried out in culture during the period of

rapid cell growth and net receptor synthesis, the distribution of ACh receptors was uniform over the cell surface. The density of receptors in the sarcolemma could thus be seen simply as a steady state determined by the relative rates of synthesis and turnover. Later in their development however, both chick (Vogel, Sykowski & Nirenberg, 1972; Fischbach & Cohen, 1973; Sytkowski, Vogel, & Nirenberg, 1973) and rat myotubes (Hartzell & Fambrough, 1973) have been found to develop discrete regions of the sarcolemma with a high density of ACh receptors, in addition to a widespread area of lower receptor density. This distribution was determined using both iontophoretic mapping (Fischbach & Cohen, 1973, Hartzell & Fambrough, 1973) and radioautography after labelling the receptors with ^{125}I- αBGT (Vogel et al., 1972, Fischbach & Cohen, 1973, Sytkowski et al., 1973; Hartzell & Fambrough, 1973). Sytkowski et al,, (1973) also examined the time course of the appearance of the dense receptor patches on chick myotubes in relation to the total number of receptors, measured as toxin-binding sites. The total number of sites increased from days 3-8 in cultures and then remained at a constant level. The dense receptor patches however appeared on about the seventh day in culture, and could be found on more than 80% of the myotubes by the eleventh day. Their appearance thus coincided with the levelling off in the total number of receptors. Of particular interest was the finding that the density of toxin-binding sites in the patches was comparable to that of the subneural membrane of adult vertebrate muscle fibres in vivo, and about ten-fold greater than the density over the remainder of the sarcolemma.

On the basis of these findings it can be concluded that the ability to develop densely-packed regions of ACh receptors is a characteristic of muscle itself which does not require any interaction with nerve processes.

It is not clear how such a non-random distribution of ACh receptors might develop, however, nor why the dense patches should only form late in the maturation of the myotube. In any case, as such a distribution has not been found for other membrane receptors (see reviews Singer & Nicolson, 1972; Nicolson, 1974) it is likely that the mechanism which produces the localization of the receptors in dense patches on cultured myotubes is related to that responsible for the development of the post-synaptic accumulation of receptors on innervated muscle cells in vivo.

Formation of the neuromuscular junction in culture

As a tool in neurobiology tissue culture had an auspicious beginning. R. G. Harrison (1910) invented the technique in order to study neural development and, in the process, firmly established the neuronal theory. It is sometimes forgotten that in the same study he demonstrated the formation of functional nerve-muscle connexions. He noted that explants of embryonic tadpole tail muscle failed to twitch spontaneously unless pieces of neural tissue were included. In subsequent years the formation of functional connexions and anatomically normal neuromuscular junctions *in vitro* has been demonstrated in a variety of tissues using modern electrophysiological and microscopic techniques. A distinction needs to be made, however, between 'tissue culture' and 'cell culture'. In the former, substantial pieces of tissue are maintained *in vitro* as an aggregate of cells, frequently retaining much of the three-dimensional structure of the original organism. In cell cultures this organization is completely destroyed to obtain a scattering of isolated cells over the substrate. This isolation of cells is required for many forms of experimental observation, particularly those involving identification of individual cells in the light microscope, but distorts a large number of regulatory processes presumably mediated by cell contact. This introduces problems which are usually observed as the failure of cells to either mature normally or survive. Such difficulties are encountered eventually in all 'primary' cultures, but may pose particular problems for the study of synapse formation in cell culture.

Several studies have reported evidence for the formation of neuromuscular junctions in explant cultures of skeletal muscle and spinal cord (Bornstein,

Iwanami, Lehrer & Breitbart, 1968; James & Tresman, 1968, 1969; Nakai, 1969; Veneroni & Murray, 1969; Peterson & Crain, 1970; Crain & Peterson, 1971; Kano & Shimada, 1971a, b; Pappas, Peterson, Masurovsky & Crain, 1971; Robbins & Yonezawa, 1971a, b; Cohen, 1972; Giller, Schrier, Schainberg, Fisk & Nelson, 1973). Some of these studies also found relatively mature nerve-muscle junctions as determined either by ultrastructural criteria (Peterson & Crain, 1971; Pappas et al., 1971) or by the development of junctional acetylcholinesterase (Bornstein et al., 1968; Peterson & Crain, 1970; Pappas et al., 1971). Other studies have examined monolayers of avian and mammalian myotubes cultured with spinal cord either as small explants or dissociated cells. While numerous cases of functional innervation were observed (Fischbach, 1970, 1972; Robbins & Yonezawa, 1971a, b; Fischbach & Cohen, 1973; Shimada & Fishman, 1973; Fambrough, Hartzell, Rash & Ritchie, 1974), there was little evidence for either ultrastructural specialization (Shimada, Fishman & Moscona, 1969a, b; Fambrough et al., 1974) or the development of junctional acetylcholinesterase (Fischbach, 1972). Both the frequency of miniature endplate potentials and the mean quantal content of evoked end-plate potentials were also low in comparison with adult junctions in vivo (Robbins & Yonezawa, 1971b; Fischbach, 1972; Cohen & Fischbach, 1973). However one important 'structural' alteration was observed in these studies. An increased sensitivity to iontophoretically applied ACh was found on the cultured myotubes at sites of functional innervation (Kano & Shimada, 1971; Fischbach & Cohen, 1973). Similarly an increased density of ^{125}I-αBGT binding sites was found on the myotubes in the vicinity of functional nerve-muscle contacts (Fambrough, et al, 1974). In the latter study a large

number of non-functional contacts showed no increase in receptor density. It does therefore appear that cell cultures of nerve and muscle develop characteristics of immature junctions, such as the release of transmitter, and a rudimentary subsynaptic membrane with an increased density of ACh receptors.

It is not clear, however, in what manner functional nerve terminals become associated with regions of the sarcolemma that contain a high density of ACh receptors. Since muscle fibres can develop local regions of high receptor density in the absence of neural tissue (Vogel et al., 1972; Fischbach & Cohen, 1973; Hartzell & Fambrough, 1973; Sytkowski et al., 1973) there are two fundamentally different mechanisms which could explain this association. These two alternatives reflect quite different means of neural influence, and thus have important implications for an understanding of the neural regulation of receptor distribution in the adult as well.

In one case (see Sytkowski et al., 1973) it is the muscle cell which initiates the process of synaptogenesis by providing discrete regions of high ACh receptor density. The action of the nerve process in this situation would be to search for the appropriate sites specified by the muscle cell. After synaptic contact is established the role of the nerve terminal would thus be expected to remain passive, perhaps simply to protect receptors in the contacted patch from the process of turnover. As the junction matured, the limiting effect of muscle activity on receptor metabolism might lead to a reduction in the extrajunctional receptor density resulting in the characteristic receptor distribution of normal adult muscle fibres. The distinguishing feature of this 'model' is the very limited role assigned to the process of neural induction in determining the

distribution of ACh receptors on the muscle cell.

The principal alternative hypothesis is that an inductive influence provided by the developing nerve causes the development of a new region of high receptor density in the subneural membrane. This view in fact assigns a more dominant role to the developing nerve, and is directly related to the concept of an inductive or 'trophic' effect of nerve contact in the regulation of extrajunctional ACh receptors in adult muscle. As mentioned earlier, the involvement of such an inductive process in the regulation of ACh receptor distribution is still speculative, and the evidence is based largely on the fact that muscle activity cannot alone account for all the experimentally observed changes in the density of extrajunctional receptors. However, other post-synaptic features such as the complex folding of the sub-synaptic membrane and the high level of cholinesterase activity develop on muscle cells only in response to innervation. They therefore provide evidence that innervation causes the development of local structural specialization by a poorly understood inductive mechanism. It is therefore not implausible that a similar mechanism might also be responsible for the development of a high local density of junctional ACh receptors.

Formulation of the problem

As described in the preceding account, several studies have shown that functionally innervated myotubes in cell culture have local regions of either high chemosensitivity (Kano & Shimada, 1971; Fischbach & Cohen, 1973) or high ACh receptor density (Fambrough et al., 1974) at the site of nerve contact. It is not known, however, whether developing nerve processes become associated with pre-existing patches of high receptor density, or alternatively the high receptor density at the site of innervation is induced by the nerve. The present investigation is an attempt to answer this question, and also to gather further information concerning the molecular mechanisms involved in the changes which occur in ACh receptor distribution during development of the muscle cell.

As a preliminary to describing the rationale behind the present study it is instructive to consider the reasons why the above questions have not been answered by previous investigations. In my view previous studies have been limited by at least two important technical difficulties. Firstly, a deficiency was observed in the systems of cell culture available for the study of the developmental changes that occur as a result of innervation. Consequently, only a limited amount of morphological differentiation took place at the developing nerve-muscle junctions (see Fischbach & Cohen, 1973; Fambrough et al., 1974), and it was not possible to determine whether the limited changes which were observed in the distribution of ACh receptors, were due to a lack of contractile activity in the muscle cells, or to the other inherent limitations of the culture system. Perhaps of even greater importance, the techniques available for examining the distribution of ACh receptors have severely limited the nature of the observations which can

be made. Iontophoretic mapping, for example, is very time consuming and not really suited for the systematic sampling of a large number of cells. Radioautography does not share this particular limitation, but can only be carried out on fixed dried cells. It cannot therefore be used for examining changes in the distribution of receptors on identified single cells. In addition, both techniques have a limited spatial resolution which can only be overcome by very laborious means (see Kuffler & Yoshikami, 1975; Fertuck & Salpeter, 1976).

The present study has thus concentrated on an attempt to circumvent both of these limitations by developing new experimental techniques for determining the distribution of ACh receptors on muscle cells, and also for studying the formation of nerve-muscle junctions in cell culture. The investigation can be divided into four separate sections: (1) the development of a simple fluorescent staining technique for examining the distribution of ACh receptors on muscle cells, (2) the design of a new monolayer culture system for nerve and muscle, based upon cells obtained from rapidly developing amphibian embryos, (3) the characterization of the different patterns of receptor distribution on both innervated and non-innervated muscle cells in culture, and (4) an examination of the mechanism whereby changes occur in the distribution of ACh receptors in the sarcolemma of cultured embryonic muscle cells, both as a result of innervation and in the absence of neural influence.

The fluorescent staining technique developed in the first section of this study (Chap. 2) has a more limited sensitivity than either radioautography or iontophoretic mapping, but is quite adequate for

revealing the topographical distribution of ACh receptors in regions of high packing density such as the subneural membrane. Furthermore, fluorescent staining has several advantages which have been of great significance for the present study: (1) it allows the distribution of ACh receptors to be examined visually and then recorded in a brief photographic exposure, (2) in routine use the spatial resolution is superior to that obtainable with either radioautography or iontophoretic mapping, (3) it allows the distribution of ACh receptors to be examined directly on living muscle cells, thus combining important advantages of both previous techniques for determining ACh receptor distribution, and (4) it is an irreversible stain for individual receptor 'molecules' in the sarcolemma, and can thus be used to follow changes in their location with time on single identified muscle cells.

The second portion of this study (Chap. 3) describes some interesting features of the new cell culture system and then goes on to examine the effect of innervation on the distribution of ACh receptors on the cultured muscle cells. The culture system, which uses myotomal muscle and neural tube cells from rapidly developing amphibian embryos, turns out to be a useful preparation for the study of changes in ACh receptor distribution. Like other vertebrate skeletal muscle cells in culture, myotomal cells develop discrete patches of high receptor density which, according to my results, can readily be visualized with fluorescent staining. Furthermore, when neural tube cells are included, they form functional nerve-muscle junctions, and develop a dense array of ACh receptors in the subsynaptic membrane. Conveniently, only the functionally innervated cells in this system undergo spontaneous contractions, which allows them to be identified even without the use of electrical recording techniques (see Cohen, 1972). This can be

contrasted with cultured myotubes from higher vertebrates which fibrillate spontaneously. The most dramatic feature of these cultures is, however, the great speed with which they differentiate. Since cells in culture are inevitably growing under sub-optimal conditions, such a rapid development allows a much greater degree of differentiation before the eventual period of decline. This difference in the rate of development is demonstrated most directly by the time required for the cells to develop dense patches of ACh receptors. While cultured chick myotubes need seven or more days of development in vitro before receptor patches can be detected (Sytkowski et al., 1973), they are found on myotomal muscle within one day of plating the cells, and only two days after egg fertilization. This rapid differentiation relative to the rate of cell metabolism (which would be expected to be lower in cold-blooded amphibian tissue than in either birds or mammals) provides a unique opportunity to observe developmental processes which would otherwise be very difficult to detect.

The last portion of this study (Chap. 3 and 4) involves an analysis of the mechanism which produces changes in the distribution of ACh receptors during innervation. The analysis has proceeded along three main lines. Firstly I have tested the effects of neuromuscular blocking agents, which prevent both neurogenic electrical activity and muscle contraction, on the changes in receptor distribution brought about by innervation. Secondly, I have examined the effects of delaying innervation by a further 2-3 days to determine whether neural influence is directed at the process whereby newly synthesized receptors are inserted into the sarcolemma, or alternatively whether it affects the population of receptors already in the membrane. Lastly, I have carried out sequential observations of the

same receptor population on individual identified muscle cells, both in the absence of any neural influence and during the process of innervation. The results demonstrate the existence of a nerve-induced process of receptor redistribution whereby extrajunctional receptor patches disappear, and receptors aggregate in the sarcolemma at the site of innervation. They also demonstrate the involvement of a spontaneous receptor redistribution in the formation of the dense ACh receptor patches on non-innervated cells.

CHAPTER 2

FLUORESCENT STAINING OF ACETYLCHOLINE RECEPTORS IN VERTEBRATE SKELETAL MUSCLE

SUMMARY

1. α-Bungarotoxin was labelled with fluorescent dyes and used as a stain for visualizing the distribution of acetylcholine receptors in vertebrate skeletal muscle fibres.

2. Dye-toxin conjugates had the same pharmacological properties as native toxin, but their potencies were lower.

3. Fluorescent staining was examined in teased muscle fibres. The stain was found to be confined to the neuromuscular junction and associated with the subsynaptic membrane.

4. Staining intensity was reduced by curare and even more so by carbachol, but not by atropine or neostigmine. Pretreatment of muscles with unlabelled α-bungarotoxin entirely prevented staining.

5. The staining at amphibian neuromuscular junctions was characterized by a pattern of intense transverse bands occurring at intervals of approximately 0.5-1 u, with fluorescence of lower intensity between them. Fluorescent staining was not detected on adjacent, extrasynaptic, muscle membrane. In side views the staining appeared as a fine line with small protuberances occurring at the same intervals as the intense bands seen face-on. These results indicate that acetylcholine receptors are associated with the entire subsynaptic membrane, including the membrane of the junctional folds and that their density changes abruptly at the border between synaptic and extrasynaptic muscle membrane.

INTRODUCTION

The distribution of acetylcholine receptors in vertebrate skeletal muscle has for a long time been studied by measuring muscle responses to topical application of acetylcholine and its analogues (Langley, 1907). Several refinements, including the use of single muscle fibres (Buchthal & Lindhard, 1937; Ginetzinsky & Shamarina, 1942; Kuffler, 1943), the development of the ionophoretic method of applying acetylcholine (Nastuk, 1953; del Castillo & Katz, 1955), and the application of Nomarski optics (Peper & McMahon, 1972) have permitted a high degree of resolution to be achieved with this approach. It has thus been shown that sensitivity to acetylcholine is greatest at the sites of neuromuscular contact; the chemosensitivity of adjacent, extrasynaptic, muscle membrane is very low, though detectable levels exist for a few hundred microns beyond the synaptic region (Miledi, 1960; Feltz & Mallart, 1971; Peper & McMahon, 1972). Amphibian muscle fibres and mammalian slow muscle fibres are also sensitive to acetylcholine close to the muscle-tendon junction (Katz & Miledi, 1964; Miledi & Zelena, 1966).

Other approaches have recently been developed which involve the use of cholinergic agents labelled with radioactive isotopes. In this regard the discovery of several snake toxins which combine with high affinity to acetylcholine receptors has been especially valuable (Chang & Lee, 1963; Tamiya & Arai, 1966; Lester, 1970; Lee, 1972). One of these is α-bungarotoxin, a basic polypeptide with a molecular weight of about 8,000 (Mebs, Narita, Iwanaga, Samejima & Lee, 1971). It has been used to estimate the number of receptors on single cells in skeletal muscle (Barnard, Wieckowski & Chiu, 1971; Miledi & Potter, 1971;

Berg, Kelly, Sargent, Williamson & Hall, 1972; Chang, Chen & Chuang, 1973) and in electric organs (Changeux, Kasai & Lee, 1970; Miledi, Molinoff & Potter, 1971) and to visualize their distribution by autoradiography (Lee & Tseng, 1966; Porter, Chiu, Wieckowski & Barnard, 1973). Quantitative estimates of autoradiographs have indicated that the density of receptors falls about a thousand-fold within 150 u of the neuromuscular junction (Fambrough & Hartzell, 1972; Hartzell & Fambrough, 1972).

A brief report has also appeared in which the distribution of acetylcholine receptors is visualized by an indirect immunofluorescent technique, based on the use of α-bungarotoxin as an antigenic agent (Bougeouis, Tsuje, Boquet, Pillot, Ryter & Changeux, 1971).

In the present study we have labelled α-bungarotoxin directly with fluorescent dyes and have used these dye-toxin conjugates to view the distribution of receptors with fluorescent microscopy. The method has proven to be relatively simple and rapid, and has revealed that the receptors are distributed throughout the subsynaptic muscle membrane, including the membrane of the junctional folds. A brief account of part of this work has been reported (Anderson & Cohen, 1973).

METHODS

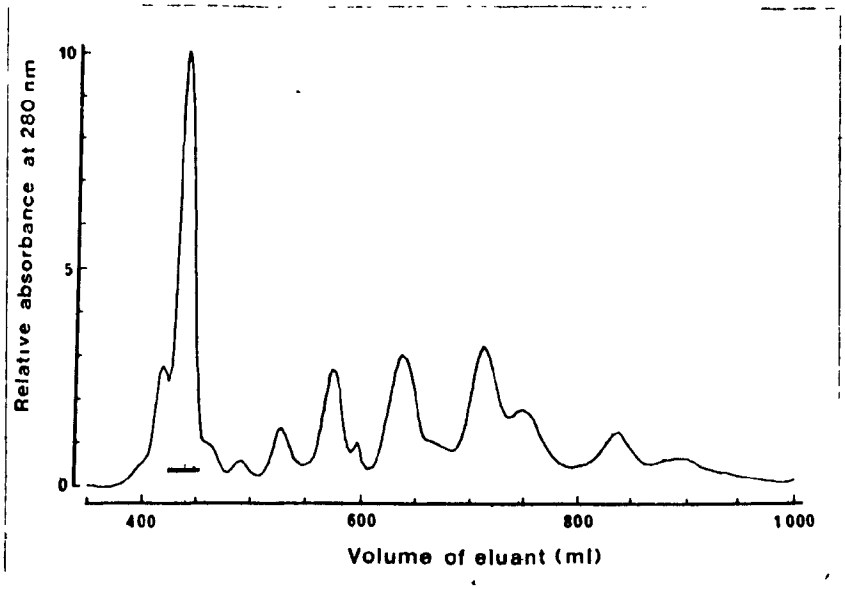
Preparation of α-bungarotoxin

α-Bungarotoxin was prepared from lyophilized venom of Bungarus multicinctus (obtained from Miami Serpentarium, Miami, Florida) essentially by the procedure previously described by others (Changeux et al., 1970; Berg et al., 1972; Bosmann, 1972; Lee, Chang, Kau & Luh, 1972). The venom (200 mg) was dissolved in 0.1 M ammonium acetate buffer, pH 5.0, and passed through a column (100 x 2.5 cm) of Sephadex G-50 at 4°C. Of the four protein peaks obtained, the middle two were concentrated with an Amicon Diaflo filter (UM05) and applied to a column (100 x 2.5 cm) of carboxymethyl-Sephadex C-50 equilibrated with 0.05 M ammonium acetate buffer, pH 5.0. The material was eluted with a linear gradient from 0.05 M, pH 5.0, to 1.0 M, pH 7.0, ammonium acetate buffer in a volume of 1 litre at 4°C. The major protein peak (Text-fig. 1) was lyophilized and later dissolved in 0.05 M ammonium acetate buffer, pH 7.0, to a final concentration of 1 mg/ml. This solution was sterilized by passage through a Millipore filter (pore size: 0.22 u) and stored at 4°C.

Conjugation of α-bungarotoxin with fluorescent dye

α-Bungarotoxin was labelled with fluoroscein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) by modification of the method of Marshall, Eveland & Smith (1958). Toxin (1-2 mg) was equilibrated with 0.05 M sodium carbonate buffer, pH 9.5, and concentrated by ultrafiltration to a final volume of about 2 ml. Dye, dissolved in 0.5 ml of the same buffer, was then added. Concentration ratios of about 200 ug FITC/mg toxin and 1 mg TRITC/mg toxin were routinely used, although

TEXT - fig. 1



Elution profile of the venom of Bungarus multicinctus chromatographed on CM-Sephadex C-50. After partial fractionation of 200 mg of venom on a column of Sephadex G-50, the two middle peaks were placed on a column of CM-Sephadex C-50 and eluted as described in the Methods. The flow rate was 12.3 ml/hr, and samples of 6.2 ml were collected. No material was detected in the first 350 ml of eluant. The eluant indicated by the bar was pooled and found to contain α-bungarotoxin.

five-fold smaller ratios also yielded conjugates which were effective for fluorescent staining. The mixture was stirred overnight at 4°C and then re-equilibrated with 0.05 M ammonium acetate buffer, pH 7.0, and concentrated by ultrafiltration. Dye-toxin conjugate was separated from free dye by passage through a column (30 x 1.5 cm) of G-25 Sephadex and was stored at 4°C for subsequent use.

Toxin concentrations were determined by the method of Lowry using bovine serum albumin (fraction V) as primary standard (Lowry, Rosebrough, Farr & Randall, 1951). Absorbances were determined at 760 nm with a Zeiss PMQ II spectrophotometer.

Material and general procedure

Amphibian muscles were stained in vitro at room temperature (20-23°C) by exposing them for 15-60 min to dye-toxin conjugate at concentrations of 10^{-6} - 10^{-5} gm/ml in Ringer solution. After rinsing for 1-2 hr with several changes of Ringer solution the muscles were fixed in 50-95% ethanol at -16°C for up to 2 days. Muscles stained with tetramethylrhodamine-labelled toxin were sometimes fixed instead with 10% neutral formalin at 4°C for up to 16 hr. The fixed muscles were washed for several hours with Ringer solution at 4°C and then single fibres, or small bundles of fibres, were teased out and mounted on glass cover slips for fluorescent microscopy. The mounting medium for fibres stained with fluorescein-labelled toxin consisted of 0.1 M sodium carbonate in 90% glycerol. Fibres stained with tetramethylrhodamine-labelled toxin were mounted in pure glycerol.

Muscles of rats and mice were treated in the same way except that Locke solution containing 10% foetal calf serum was used instead of Ringer solution. In a few cases mouse muscles were stained by injecting dye-toxin

conjugate intramuscularly. The muscles were removed 2-5 hr after the injection, rinsed, and then treated as above.

For most experiments the following muscles were used; sartorius of Rana pipiens and Xenopus laevis, tail musculature of Xenopus laevis tadpoles, mouse soleus and plantaris, and rat diaphragm. Several other muscles were also examined including extensor longus digitorum IV, rectus abdominis, rectus femoris anticus, and cutaneous pectoris of Rana pipiens and Xenopus laevis, and interhyoideus, and levator mandibularis of Xenopus tadpoles. Amphibians were anaesthetized with tricaine. Mice and rats were anaesthetized with ether.

To produce degeneration of motor nerve terminals in frog sartorius muscles a 5-10 mm length of sciatic nerve was excised high in the leg. Muscles were removed for staining 2-4 weeks later (see Birks, Katz & Miledi, 1960). Degeneration of motor nerve terminals in mouse soleus and plantaris was produced by excising a 3-6 mm length of sciatic nerve high in the leg. Muscles were removed for staining 1-3 weeks later (see Miledi & Slater, 1968).

Conventional methods were used for intracellular recordings from amphibian muscle fibres. Muscle tension was measured with a force transducer and displayed on a Grass polygraph. Contraction responses of Xenopus tadpole tails were viewed through a dissecting microscope at a magnification of 25 times.

The composition of the Ringer solution in mM. was: NaCl, 111; KCl, 3; $CaCl_2$, 1.8; tris maleate adjusted to pH 7.4 with NaOH, 5. The composition of the Locke solution in mM. was: NaCl, 150; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 1; $NaHCO_3$, 12; glucose 11. The solution was bubbled with a mixture of 95% O_2 and 5% CO_2.

Fluorescent microscopy

Teased muscle fibres were examined with a Zeiss Ultraphot II microscope, using an HBO200W/4 lamp as the excitation source. For fibres stained with fluorescein-labelled toxin the exciting light was passed through a heat-absorbing filter (KG1) and a BG 12 blue filter. The emitted light was filtered with a No. 50 barrier filter. For fibres stained with tetramethylrhodamine-labelled toxin an intereference filter transmitting at 546 $\pm$ 5 nm was placed in the excitation path together with the heat-absorbing filter. The emitted light was usually filtered with a Kodak No. 23A light red filter but in some cases a Kodak No. 25A dark red filter was used instead. Several objectives, with magnifications between 6.3 and 100 times were employed. The fluorescence was photographed with Kodak Tri-X 35 mm black and white film. Exposure times varied between 15 sec and 4 min.

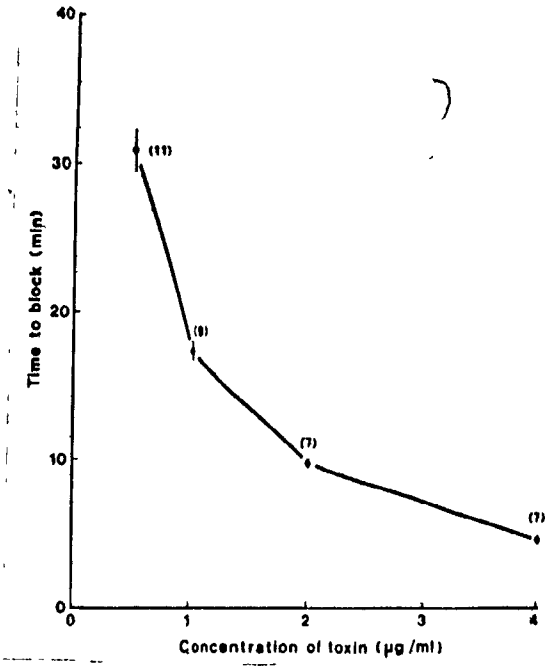
RESULTS

Biological activity of dye-toxin conjugates

Dye-toxin conjugates, like native toxin, blocked the depolarizing action of acetylcholine. Miniature end-plate potentials, end-plate potentials, and depolarization responses to bath applied carbachol were abolished in surface muscle fibres within a few minutes after adding the conjugate to the bathing fluid (final concentration: 10^{-5} gm/ml). However the conjugates turned out to be less potent than native toxin. Quantitative estimates of these differences in potency were determined as follows. Skinned tails of Xenopus tadpoles (stages 47-48 of Nieuwkoop and Faber, 1956) were exposed to several concentrations of native toxin and their contraction responses to bath applied carbachol (10^{-4} gm/ml) were tested at appropriate intervals. Each tail was tested only once and the time required for complete block was noted. From these measurements a dose-response curve was plotted as illustrated in Text-fig.2. Similar determinations of times to block were then made with dye-toxin conjugates at concentrations of 10^{-5} or 5×10^{-5} gm/ml and the "effective" concentration of the conjugate was obtained from the relationship in Text-fig. 2. In this way it was determined that dye-toxin conjugates which were effective for fluorescent staining had relative potencies of 1-50%. The loss of potency tended to vary with the dye/toxin ratio in the initial reaction mixture. Reductions in potency have previously been noted after conjugating fluorescent dyes to other types of substances, including antibodies, enzymes, and hormones (Goldman, 1968).

Loss of potency was also revealed by comparing the toxicity of dye-toxin conjugates with that of native toxin. For these experiments the toxin was injected intramuscularly into the legs of mice and the

TEXT - fig. 2



Relation between concentration of α-bungarotoxin and the time required for the toxin to completely abolish the contraction response of tadpole tails to 10^{-4}gm/ml carbachol. Tails of stage 47-48 Xenopus tadpoles were skinned and exposed to α-bungarotoxin, until tested with carbachol. Contraction responses were viewed through a dissecting microscope at a magnification of 25 times. Each tail was tested only once. Means ± 1 S.E.M. are shown, and the number of determinations at each concentration of toxin is indicated in parenthesis.

animals were observed for the next several hours. Doses of native toxin above 0.3 ug/gm body weight killed all mice within 4 hr whereas doses below 0.1 ug/gm did not cause any deaths. This range of lethal and sub-lethal doses is similar to that found in previous studies (Chang & Lee, 1963; Lee et al., 1972). Dye-toxin conjugate which had a relative potency of 10%, as determined on tadpole tails by the method described above, caused death only in doses above 1 ug/gm.

Conjugates were also tested for their reversibility of action, using sartorius muscles of Xenopus laevis. Following complete block of neuromuscular transmission and of the contraction response to 10^{-4} gm/ml carbachol, the muscles were washed with Ringer solution for about 1 hr at room temperature and subsequently at 4°C. Periodic tests were made at room temperature for up to 60 hr without any sign of recovery of the response to carbachol, although the muscles remained responsive to direct stimulation and control muscles continued to respond to carbachol during the same period. Similar observations have previously been made with native toxin (Miledi & Potter, 1971). The results thus indicate that, in spite of their reduced potency, dye-toxin conjugates act in the same way as native toxin. Further evidence in support of this conclusion was obtained by examining the specificity of the fluorescent staining produced by the conjugates (see below).

Localization of fluorescent stain

The patterns of fluorescence on single muscle fibres after treatment with dye-toxin conjugates were similar to those seen with other methods which permit visualization of the subsynaptic muscle membrane or the motor nerve terminals (Kuhne, 1887; Couteaux, 1947; Couteaux & Taxi, 1952;

McMahon, Spitzer & Peper, 1972). In frog and Xenopus the patterns consisted of long narrow branches oriented mainly parallel to the long axis of the muscle fibres (Pl. 1 a-c). The width of these branches was fairly constant (2-4 μ) but their number, length (up to several hundred microns), and degree of branching varied considerably in different muscle fibres. In rat and mouse the fluorescent patterns were also composed of narrow branches, a few microns wide, but were always more compact (Pl. 1 d-h). Individual branches were often less than 20 u long and usually did not show any preferred orientation. In most examples their borders were more intensely fluorescent than their interiors. A similar characteristic outlining has previously been observed with other methods which stain the subsynaptic muscle membrane (Couteaux, 1947, 1955; Couteaux & Taxi, 1952).

The neuromuscular localization of fluorescent stain was further revealed by comparing the distribution of flurescence and cholinesterase on the same muscle fibres. For these experiments Xenopus muscles were exposed to dye-toxin conjugate and then stained for cholinesterase, using Karnovsky's method (1964). The reaction product in the latter method preferentially accumulates at the edges of the neuromuscular junction and thereby outlines it (McMahon et al., 1972). The patterns of staining on single muscle fibres proved to be virtually identical for both methods, with the fluorescence being confined within the boundaries of the cholinesterase stain (Pl. 2).

Studies of the prejunctional effects of acetylcholine and of anticholinesterases have raised the possibility that motor nerve terminals may also contain acetylcholine receptors (Hubbard, Schmidt & Yokota, 1965; Katz, 1969). If these receptors contributed significantly to the

fluorescent staining then a decrease in staining intensity would be expected following degeneration of the terminals. However no obvious decreases in intensity were observe in frog and mouse muscles which had been denervated for sufficient periods of time to allow degeneration of the terminals (see Methods). It follows that in normal muscles the fluorescent stain was associated mainly with the subsynaptic muscle membrane and not with the motor nerve terminals. Other features of the distribution of fluorescent stain in denervated muscle will be presented later (Cohen & Anderson, in preparation).

Amphibian muscle fibres and mouse soleus muscle fibres were also examined for fluorescence in the vicinity of their tendons since ionophoretic methods have revealed a significant sensitivity to acetylcholine here, albeit some hundred- to thousand-fold less than that at the neuromuscular junction (Katz & Miledi, 1964; Miledi & Zelena, 1966). However no obvious fluorescent staining was observed.

Specificity of fluorescent stain

On the basis of previous findings which have demonstrated that α-bungarotoxin binds irreversibly to nicotinic acetylcholine receptors some predictions can be made concerning the effects of various procedures on the intensity of the fluorescent stain. Pre-exposure to native toxin for a sufficient period of time should entirely prevent staining. Reversible nicotinic agents, such as curare and carbachol, should reduce the intensity of staining whereas muscarinic agents and anticholinesterases, should not have any significant effect. These predictions were experimentally tested, most extensively on the tail musculature of stage 47-49 Xenopus tadpoles. The tails are relatively thin and are composed

of a series of myotomes, each containing muscle cells whose ends are innervated and very closely aligned (Lewis & Hughes, 1960). These unique features permitted the fluorescent staining to be viewed in hemisected tails and effectively reduced the variability in staining intensity that can occur when sampling single muscle fibres. In order to examine the effects of reversible agents on the fluorescent staining, tails were skinned and exposed for 20 min to Ringer solution containing the agent at a concentration of 10^{-4} gm/ml. Dye-toxin conjugate was then added to a final concentration of 10^{-5} gm/ml. After a further 20 min the tails were transferred to Ringer solution of the original composition and were rinsed for 60 min. The staining in these experimental tails was compared with controls which were treated in the same manner except for the absence of the reversible agent.

Pl. 3 illustrates some typical results. Curare diminished the fluorescent staining and carbachol virtually abolished it (Pl. 3 a-c). These observations are in line with previous studies which have shown that carbachol is more effective than curare in inhibiting the binding of toxin to muscle (Miledi & Potter, 1971; Berg et al., 1972). As further predicted, atropine and neostigmine did not have any significant effect (Pl. 3 d, e). Pretreatment with native toxin (10^{-5} gm/ml for 15 min) entirely prevented staining in tadpole tails even if the exposure time to dye-toxin conjugate was increased to 60 min. In addition, the binding of stain to muscle was not readily reversible; satisfactory fluorescent staining was observed even when tails were rinsed in Ringer solution for 30 hr, at 4°C, after exposure to dye-toxin conjugate. Qualitatively similar observations were made on several other amphibian

and mouse muscles. On the basis of these results it seems reasonable to conclude that the fluorescent staining is due to binding of dye-toxin molecules to acetylcholine receptors.

Fine distribution of fluorescent stain on subsynaptic membrane

At high magnification, face-on views of the fluorescent staining in frog and Xenopus muscle fibres revealed that individual branches of fluorescence were composed of a pattern of intense transverse bands occurring at intervals of approximately 0.5-1 u, with fluorescence of lower intensity between them (Pl. 4 a, b). The intense bands were observed even when muscles were rinsed with Ringer solution for over 24 hr at 4°C following exposure to dye-toxin conjugate, but were prevented by the presence of carbachol (10^{-4} gm/ml) or by pretreatment with native toxin. They are therefore due to specific membrane-bound stain rather than non-specific trapping of stain. A similar pattern has previously been observed in amphibian muscle fibres stained for cholinesterase (Couteaux & Taxi, 1952). In the light of subsequent studies in which cholinesterase staining has been viewed with the electron microscope it is clear that the intense bands reflect the stain within the half- to one-micron deep junctional folds (Lehrer & Ornstein, 1959; Barnett, 1962; Couteaux, 1963). The same interpretation presumably applies in the present case and is in line with the observation that the intervals between the intense fluorescent bands are similar to the intervals between junctional folds in amphibian muscle (Birks, Huxley & Katz, 1960; McMahon et al., 1972). The width of the intense bands is greater than the width of the junctional folds (about 0.1 u) but this is not unexpected in view of the fact that folds are often branched (Birks et al., 1960; McMahon et al., 1972). The

less intense fluorescence between the intense bands presumably reflects the staining of receptors associated with the subsynaptic membrane between the folds. On the other hand, fluorescent staining was not detected on adjacent, extrasynaptic, muscle membrane. Instead the fluorescence declined abruptly to background levels at the border between synaptic and extrasynaptic muscle membrane.

Additional support for the above interpretations was obtained from side views of the fluorescent staining. In this orientation the staining appeared as a fine line with small protuberances (less than 1 u) occurring at the same intervals as the intense bands seen face-on (Pl. 4 c, d). The simplest explanation of the protuberances is that they are due to staining of receptors associated with the membrane of the junctional folds. The fluoresence between the protuberances suggests that the synaptic membrane between folds also contains receptors.

A pattern of intense fluorescent bands of the sort seen in amphibian muscle fibres was rarely resolved in rat and mouse muscle fibres. The basis for this difference presumably does not result from basic differences in the distribution of acetylcholine receptors in amphibian and mammalian muscle fibres but rather from the fact that the orientation of folds at mammalian neuromuscular junctions is variable and irregular so that the chances of "visualizing" them in teased preparations are very low (Couteaux, 1958; Andersson-Cedergren, 1959; Lehrer & Ornstein, 1959). Similarly, the characteristic intense fluorescent outlining which occurred in mammalian, but not in amphibian, muscle fibres (Pl. 1) presumably reflects the fact that the junctional gutters are considerably deeper in mammalian muscle than they are in amphibian muscle (Couteaux, 1955).

DISCUSSION

The present study has demonstrated that conjugation of fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate to α-bungarotoxin provides specific fluorescent stains for acetylcholine receptors in vertebrate skeletal muscle. Although the dye-toxin conjugates were less potent than native toxin they retained the same action of blocking acetylcholine receptors irreversibly. The lower potency of the conjugates appears to be related to the fact that the dyes react with ε -amino groups of basic amino acid residues, such as lysine, and affect their charge (Goldman, 1968). Evidence has been obtained which indicates that at least some lysine residues in α-bungarotoxin are important in determining its potency (Lee, 1972). On the other hand hydrophobic amino acid residues, with which the dyes do not react, appear to be important in conferring upon α-bungarotoxin its irreversibility of action (Lee, 1972). It is therefore not unexpected that the dye-toxin conjugates also acted irreversibly.

The method of fluorescent staining revealed that acetylcholine receptors are associated with the membrane of the junctional folds, and probably with the entire subsynaptic muscle membrane. Since the folds increase the area of subsynaptic membrane by an estimated factor of 4-5 times (Andersson-Cedergren, 1959), many more receptors can be accommodated than would otherwise be the case and this in turn may permit larger synaptic currents to be generated (Eccles & Jaeger, 1958). In this respect it is probably significant that synaptic vesicles, from which acetylcholine is presumably released, tend to be accumulated opposite junctional folds (Birks et al., 1960; McMahon et al., 1972). However, the junctional folds

also contain cholinesterase (Couteaux, 1958; 1963) and it is not known to what depth the molecules of a released packed of acetylcholine can diffuse before they are entirely hydrolyzed.

Whereas subsynaptic membrane was brightly stained by dye-toxin conjugates, fluorescent staining was not detected on adjacent extrasynaptic muscle membrane. We have not attempted to quantify these observations and therefore have no estimate of the relative decline in receptor density. Nevertheless, our results indicate that the density of acetylcholine receptors changes abruptly at the border between subsynaptic and extrasynaptic membrane. Similarly, Peper & McMahon (1972) have elegantly demonstrated a very steep fall in acetylcholine sensitivity immediately outside the subsynaptic membrane. The abrupt change in receptor density makes it unlikely that synaptic receptors migrate into adjacent extrasynaptic membrane. This is in contrast with the finding that some surface antigens on cultured myotubes do move continuously at a slow rate (Edidin & Fambrough, 1973). Whether acetylcholine receptors move within the limits of the subsynaptic membrane or whether they are rigidly fixed may be possible to determine by staining a portion of the subsynaptic membrane and observing if the fluorescence spreads with time.

Although fluorescent staining of extrasynaptic muscle membrane was not detected in the present study, the existence of extrasynaptic receptors is well established. In rat diaphragm extrasynaptic sensitivity to acetylcholine declines with distance away from the neuromuscular junction and at distances of 100-200 u can be several thousand times less than the synaptic sensitivity (Miledi, 1960). Likewise, estimates based on autoradiography with I^{125}-labelled α-bungarotoxin indicate that the density of receptors falls about a thousand-fold within 150 u of the

neuromuscular junction (Hartzell & Fambrough, 1972). It is unlikely that the method of fluorescent staining could detect such low densities in teased muscle fibres. In the frog, extrasynaptic receptors are most abundant during the winter and absent during the summer (Feltz & Mallart, 1971). However our sample of observations on muscle fibres of "winter" frogs is too small at this point to allow any conclusions concerning detection of amphibian extrasynaptic receptors by fluorescent staining. A limitation in the sensitvity of the method may also account for the absence of staining at the myotendinous junction. Acetylcholine sensitivity at this site tends to be variable and is often several thousand times less than at the neuromuscular junction (Katz & Miledi, 1964; Miledi & Zelena, 1966). On the other hand we are not aware of any studies which have demonstrated extra binding of α-bungarotoxin at the myotendinous junction.

Despite the possible limitations with regard to sensitivity there are clearly many problems to which the method of fluorescent staining may be profitably applied. Many vertebrate and invertebrate neurons contain nicotinic acetylcholine receptors and recent studies have indicated that α-bungarotoxin binds to these receptors in guinea pig cerebral cortex and on chick sympathetic neurons (Bosmann, 1972; Greene, Sytkowski, Vogel & Nirenberg, 1973). Fluorescent staining may reveal more precisely the distribution of receptors on such neurons as well as the numbers and location of "nicotinic" neurons in the central nervous system. The fact that α-bungarotoxin can be labelled with fluorescent dyes of different colour may also permit one to determine if synaptic contacts turn over or if they can be made to do so by experimental modification of neuronal activity. It will also be of interest to use as fluorescent stains other toxins, such as β-bungarotoxin (Chang & Lee, 1963; Chang, Chen &

Lee, 1973), which act irreversibly on other components of the nervous system.

PLATE I

Examples of fluorescent staining on single muscle fibres. (a-c) Xenopus sartorius; (d) mouse plantaris; (e,f) mouse soleus; (g,h) rat diaphragm. Muscles were stained with tetramethylrhodamine-labelled toxin (a-f) and with fluorescein-labelled toxin (g,h).

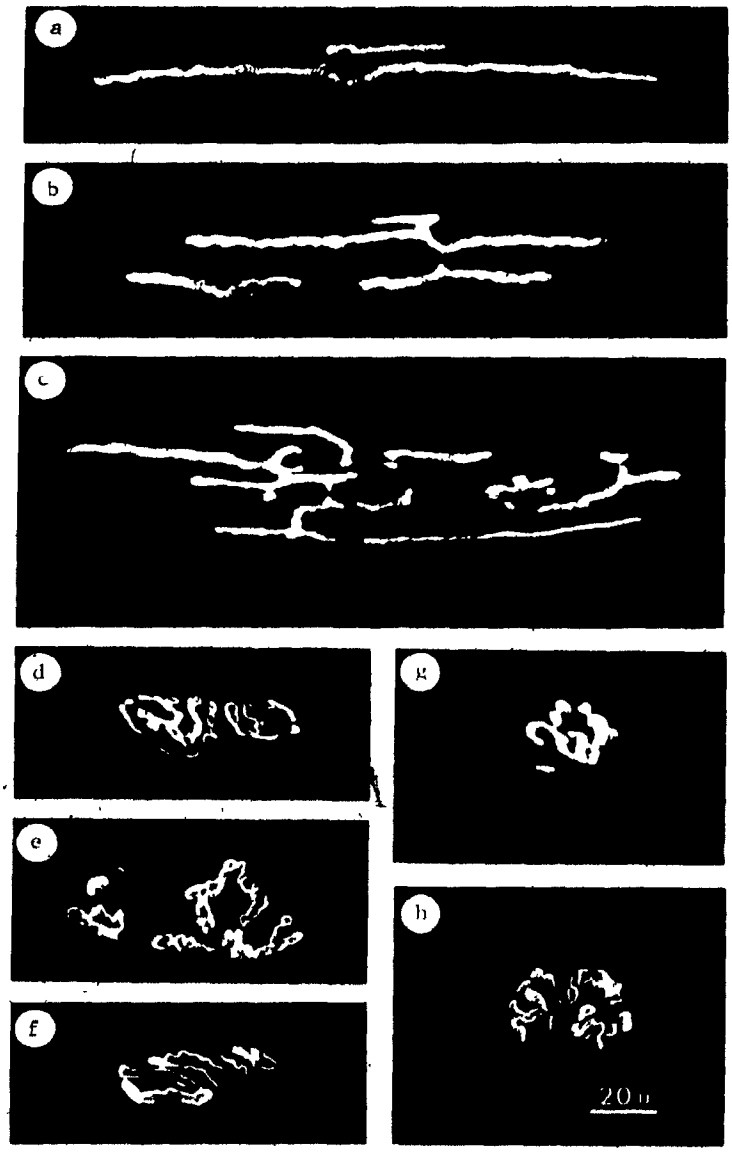

a
b
c
d
e
f
g
h
20 μ

PLATE 2

Fluorescent staining and cholinesterase staining on the same muscle fibres. (a) Xenopus sartorius muscle fibre stained with tetramethlyrhodamine-labelled toxin. (b) Same muscle fibre as in (a), stained for cholinesterase by the method of Karnovsky (1964) and viewed with brightfield optics. The cholinesterase stain accumulates preferentially at the edges of the neuromuscular junction and outlines it (see McMahon, Spitzer & Peper,1972). Comparison of (a) and (b) confirms that the fluorescent stain is confined to the neuromuscular junction. (c,d) Another muscle fibre, from rectus femoris anticus of Xenopus, stained as in (a) and (b) respectively.

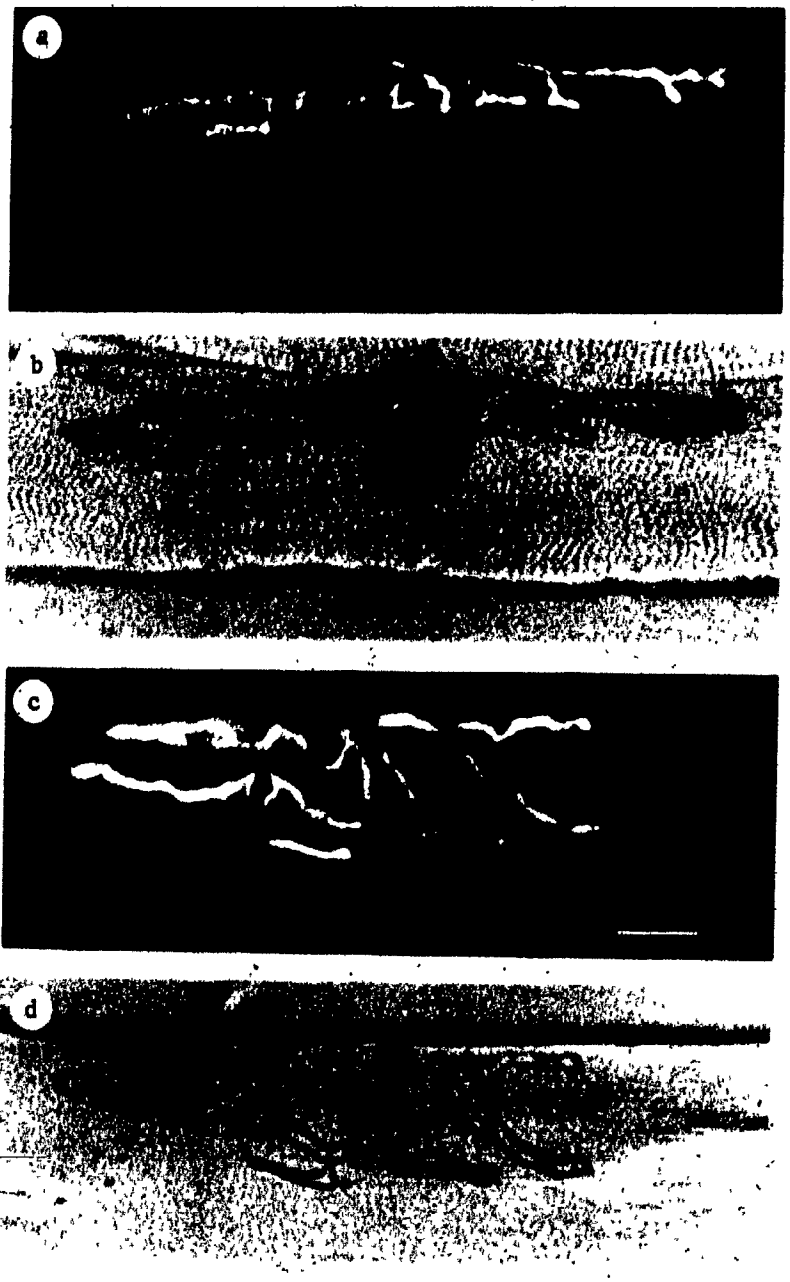

a
b
c
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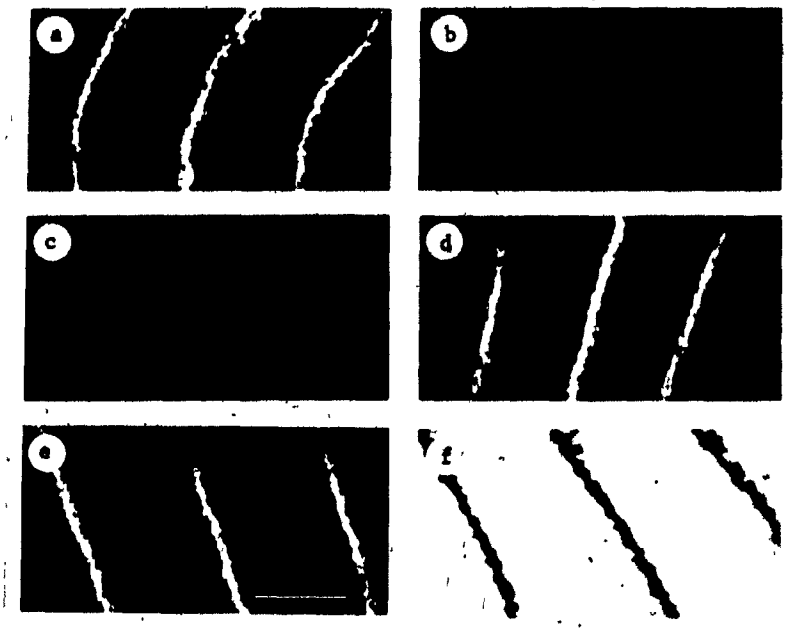
PLATE 3

Effects of cholinergic agents on fluorescent staining of tail musculature from stage 47-49 Xenopus tadpoles. (a) Hemisected tail, stained for 20 min with 10^{-5} gm/ml tetramethylrhodamine-labelled toxin. (b) Another hemisected tail, treated as in (a) but 10^{-4} gm/ml d-tubocurarine chloride was present for 20 min before, during, and for the rinsing period after exposure to dye-toxin conjugate. (c-e) Hemisected tails, treated as in (b) but instead of curare the same concentration of carbachol chloride (c), neostigmine bromide (d), or atropine sulphate (e) was present. Photographic conditions were identical in each case. Note that the fluorescent staining was decreased by curare and virtually eliminated by carbachol, but was unaffected by neostigmine or atropine. (f) Hemisected tail, stained for cholinesterase and indicating a similar pattern of distribution as the fluorescent stain.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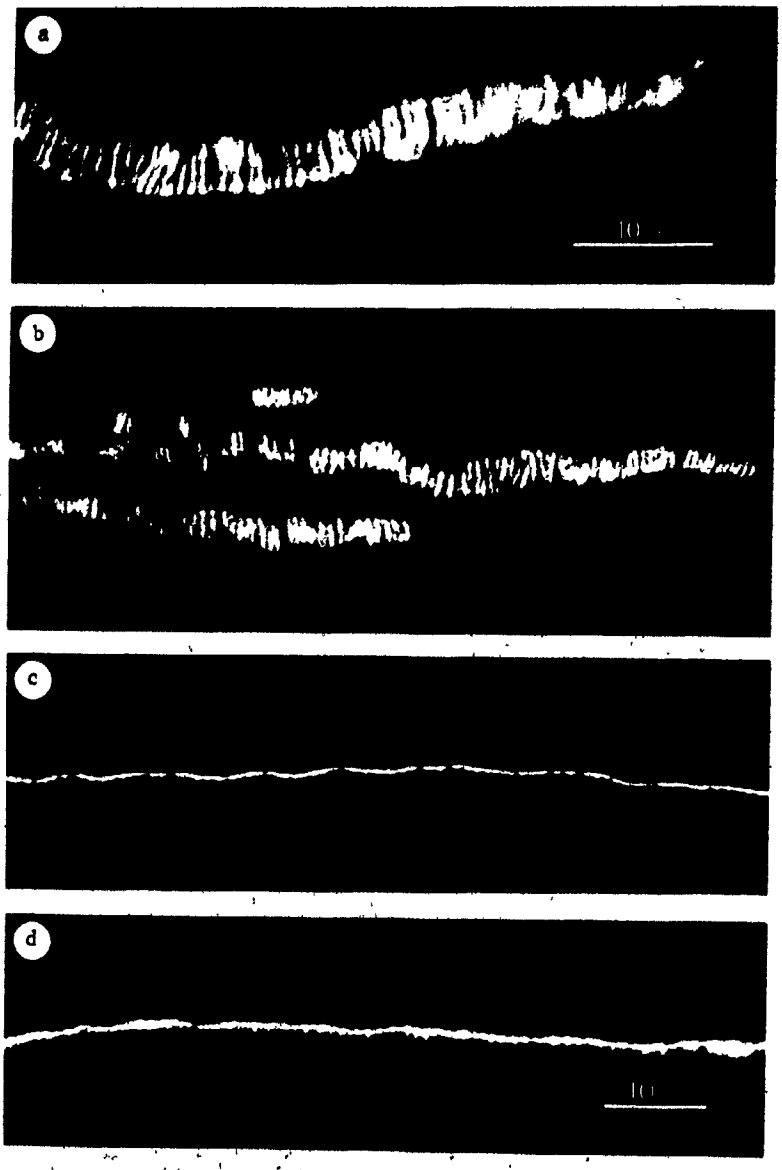
f

PLATE 4

Fine distribution of fluorescent stain at amphibian neuromuscular junctions. Face-on views of part of frog sartorius neuromuscular junction stained with fluorescein-labelled toxin (a) and Xenopus sartorius neuromuscular junction stained with tetramethylrhodamine-labelled toxin (b). Note the intense transverse bands of fluorescence, and the fluorescence of lower intensity between them. Note also the absence of staining beyond the synaptic borders. (c,d) Side views of part of Xenopus sartorius neuromuscular junctions stained with tetramethylrhodamine-labelled toxin. In each case the muscle fibre occupies the lower portion of the field. The fluorescent staining consists of a fine line with small protuberances occurring at intervals similar to those of the intense transverse bands in (a) and (b). The intense bands (a,b) and the protuberances (c,d) probably reflect the staining of acetylcholine receptors on the membrane of the junctional folds. The fluorescence between the intense bands and between the protuberances probably reflects stain associated with acetylcholine receptors on the subsynaptic membrane between junctional folds. Scale in (d) also applies to (b) and (c).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CHAPTER 3

EFFECTS OF INNERVATION ON THE DISTRIBUTION OF ACETYLCHOLINE RECEPTORS ON CULTURED AMPHIBIAN MUSCLE CELLS

SUMMARY

1. Myotomal muscle cells from embryos of Xenopus laevis were cultured as a monolayer either alone or together with neural tube cells from the same embryos.

2. Spontaneous twitching and contractions evoked by electrical stimulation of neuronal perikarya were observed only in nerve-contacted muscle cells, and could be abolished by curare or α-bungarotoxin.

3. Within 2 days in culture muscle cells not contacted by nerve developed one or more discrete patches of acetylcholine (ACh) receptors as revealed by staining with fluorescent conjugates of α-bungarotoxin. Similar patches were also seen when staining was carried out after paraformaldehyde fixation, suggesting that they were not induced by the dye-toxin conjugate.

4. Radioautography after labelling with ^{125}I- α-bungarotoxin revealed patches with grain densities approximately 25-fold greater than over the remainder of the cell.

5. Fluorescent stain on innervated cells was restricted to the path of nerve-muscle contact and sometimes extended for greater lengths than the largest patches seen on non-contacted muscle cells.

6. Similar long bands of stain associated with nerve-muscle contacts were observed when cultures were grown in high concentrations of curare and carbachol which prevented spontaneous twitching. They were also seen in cultures in which the addition of neural tube cells was delayed for 2-3 days.

7. It is concluded that innervation caused receptors to accumulate at sites of nerve-muscle contact and that this process can operate independently of muscle activity.

INTRODUCTION

It has been known for many years that in skeletal muscle sensitivity to ACh is localized almost exclusively at the region of innervation (Langley, 1907; Kuffler, 1943). The iontophoretic technique (Nastuk, 1953; del Castillo & Katz, 1955) and the subsequent application of Nomarksi optics (Peper & McMahon, 1972) have greatly improved the spatial resolution of such measurements and it is now clear that ACh sensitivity is restricted largely to the subsynaptic membrane. For example, sensitivity has been found to decline fifty-fold within 2 um of the site of nerve contact (Kuffler & Yoshikami, 1975) and a thousand-fold or more within a few hundred microns (Miledi, 1960b; Dreyer & Peper, 1974). Other investigations, in which ACh receptors have been labelled with conjugates of α-bungarotoxin and their distribution examined by radioautography, have further indicated that receptor density is very much higher in the subsynaptic membrane than in the remainder of the sarcolemma (Barnard, Wieckowski & Chiu, 1971; Hartzell & Fambrough, 1972; Albuquerque, Barnard, Porter & Warnick, 1974; Fertuck & Salpeter, 1974, 1976). Regions of high chemosensitivity have also been observed at sites of synaptic contact on parasympathetic neurons in the amphibian heart (Harris, Kuffler & Dennis, 1971; Roper, 1976) and at excitatory and inhibitory synapses in arthropod muscle (Takeucki & Takeuchi, 1964, 1965; Usherwood, Machili & Leaf, 1968). On the basis of these findings, it seems likely that a high receptor density will prove to be a common characteristic of many chemical synapses.

The manner in which receptors become localized at synapses during the course of development is an intriguing question. It has been established that at early stages of development, shortly after innervation has occurred, skeletal muscle fibres have a high sensitivity to ACh along their entire

length (Diamond & Miledi, 1962; see also Berg, Kelly, Sargent, Williamson & Hall, 1972). A number of experimental procedures also lead to the development of a widespread sensitivity in mature muscle fibres. Since these procedures restore the muscle fibre's ability to accept new innervation, it has been suggested that sensitivity to ACh may be a prerequisite for the establishment of nerve-muscle synapses (Katz & Miledi, 1964; Fex, Sonesson, Thesleff & Zelena, 1966). More recently it has been found that avian and rat myotubes in cell culture develop not only a widespread distribution of receptors, but also patches of even greater density (Vogel, Sytkowski & Nirenberg, 1972; Fischbach & Cohen, 1973; Sytkowski, Vogel & Nirenberg, 1973; Hartzell & Fambrough, 1973; Vogel & Daniels, 1976). This has raised the possibility that receptor patches are potential sites of innervation which growing nerve fibres seek out (Sytkowski et al., 1973; Fischbach & Cohen, 1973). On the other hand, the distribution of ACh receptors prior to innervation has not been determined for the case of normal embryonic development. It is therefore necessary to consider the alternative possibility that innervation itself induces a high density of ACh receptors at the site of contact, just as it is known to induce junctional folds and cholinesterase (Miledi, 1962; Couteaux, 1963).

We have explored these possibilities in cell cultures of myotomal muscle and neural tube derived from embryos of Xenopus laevis. The muscle cells and neural tube cells develop rapidly in culture and establish many functional synaptic contacts. The cultures have other useful features; they can be maintained under relatively simple conditions at room temperature without antibiotics, and with little contamination by other cell types. In addition the muscle cells remain mononucleated and do not proliferate. It is of interest to note that the first indication of nerve-muscle synapse

formation *in vitro* is found in R. G. Harrison's (1910) classical study in which he used explants of these tissues to demonstrate axonal growth.

In the present study we have examined the distribution of ACh receptors with fluorescent conjugates of α-bungarotoxin (Anderson & Cohen, 1974). This paper describes the rapid and dramatic changes in receptor distribution which occur when the muscle cells become innervated. The following paper considers the manner in which these changes are brought about (Anderson & Cohen, 1976). Brief accounts of this work have been reported (Anderson & Cohen, 1975; Anderson, Cohen & Zorychta, 1976).

METHODS

Preparation of cultures

Mating was induced in mature Xenopus laevis toads by injection of chorionic gonadotrophin (Ayerst) into the dorsal lymph sacs as described by Nieuwkoop & Faber (1956). Approximately 24 hr after fertilization normally developing embryos were transferred to a sterile dissecting medium and thereafter all procedures were carried out under sterile conditions. Dorsal portions of stage 22-23 embryos (Nieuwkoop & Faber, 1956) were isolated by dissection and incubated for 30-60 min in a collagenase solution in order to facilitate the separation of neural tube, myotomal muscle and notochord (Text-Fig. 1). Either of the following three types of culture was then prepared. (1) Mixed nerve and muscle cultures: Isolated neural tubes and myotomal muscle were bathed in a trypsin-EDTA solution for about 1 hr and then gently agitated in order to enhance their dissociation. The cells were then plated directly in the same culture chamber. One day later the plating medium was replaced by a maintenance medium, and thereafter the cultures were left undisturbed until required for experimentation. (2) Muscle cultures, free of nerve: In this case only isolated muscle was dissociated and plated. Otherwise, the procedures were the same as for the mixed cultures. (3) Muscle cultures to which neural tube cells were added after 2-3 days: Muscle cultures were prepared as above and isolated neural tubes were stored at 4-10°C in plastic Petri dishes (Falcon) containing plating medium. After 2-3 days the neural tubes were dissociated and added. As described below the maintenance medium was modified immediately before neural tube cells were added in order to facilitate their adhesion to the culture dish.

TEXT - fig. 1

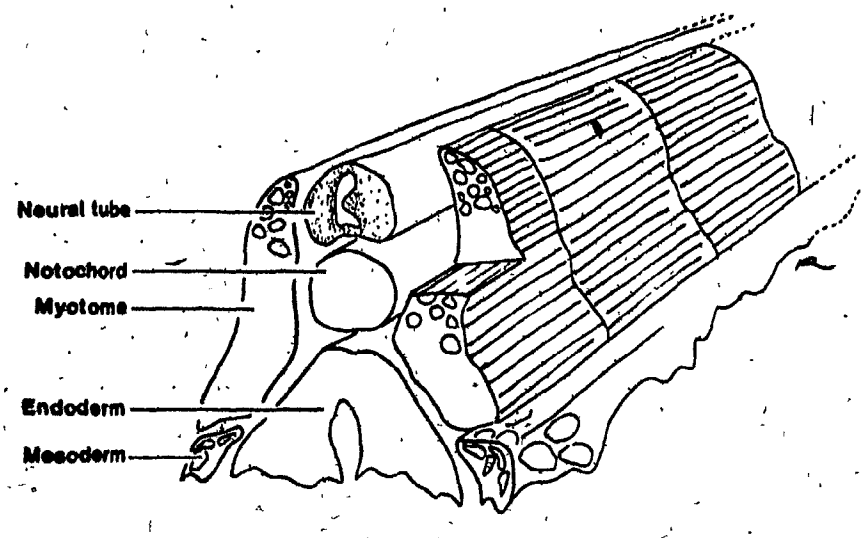


Diagram illustrating a portion of the dorsal region of a stage 22-23 Xenopus embryo after removing the skin. On either side of the neural tube and notochord there are 9-12 myotomes only three of which are shown. Each myotome consists of about 100 muscle cells, approximately 100 um long and 10 um in diameter. The cells are laden with yolk granules and innervation is just beginning (Nieuwkoop & Faber, 1956; Muntz, 1975). Treatment with collagenase allows the myotomes and neural tube to be separated from each other and from the notochord.

All procedures were carried out at room temperature (21-24°C).

(Text-fig. 1 near here)

Solutions and culture media

Dissecting medium: L-15 (Gibco), 60% (v/v); dialyzed horse serum (Gibco), 5% (v/v); Mycostatin (Gibco), 50 units/ml; gentamycin (Schering), 50 ug/ml. The latter two agents are effective in eliminating the fungal and bacterial contamination normally associated with Xenopus eggs (Laskey, 1970).

Collagenase (Type 1, Sigma) was used at approximately 1 mg/ml in 60% L-15.

Dissociation medium: a solution (Gibco) of trypsin (5 mg/ml) and EDTA (2 mg/ml) was diluted to 20% (v/v) with a Ringer solution containing 67 mM NaCl, 1.6 mM KCl and 8 mM HEPES buffer (Gibco).

Plating medium: L-15, 60%; dialyzed horse serum, 5%; Holmes' α-1-protein (Gibco), 0.2 ug/ml.

Maintenance medium: L-15, 60%; Holmes' α-1-protein, 0.2 ug/ml. Serum was omitted because its continued presence at a concentration of 5% caused myotomal muscle cells to degenerate within 3-4 days. However, some serum was found to be essential for the attachment of cells to the culture dish. As a result when neural tube cells were added to 2- and 3-day-old muscle cultures the maintenance medium was supplemented at the same time with up to 1% dialyzed horse serum. These levels of serum caused granulation to occur in some muscle cells, but they remained viable for at least another four days.

For some experiments the plating and maintenance media also contained 10^{-5} - 10^{-4} g/ml carbachol chloride (Mann) or 10^{-4} g/ml d-tubocurarine chloride (Sigma).

All of the above media were prepared using sterile distilled water (Gibco) and when necessary pH was adjusted to about 7.2 with NaOH or HCl. Solutions of non-sterile agents were sterilized by double filtration through Millipore membranes (pore size: 0.22 um).

Frog Ringer contained, in mM: NaCl, 111; KCl, 3; $CaCl_2$, 1.8; Tris maleate buffer adjusted to pH 7.4 with NaOH, 5.

Culture chambers

When cultures were to be fixed after fluorescent staining a simple chamber was frequently used which consisted of a glass ring on a collagen-coated glass coverslip. After filling with medium (volume: about 1.8 ml) and adding dissociated tissue, the chamber was sealed by placing another glass coverslip on top of the glass ring. All connexions were made with heat-sterilized silicone grease (Dow Corning). After staining and fixing the cultures, the collagen-coated coverslip was removed and mounted on a glass slide for examination in the fluorescence microscope.

When fluorescent staining was to be examined in living cultures a more complex chamber was used (Text-fig. 2). The floor of the chamber also consisted of a collagen-coated glass coverslip but in this case it covered a hole (16 mm diameter) in a glass slide. On top of the slide, surrounding the hole, was a glass ring. The chamber was held together with silicone grease and sealed as above. After fluorescent staining the glass ring was removed and a coverslip set directly over the hole in the glass slide.

(Text-fig.2 near here)

Electrical stimulation

Cultures were placed on the fixed stage of an inverted phase contrast

TEXT - fig. 2

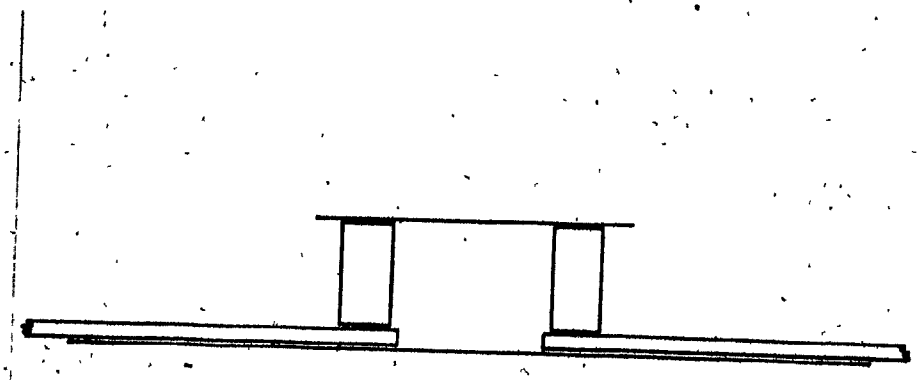


Diagram illustrating a side view of the culture chamber. The floor consists of a collagen-coated coverslip which covers a hole (15 mm in diameter) in a glass slide. A glass ring (9 mm high) rests on the slide and surrounds the hole. After filling the chamber with medium and adding tissue to it, the top is covered with a second coverslip. All connexions are sealed with silicone grease. The diagram is not drawn to scale.

Zeiss microscope and grounded by an agar-Ringer electrode. Electrical pulses of about 1 msec duration were delivered through a glass microelectrode filled with frog Ringer and having a bevelled tip of about 2 um diameter. Stimulus intensities required to evoked muscle contraction by direct stimulation were at least five-fold greater than for evoking contractions by stimulation of neuronal cell bodies.

Fluorescent staining

α-Bungarotoxin and its conjugates with fluorescein isothiocyanate (BBL) and tetramethylrhodamine isothiocyanate (BBL) were prepared as previously described (Anderson & Cohen, 1974), except that the final solutions were equilibrated with frog Ringer instead of with 0.05 M ammonium acetate buffer. Stock solutions (10^{-4} - 10^{-3} g/ml) were stored frozen at -40°C and thawed when required. Rhodamine-toxin conjugates had a potency of 5-10% relative to native toxin, as determined by tests on Xenopus tadpole tails (see Anderson & Cohen, 1974). The potency of fluorescein-toxin conjugates was 1-3%.

Cultures were stained by exposing them to fluorescent toxin (10^{-5} g/ml in plating medium) for 20-30 min at room temperature. After rinsing with 60% L-15 the cultures were either fixed by immersion in cold (-16°C) 95% ethanol or examined alive. Ethanol-fixed cultures were rehydrated briefly with frog Ringer. When fluorescein staining was to be observed the fixed cultures were mounted in 90% glycerol containing either 0.1 M sodium carbonate (pH 9.5) or 0.1 M Tris buffer (pH 9.0). When only rhodamine staining was to be observed the cultures were mounted in pure glycerol. Staining appeared brighter in fixed, glycerol-mounted, cultures than in living cultures but the staining patterns were similar.

Fluorescence microscopy

In early experiments fluorescent staining was observed with a Zeiss microscope using transmitted dark-field illumination as previously described (Anderson & Cohen, 1974). More recently we have used incident illumination, employing the Zeiss III RS epi-condenser. With this latter system the same field can also be examined directly with phase contrast optics. For excitation of fluorescein the light from an HBO200/W2 lamp was passed through an LP455 filter and a KP500 interference filter. For rhodamine KP600 and BP546 interference filters were used. The corresponding barrier filters were an LP520 for fluorescein and a Kodak No. 23A for rhodamine. Living cultures were always examined with incident illumination and for rhodamine staining an extra BP546 filter was placed in the excitation path.

Kodak Tri-X 35 mm film, processed to ASA 1600 in Acufine Developer, was used in preparing black and white micrographs. For color micrographs High Speed Ektachrome, processed to ASA 400, was used.

Radioautography

α-Bungarotoxin was conjugated with carrier-free ^{125}I (new England Nuclear) by chloramine T oxidation as described by Berg et al. (1972) and separated from the reaction mixture by chromatography on Sephadex G-25. The labelled toxin had an initial specific activity of 20,000 tpm/ng. Radioactivity was measured in a gamma scintillation spectrometer (Nuclear Chicago, Model 1185).

Cultures were exposed to the radioactive toxin (10^{-6} g/ml in plating medium) for 20 min. After rinsing they were fixed with 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0), rinsed with buffer, dehydrated

in ethanol, degreased in xylene and then returned to ethanol. The coverslips containing the cultured cells were dried in air at room temperature, coated with Kodak NTB-2 emulsion and incubated in the dark at 4°C (Kopriwa & Leblond, 1962). Radioautographs were developed in Kodak D170 at 18°C and fixed with 0.24 g/ml sodium thiosulphate.

Grains were counted at a magnification of 1250 times, and areas were determined with an eyepiece grid. For patches containing a high grain density an area of 64 um^2 or more was counted. For grains over the rest of the cell the areas that were counted were at least fifteen times larger. To estimate the area of a dense patch relative to the remainder of the cell photographic prints were cut into the two respective parts and weighed.

RESULTS

General description of cultures

Muscle cultures

Dissociated muscle cells attached to the collagen substrate and began to elongate within 12 hr. The majority reacquired a bipolar configuration, usually with several projections emerging along their length, but others developed a more stellate appearance (Pl.1). Striations, having a periodicity of about 1.9 um, were observed within one day and increased in prominence during the following 3-4 days. Over the same period most of the yolk granules originally present in the cells were consumed and the cells grew in size. Bipolar cells attained lengths of up to 300 um and widths of up to 40 um. The cells also increased in thickness and after about 4 days began to lose some of their finer processes. Throughout the period that the cultures were maintained (up to 1 week) the muscle cells remained mononucleated and did not migrate on the culture dish.

Each culture contained several hundred muscle cells as well as a few other cells, such as fibroblasts and an occasional melanocyte. Contamination by neural tube cells was rare, occurring in less than 10% of the more than 40 muscle cultures examined in this study. These contaminated cultures never contained more than four nerve cells, and were not included in further studies.

Muscle contraction did not occur spontaneously but could be evoked by direct electrical stimulation and by application of ACh and carbachol. Both d-tubocurarine and α-bungarotoxin were effective in blocking the action of the cholinergic agonists but did not affect contractions evoked by electical stimulation. Contractions of sufficient intensity caused the cells to detach from the substrate, resulting in irreversible damage.

Mixed cultures

Neural tube cells in culture occurred either in isolation or in small clusters, and had spherical perikarya which were 10-20 um in diameter. During the first day processes began to emerge from them in the manner originally described by Harrison (1910). These nerve processes continued to grow for 2-3 days and contacted many muscle cells (Pl. 1B). However, after the third day nerve growth appeared to cease. In fact fewer nerve processes and nerve-muscle contacts were observed in 4-5 day-old cultures than in 2-3 day old ones. This retraction of nerve processes was related to the absence of serum in the culture medium after the first day (see Methods). Although the continued presence of serum facilitated the growth of nerve processes it also caused the myotomal muscle cells to degenerate within 3-4 days. By withdrawing serum from the cultures after the first day the muscle cells remained viable for at least a week.

Nerve-muscle contacts seemed to have no preferred location or orientation (see Pls. 5 and 6). Frequently the nerve grew along the edges of muscle cells but in many cases it coursed over the muscle cell, either on the surface facing the collagen substrate of the culture dish or on the opposite free surface. Some of the contacts were oriented longitudinally with respect to the muscle cell axis whereas others had a transverse orientation. They occurred in virtually any region of the muscle cell and varied greatly in length.

Single electrical stimuli applied to nerve cell bodies evoked synchronous contractions in many of the contacted muscle cells. Spontaneous contractions were also observed in some of the nerve-contacted muscle cells. Their frequency was usually low but could be quickly and reversibly augmented by the addition of 1-2% serum. Both spontaneous and evoked contractions were reversibly blocked by d-tubocurarine (10^{-5} g/ml)

and by magnesium (10mM), and were irreversibly blocked by α-bungarotoxin (10^{-6} g/ml for 20 min). These findings indicate that muscle cells had become functionally innervated, as was previously shown to be the case in explant cultures of these same tissues (Cohen, 1972).

Patterns of fluorescent staining

Muscle cultures

One or more patches of fluorescent stain were observed on virtually every muscle cell in fourteen cultures examined 2-5 days after plating. Actual counts were made in seven cultures, 2-4 days old. In each case about one hundred muscle cells were examined, and out of a total of six hundred and fifty-two cells only one was observed which did not have a patch of stain. On the other hand fluorescent stain was never seen on fibroblasts, melanocytes, or other non-muscle cell types.

Several examples illustrating the diversity of the patches of stain are shown in Pls. 2, 3 and 4A. Although there was considerable variation from cell to cell in the location, number, size and configuration of these patches some generalizations can be made. Their most common location was at or near the ends of cells, or cell processes, on the surface facing the collagen substrate (Pls. 2A,B,E,F;3A,B). Another common location was on the free surface in more central regions of the cell (Pls. 3C,D; 4A). However patches on the free surface sometimes occurred near the ends of cells (Pl. 3E,F) and those on the collagen surface also occurred in central regions. Less frequently patches were also situated on the edges of cells and could not be visualized face-on (Pl. 2C,D). Patches on the same cell often belonged to more than one of these categories (Pls. 2C,D; 4A). Large cells tended to have more and larger patches and in some instances as many as seven distinct patches were seen on a single cell.

However, even on the largest cells patches usually extended for less than 20 um and never more than 40 um. When they occurred in central regions of cells they never extended from one edge of the cell to the other.

Individual patches of stain had fairly distinct boundaries but the intensity within the patch was frequently non-uniform. Thus many patches appeared to be compound structures composed of numerous lines or spots. Their substructure varied according to their location on the cell. Patches on the collagen surface were usually composed of a series of fine lines of alternating intensity generally oriented parallel to each other (Pl. 2E,F). On the other hand, patches on the free surface consisted of either compact arrays of small spots (Pls 3C; 4A) or complex arrangements of thin lines sometimes oriented in a stellate pattern (Pl. 3E). Often there appeared to be little if any fluorescent stain between the lines or spots.

Mixed cultures

Forty mixed cultures ranging in age from 2-5 days were examined in this study. In six of the cultures which were 3 days old some of the innervated muscle cells were first identified on the basis of spontaneous twitching or contractions evoked by electrical stimulation of the appropriate nerve cell bodies; they were then stained and examined alive.

In every one of 78 identified innervated muscle cells staining was restricted to the path of the innervating nerve process and there were no patches of stain elsewhere on the cell (Pl. 5). Indeed the distribution of stain in most of these examples was dramatically different from that found either on the cells in muscle cultures or on non-contacted muscle cells in these mixed cultures. Such distinctive staining patterns were seen on many of the nerve-contacted muscle cells in all mixed cultures, and a variety of examples are shown in Pls. 4B,5 and 6. The following description is based on the staining patterns seen in all 40 mixed

cultures of varying ages.

Stain was associated with nerve-muscle contacts independently of their location on the muscle cell. In some cases the staining appeared as a fine line along the edge of the cell (Pls. 5E; 6C,E) and in other cases it appeared as a narrow band either on the surface facing the collagen substrate or on the opposite free surface. These face-on examples seemed to have no preferred orientation with respect to the long axes of the muscle cells or their processes. Thus in some instances the bands of stain had a longitudinal orientation (Pls. 5A; 6D) whereas in others they extended across the cell (Pls. 5G, I; 6D) or even across several cells in succession (Pls. 4B, 6A). Interestingly the bands and lines of stain were frequently located in the central regions of muscle cells. This is in contrast to the situation in vivo where the myotoma' muscle cells are innervated exclusively at their ends (Lewis & Hughes, 1960).

Viewed at high magnification it was apparent that the distribution of stain along the path of nerve contact varied considerably. Occasionally the staining continued without interruption along the entire length of the contact (Pl. 6A,B). Most often, however, the bands were discontinuous and consisted of a series of small irregular regions of stain (Pls. 5; 6C,D). In still other examples the stain was limited to only a portion of the contact. Some bands of stain had intense borders parallel to the path of the nerve (Pls. 4B; 6A), but in the majority of examples this was not the case. Nevertheless the bands of stain always had well-defined boundaries where the staining intensity changed abruptly rather than gradually. Their widths were variable, even along the path of a single contact,and usually had a range of 2 - 5 um. However, the bands of stain often extended beyond the diameters of the corresponding nerve process

(Pl. 5A,B,I,J) suggesting that the stain was associated mainly if not entirely with the muscle cells. Indeed fluorescent stain was never found on nerve processes as they coursed over the culture dish or over other cell types such as fibroblasts.

The lengths of the lines and bands of stain were also highly variable. Thus, while many were less than 20 um some extended for more than 40 um and occasionally for as much as 100 um (Pls. 5A,C; 6C,D). This finding is of particular significance since the patches on non-contacted muscle cells were never more than 40 um in their greatest dimension. In addition, unlike the patches on non-contacted cells, the bands of stain sometimes extended entirely across the central regions of muscle cells (Pls. 4B; 5I; 6A). These examples therefore can not be explained simply in terms of the nerve having contacted pre-existing receptor patches. Instead they indicate that the localization of at least some of the receptors along the path of contact was neurally induced.

Lines and bands of stain sometimes appeared to extend beyond the region of nerve contact and in a few cases were also found on muscle cells which had no apparent nerve contact. These latter examples were seen only in regions of the culture which contained neural tissue. They can most likely be explained either by difficulty in resolving very fine nerve processes in the light microscope (see also Fambrough, Hartzell, Rash & Ritchie, 1974), by mechanical displacement of the nerve process during the staining procedure, or by spontaneous retraction of the nerve process. Some retraction clearly did occur with aging of the cultures and an example of retraction associated with a modified pattern of stain is given in the following paper (see Pl. 6 in Anderson & Cohen, 1976).

Although innervation was invariably correlated with a localization

of stain along the path of nerve contact and with an absence of stain elsewhere on the cell, such distinctive patterns of stain were not associated with all nerve-contacted cells. Instead some muscle cells had little or no stain along the path of nerve contact. In these cases there was almost always additional stain on other regions of the cell. This staining sometimes consisted of the characteristic patches associated with non-contacted muscle cells. On the other hand the staining pattern was sometimes markedly different and consisted of a scattering of small fluorescent spots over a large portion of the cell. This latter form of staining was observed more frequently in cultures where addition of neural tissue was delayed for 2 - 3 days (see below).

Addition of neural tube cells to 2-and-3-day-old muscle cultures

The results described so far indicate that contact of a growing nerve fibre with a pre-existing patch of high receptor density can not account for all of the staining that was associated with nerve-muscle contacts. Indeed since the nerve and muscle cells were plated simultaneously the receptor distribution prior to innervation was unknown and patches may never have been present. It was therefore of interest to determine whether the nerve would induce a similar localization of receptors if innervation was delayed until patches had already developed on all muscle cells. This possibility could be readily tested because all the muscle cells in "pure" muscle cultures developed characteristic receptor patches within two days of plating. Muscle cultures were therefore allowed to develop for 2 - 3 days during which time they were carefully examined to ensure that there was no neural contamination. Freshly dissociated neural tube cells were then plated. Within one day growing nerve processes contacted many of the

muscle cells, some of which began to twitch spontaneously, indicating that innervation had occurred. Altogether six such cultures were stained with fluorescent toxin 1-2 days after the addition of neural tube cells.

In each of these cultures the staining patterns on nerve-contacted cells were similar to those described for cultures where neural tube cells were present from the start. Most significantly lines and bands of stain were associated with paths of nerve-muscle contact, and some of these extended for more than 40 um (Pl. 7A,C) or crossed the entire width of the cell. In such cases there were usually no characteristic patches elsewhere on the cell. One interesting difference, however, between these cultures and those in which neural tissue was present from the start, was that in many more instances there was a widespread speckling of small spots of fluorescent stain (Pl. 7A,E), particularly on the first day after adding neural tissue. This speckling was usually most extensive on those muscle cells which had relatively little stain along the path of nerve-muscle contact. Furthermore, it was never seen on non-contacted muscle cells in regions of the culture devoid of neural tube cells, or in two additional cultures which were treated in the same manner (see Methods) except that neural tube cells were not added. Instead the non-contacted muscle cells had only the characteristic patches of stain.

These observations therefore indicate that the nerve can cause both a rapid accumulation of receptors at sites of contact, and the development of scattered recptor clusters, on muscle cells which have receptor patches before contact is made.

Mixed cultures grown in curare and carbachol

Previous studies have indicated that neuromuscular connections can become established in the presence of cholinergic agents which block neuromuscular transmission (Cohen, 1972; Jansen & Van Essen, 1975). On

the other hand supression of neuromuscular activity in adult muscle results in the appearance of extrajunctional ACh receptors (Thesleff, 1960; Lomo & Rosenthal, 1972; Berg & Hall, 1975a; Chang, Chuang & Huang, 1975; Lavoie, Collier & Tenenhouse, 1976; Pestronk, Drachman & Griffin, 1976). Studies of synapse formation in cell culture have led to the suggestion that contractile activity may also be required for nerve contacts to bring about changes in the distribution of extrajunctional receptors during development (Fischbach & Cohen, 1973; Cohen & Fischbach, 1973). It was therefore of interest to determine whether the spontaneous neuromuscular activity in the present experiments was necessary for the development of the distinctive staining patterns on nerve-contacted cells. For this purpose mixed cultures were grown from the start, for 3-4 days, in the presence of either d-tubocurarine chloride (10^{-4} g/ml; 3 cultures) or carbachol chloride (10^{-5} - 10^{-4} g/ml; 6 cultures). In addition, neural tube cells were added to 3-day-old muscle cultures in the presence of d-tubocurarine chloride (10^{-4} g/ml; 3 cultures) and allowed to develop for 1-2 days. As in drug-free cultures, nerve processes developed rapidly and within one day many could be seen in contact with muscle cells. However, as expected, contractions did not occur spontaneously under these conditions and could not be evoked by electrical stimulation of neural tube cells. On the other hand, both spontaneous and nerve-evoked contractions were observed within 10-20 min after withdrawing the drug. When such cultures were stained after rinsing out the drug the usual patterns of fluorescence were observed. Muscle cells not contacted by nerve had characteristic patches of stain, whereas some of those which were contacted had the distinctive lines and bands without any patches elsewhere (Pl. 8D,F). Likewise, muscle cells which had little or no stain

along the path of nerve contact sometimes had a widespread scattering of small fluorescent spots. As in cultures grown in the absence of the drugs, this feature was more common in cultures where the addition of neural tube cells was delayed. These results therefore indicate that nerve-induced changes in the distribution of ACh receptors occurred by a mechanism which is independent of contractile activity. This finding is extended in the following paper which demonstrates similar patterns of stain even when cultures are grown in the presence of α-bungarotoxin (Anderson & Cohen, 1976).

Control experiments

Specificity of fluorescent staining

In a previous study it was established that α-bungarotoxin retains its specificity for ACh receptors after it has been labelled with fluorescent dyes (Anderson & Cohen, 1974). As indicated in Table 1, specificity tests were also carried in the present study, with similar results. Fluorescent stain was barely detectable when the staining procedure was carried out in the presence of d-tubocurarine (10^{-4}g/ml) and was not seen when cultures were pretreated with native α-bungarotoxin (10^{-6}g/ml for 20 min). Carbachol was also tested but in this case the cultures were grown in the drug from the start in order to avoid muscle contracture and damage. At a concentration of 10^{-4}g/ml it completely inhibited fluorescent staining. As expected the blocking effect of native toxin was not overcome even when the cultures were rinsed extensively with toxin-free medium for up to 1 hr. On the other hand the blocking effects of curare and carbachol were quickly reversible. For example in one type of experiment cultures were exposed for 30 min to rhodamine-toxin (10^{-5}g/ml) in the presence of curare (10^{-4}g/ml). They were then rinsed for 20 min with a medium free of both agents and exposed to fluorescein-toxin (10^{-5}g/ml) for a further 30 min.

Table 1. Effects of cholinergic agents on fluorescent staining. F and R indicate fluorescein and tetramethylrhodamine respectively. Concentrations are given in text.

Culture Medium	Staining Procedure			Fluorescent Staining	
				F	R
standard	F-toxin	--	--	bright	none
standard	R-toxin	--	--	none	bright
standard	toxin	R-toxin	F-toxin	none	none
standard	curare	curare, R-toxin	F-toxin	bright	barely visible
plus carbachol	carbachol, R-toxin	F-toxin	--	bright	none
plus carbachol	carbachol F-toxin	R-toxin	--	none	bright

Upon examination in the fluorescence microscope only the green stain due to the fluorescein conjugate was observed (Pl. 8A-C). Similar results were obtained when carbachol was used instead of curare (Pl. 8D-F; Table 1). All of these tests therefore indicate that the staining is specific for ACh receptors.

Receptor staining was readily distinguishable from yolk granules which proved to be autofluorescent. The granules had a smooth appearance (Pl. 3C) with no single plane of focus, and when viewed with fluorescein optics they appeared yellow rather than green. Their presence in large numbers resulted in a bright glow which obscured the fluorescent stain, and because of this we restricted our observations to cultures which were 2 or more days old. Smaller bright refractile spots, also readily distinguishable from receptor staining, were seen in early experiments in which transmitted light was used for excitation (Pl. 4). These spots were not observed with incident light fluorescence.

Staining after fixation with paraformaldehyde

Although the patterns of fluorescent stain reflect the distribution of ACh receptors, it is less clear that they also correspond to the distribution of receptors prior to binding with fluorescent toxin. For example, other ligands such as immunoglobulins and lectins can cause their receptors to aggregate in the plasmalemma (Taylor, Duffus, Raff & de Petris, 1971; Rosenblith, Ukena, Yin, Berlin & Karnovsky, 1973). This effect appears to depend upon the fact that these ligands are multivalent and can therefore cross-link their previously dispersed receptors. α-Bungarotoxin on the other hand is a small univalent ligand (Mebs, Narita, Iwanaga, Samejima & Lee, 1971; Lee, 1972) and would therefore not be expected to cause changes in the distribution of ACh receptors. In any event we have attempted to examine this possibility experimentally, and have based the

test on the fact that ligand-induced changes in receptor distribution can be prevented if the cells are first fixed with paraformaldehyde (Rosenblith et al., 1973; Ryan, Borysenko & Karnovsky, 1974).

For these experiments muscle cultures were fixed at room temperature for 1 hr with 3×10^{-2} g/ml paraformaldehyde in 0.1 M phosphate buffer at pH 7.0. The cultures were then rinsed with plating medium for at least 30 min and stained with fluorescent toxin. In some cases the rinse and staining were carried out in the presence of carbachol (10^{-4}g/ml). Upon examination with fluorescence optics the cells generally appeared brighter than usual, but had characteristic patches similar to those on living cells (Pl. 9A,B). Cells stained in the presence of carbachol also appeared brighter than usual, suggesting some non-specific uptake of dye-toxin conjugate, but had no patches of fluorescent staining. These results indicate that staining specificity for ACh receptors was retained after paraformaldehyde fixation, and suggest that receptor patches existed on the cells before exposure to the toxin. Similar conclusions have been drawn for the patchy distribution of toxin-binding sites on cultured chick myotubes (Sytkowski et al, 1973).

Radioautography with ^{125}I-labelled toxin

Although fluorescent staining can reveal areas of high receptor density the sensitivity of the method may not be sufficiently great to reveal lower densities of receptors (Anderson & Cohen, 1974). It was therefore not possible to determine by fluorescent staining whether all receptors on muscle cells were localized in patches or whether some were also distributed elsewhere on the sarcolemma. In order to check this possibility experiments were carried out in which receptor distribution

was assessed by radioautography using ^{125}I-labelled α-bungarotoxin. For these experiments muscle cultures were exposed to the radioactive toxin (10^{-6}g/ml) for 20 min, rinsed, and then processed as described in the Methods. As expected, patches of high grain density were observed which were similar both in size and distribution to the patches of fluorescent staining on the free surface of the muscle cells (Pl. 9C,D,E). In addition to these patches a lower density of grains was observed over the remainder of the cell. Most of the grains appeared to be due to specific binding of radioactive toxin to ACh receptors: thus in a sister culture which was incubated for 20 min with 10^{-6}g/ml native toxin before being exposed to the radioactive toxin the grain counts on the cells were reduced by 92% (Table 2). In one culture grain counts were made on 13 muscle cells having only one obvious patch and the grain densities were found to be about 25 times higher in the patches than on the rest of the cell (Table 2). Estimates based on measurements of relative patch and cell areas further indicated that about 44% of all the grains on these cells were localized in patches (Table 2). It follows from these experiments that ACh receptors occurred not only in patches but also over the entire cell. The density of receptors in this widespread phase, however, was too low to be detected by the fluorescent staining technique.

Table 2. Radioautography with ^{125}I-labelled α-bungarotoxin in 2-day-old muscle cultures.

A. Inhibition by pre-incubation with native toxin (10^{-6} gm/ml for 20 min). Isolated cells were chosen at random and all grains over at least seventy percent of each cell were counted. Similar areas adjacent to the cells were also counted in order to obtain the background grain density. The results (mean ± standard error) in each case are based on fourteen cells or culture dish areas. Percent inhibition was calculated after correction for background grains on the culture dish.

Grain density (grains/64 um^2)				Percent inhibition by native toxin
Pre-incubated with native toxin		No pre-incubation		
Culture dish	Cell	Culture dish	Cell	
0.4 ± 0.04	0.8 ± 0.1	0.4 ± 0.03	5.4 ± 0.4	92

B. Distribution of grains. Isolated cells were chosen which had only one patch of high grain density. Grain densities are corrected for background grains on the culture dish.

No. of cells	Grain density (grains/64 um^2)		Grain density in patch relative to rest of cell	Area of patch relative to rest of cell	Percentage of grains in patch
	high density patch	rest of cell			
13	48 ± 2	2 ± 0.2	26 ± 3	0.034 ± 0.004	44 ± 3

DISCUSSION

Patches of ACh receptors on non-innervated cells

The present study has demonstrated distinctive patterns of ACh receptor distribution on myotomal muscle cells cultured with and without nerve. Non-innervated muscle cells develop characteristic patches of receptors which tend to occur in preferred locations, such as near the tips of cell processes facing the collagen substrate and in central regions on the opposite side of the cell. They occupy a small percentage of the cell area but contain a significant fraction of the receptors. Radioautographic experiments indicated that single patches which occupy only about 3-4% of the cell area contain almost 50% of the receptors. Presumably these patches reflect regions of sarcolemma where the receptor density is relatively high. This has recently been found to be the case for ACh receptor patches on cultured chick myotubes in experiments which combined an immunoperoxidase technique with electron microscopy (Vogel & Daniels, 1976). The alternative possibility, that receptor density in the sarcolemma is uniform and that the patches reflect regions of extensive membrane folding, seems unlikely. For example bright fluorescent staining was observed on fine cell processes which were less than 1 um in diameter (see Pl. 3A) and which would not be expected to have extensive membrane folding. Furthermore, radioautography revealed patches with grain densities which were on the average 25-fold greater than over the remainder of the cell. In individual examples the factor was as large as 40-fold. To increase the surface area by such a large factor would require a degree of membrane folding which has never been seen in normal or cultured muscle cells. For example the complex folds at the mammalian neuromuscular

junction are estimated to increase membrance area by only 4-5 times (Andersson-Cedergren, 1959). In view of these considerations it seems likely that the receptor patches on cultured myotomal muscle cells, like those on cultured chick myotubes, reflect regions of sarcolemma containing a high receptor density.

Another question is whether the patches of receptors on non-innervated cells are a normal feature of the sarcolemma. This matter is of considerable relevance since other ligands such as immunoglobulins and lectins have been shown to induce the aggregation of their receptors (for reviews see Raff & de Petris, 1974; Nicolson, 1974). However, discrete regions of high ACh receptor density have been revealed in cultures of chick and rat myotubes by the method of iontophoresis which is carried out in the absence of α-bungarotoxin (Cohen & Fischbach, 1973; Hartzell & Fambrough, 1973). Receptor patches were also observed in the present study as well as on chick myotubes (Sytkowski et al., 1973) when receptor labelling was carried out after formaldehyde fixation, a procedure which is known to prevent ligand-induced clustering of receptors in other systems (Rosenblith et al, 1973; Ryan et al., 1974). It therefore appears that ACh receptor patches are a normal feature of the sarcolemma and do not merely reflect an aggregation of receptors induced by toxin binding.

It is possible nevertheless that the formation of ACh receptor patches may reflect processes similar to those which have been implicated in the aggregation of immunoglobulin and lectin receptors. For example, several studies have suggested that the mobility of some membrance receptors is controlled by attachement to cytoskeletal elements composed of microfilaments and microtubules (for reviews see Nicolson, 1974; Raff & de Petris, 1974;

Edelman, 1976). Similar cytoplasmic structures may also interact with ACh receptors. The resolution afforded by fluorescent staining has revealed that ACh receptor patches are highly ordered structures with distinctive patterns. Furthermore, the complex substructure of the patches on the surface of the cell in contact with the collagen substrate is distinctly different from that of patches on the free surface. This difference in organization presumably reflects the fact that the sarcolemma is attached in some regions to the substrate. The complexity of receptor patches on the free surface is particularly difficult to rationalize if one assumes the membrance is a simple "fluid-mosaic" in which integral glycoproteins exist in a state of diffusional equilibrium (see Singer & Nicolson, 1972). These ACh receptor patches usually consist of either an aggregate of small receptor clusters (Pl. 3C) or a stellate pattern of thin lines, sometimes interspersed with small clusters (Pl. 3E). It is unlikely that the components of these patches are held together in such non-random arrays by simple intermolecular forces between receptor units. Instead it seems more probable that small ACh receptor clusters are anchored to cytoplasmic elements which might then provide a structural basis for the complex patch substructure. In this vein it is interesting also to note that the cable-like bundles of contractile proteins which form part of the cytoskeleton in non-muscle cells have been found to assume complex networks (Lazarides, 1976) with foci not unlike the stellar arrangements of some ACh receptor patches.

Distribution of ACh receptors on innervated cells

The most distinctive feature of the receptor staining on innervated myotomal cells was its localization along the path of nerve contact. Since staining was never seen on nerve processes which were not in contact with muscle cells, and in many cases bands of stain were broader than the corresponding nerve, the pattern of stain at sites of nerve contact almost certainly reflects the development of a high receptor density in the subsynaptic membrane. This conclusion can be drawn most directly from the results of the following paper where similar patterns of stain were found to develop even when muscle cells were exposed to fluorescent toxin prior to the addition of nerve (Anderson & Cohen, 1976). It is also in agreement with other studies which have demonstrated either a high sensitivity to ACh (Harris, Heinemann, Schubert & Tarakis, 1971; Kano & Shimada, 1971a; Fischbach & Cohen, 1973) or a high density of ^{125}I- α-bungarotoxin binding sites (Fambrough et al., 1974) in the vicinity of nerve-muscle contacts in cell culture.

The resolution afforded by fluorescent staining revealed a large diversity in the pattern of receptor distribution along the path of nerve-muscle contact. In some cases the staining along the path of contact was continuous and uniform but in the majority of examples the bands of stain were discontinuous and irregular. Of particular significance is that in all cases there were abrupt changes in staining intensity, and hence in receptor density, along the edges of the bands of stain. Similar abrupt changes in receptor density occur at the border between synaptic and extrasynaptic membrane in normal adult muscle (Peper & McMahon, 1972; Hartzell & Fambrough, 1972; Anderson & Cohen, 1974; Kuffler & Yoshikami, 1975; Fertuck & Salpeter, 1976). The present study has indicated that

they can also occur elsewhere on the muscle cell. For example receptor patches on non-contacted muscle cells had well-defined boundaries, as did the small receptor clusters which were seen on some of the nerve-contacted cells. It is apparent therefore that abrupt changes in receptor density are not a unique feature which is dependant upon innervation. Instead they are more likely to reflect a mechanism within the muscle cell that can organize ACh receptors into two distinct phases which have markedly different packing densities and are separated by distinct borders.

Since non-contacted muscle cells developed patches of high receptor density analogous to those which have been observed on chick (Vogel et al, 1972; Fischbach & Cohen, 1973; Sytkowski, et al., 1973; Vogel & Daniels, 1976) and rat myotubes (Hartzell & Fambrough, 1973), the question can be raised as to whether these structures play a role in synaptogenesis, perhaps by providing a site which growing nerves seek out. To answer this question it would be necessary to determine whether any part of the subneural membrane contained a high density of ACh receptors prior to the establishment of synaptic contact. The results of the present study therefore do not exclude this possibility. However they do indicate the existence of an alternative mechanism by which receptors become localized at sites of innervation. For example, the lengths of the bands of staining at sites of nerve contact were sometimes too great to be explained simply by the nerve having contacted a pre-existing patch. Even if a patch was contacted, the staining pattern on innervated myotomal cells can thus only be rationalized by assuming that new regions of densely packed receptors formed along the path of the nerve. In fact, such an inductive process could alone account for all the changes in ACh receptor distribution at sites of innervation. Taken together, these considerations imply that the

formation of dense receptor patches may not be a requirement for synapse formation. Instead the formation of patches may simply reflect a mechanism in muscle cells which normally mediates the nerve-induced localization of ACh receptors.

The nerve-induced changes in receptor distribution demonstrated in the present study involved not only an accumulation of receptors in the subneural membrane, but also in some cases the development of a widespread scattering of small receptor clusters. Similar changes occurred even when the cultures were grown in high concentrations of curare or carbachol which block all spontaneous twitching. Indeed, the concentration of curare used in these experiments has been shown to abolish all neurogenic electrical activity in cultured myotomal muscle cells (Cohen, 1972). This indicates that the development of new regions of high receptor density at the site of innervation, and the extensive changes in the distribution of 'extrajunctional' receptors, were brought about by some neural factor independant of synaptic or contractile activity. The same conclusion can be drawn from the results of the following study where corresponding changes in receptor distribution were observed when development took place in the presence of α-bungarotoxin (Anderson & Cohen, 1976). These observations thus indicate that changes in receptor distribution must have been induced either by the release of a neural substance or by direct interaction between the surface membranes at the site of contact.

The inductive process involved in the change in receptor distribution could operate in at least two conceptually distinct ways. In one case nerve contact might provide a spatial component to receptor metabolism, leading to a gradual build-up of receptors synthesized after the contact is made. For example newly synthesized receptors might be inserted preferentially

at the site of contact or receptors in the region of contact might be protected from degradation. The latter possibility is in line with recent studies which have revealed that junctional receptors are more stable metabolically than extrajunctional receptors (Berg & Hall, 1974, 1975b; Chang & Huang, 1975; Frank, Gautvik & Sommerschild, 1975). The other principle alternative would be that nerve contact produces a rearrangement of receptors in the sarcolemma such that mobile receptors aggregate at the site of innervation. This possibility is supported by the finding of the present study that the inductive effect of innervation on receptor distribution is not restricted to the vicinity of the nerve contact, but can also lead to the appearance of small receptor clusters over large areas of extrasynaptic muscle membrane. More direct evidence in support of a process of receptor redistribution is provided in the following paper (Anderson & Cohen, 1976).

EXPLANATION OF PLATES

Except where stated otherwise examples of fluorescent staining were obtained with incident illumination. Bars represent 20 um.

Plate 1

Low magnification phase contrast views of living cultures. A: A region of a 3-day-old muscle culture. By this time in culture the myotomal muscle cells have developed striations. The cells remain mononucleated. B: A region of a 2-day-old mixed, nerve and muscle culture. Nerve processes emanating from a cluster of neural tube cells have contacted several myotomal muscle cells. Most of the muscle cells still have numerous yolk granules in their nuclear region and have less prominent striations than in A.

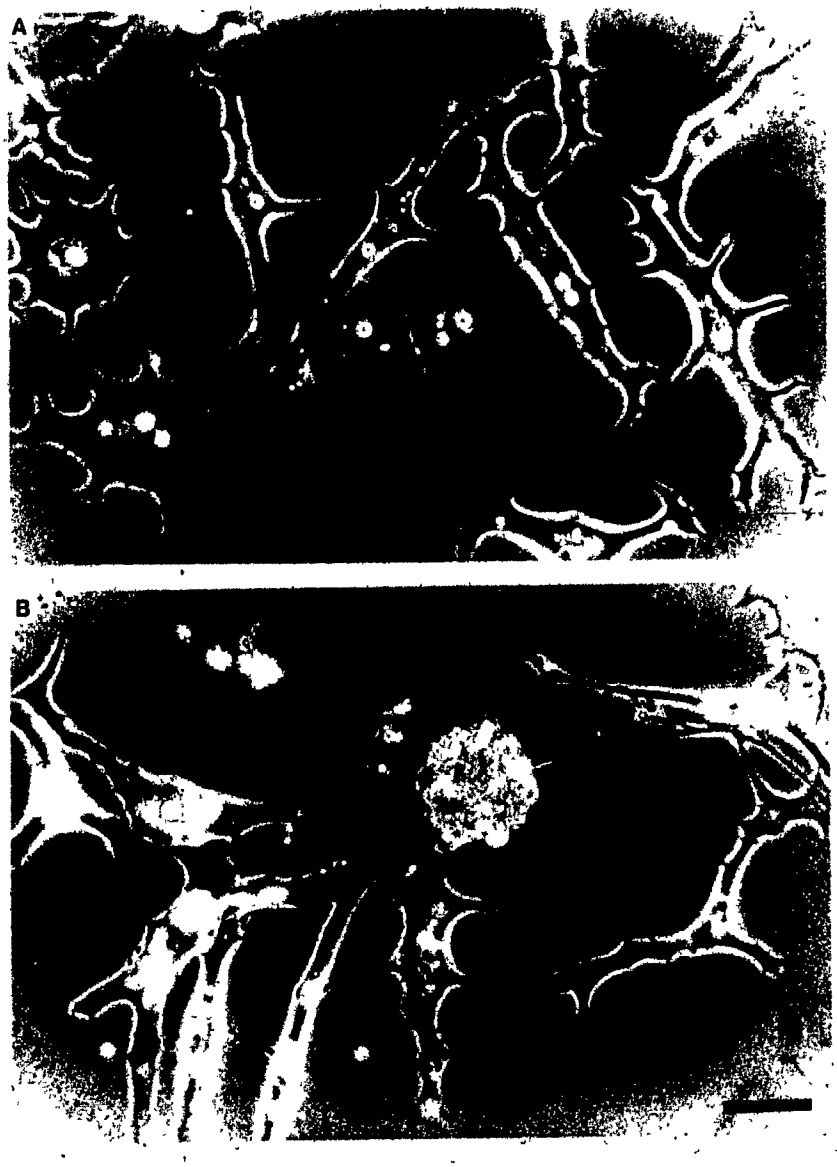
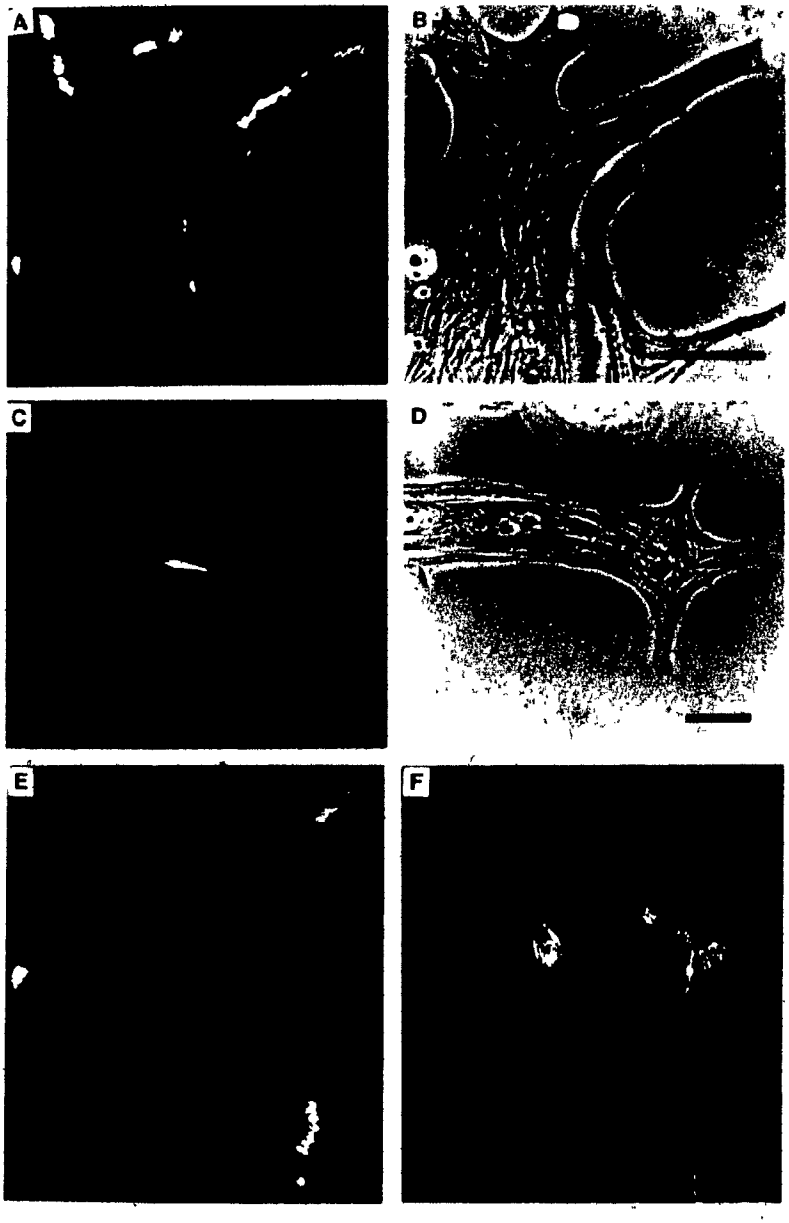

A
B

Plate 2

Patches of fluorescent stain on muscle cells cultured without neural tissue. Cultures were stained with rhodamine-labelled toxin, fixed in ethanol and mounted in glycerol. A, B: Fluorescence and phase contrast views of the same field in a 5-day-old culture. Patches of stain are on the collagen surface of the cells. The bright profile near the left hand edge of A is autofluorescence associated with the unidentified particle in B. Cell outlines (A) have been traced in. C,D: Fluorescence and phase contrast of a field in a 3-day-old culture. A patch of stain is located on the edge of the cell. Other patches on the collagen surface of the cell are not in the plane of focus and appear faint. E: Patches of stain on collagen surface at ends of cell processes. Fluorescence and phase contrast views are superimposed. 3-day-old culture. Scale as in D. F: Same as E, but from another 3-day-old culture. Note the non-uniformities within each patch. Scale as in B.


A
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C
D
E
F

Plate 3

Patches of fluorescent stain on muscle cells cultured without neural tissue. 4-day-old cultures were stained with rhodamine-labelled toxin and examined alive. Each pair of micrographs shows the same field viewed with fluorescence and phase contrast optics. A, B: Patches on collagen surface of cell. Note in particular the staining associated with some of the very fine processes at the cell edge. C, D: Patches (see arrows) of stain on the free surface of the cell. Note that the lower patch consists of an aggregation of small fluorescent spots. Several autofluorescent granules are present in the perinuclear region. E,F: Patch, on free surface of cell, consisting of a network of fine lines and spots.

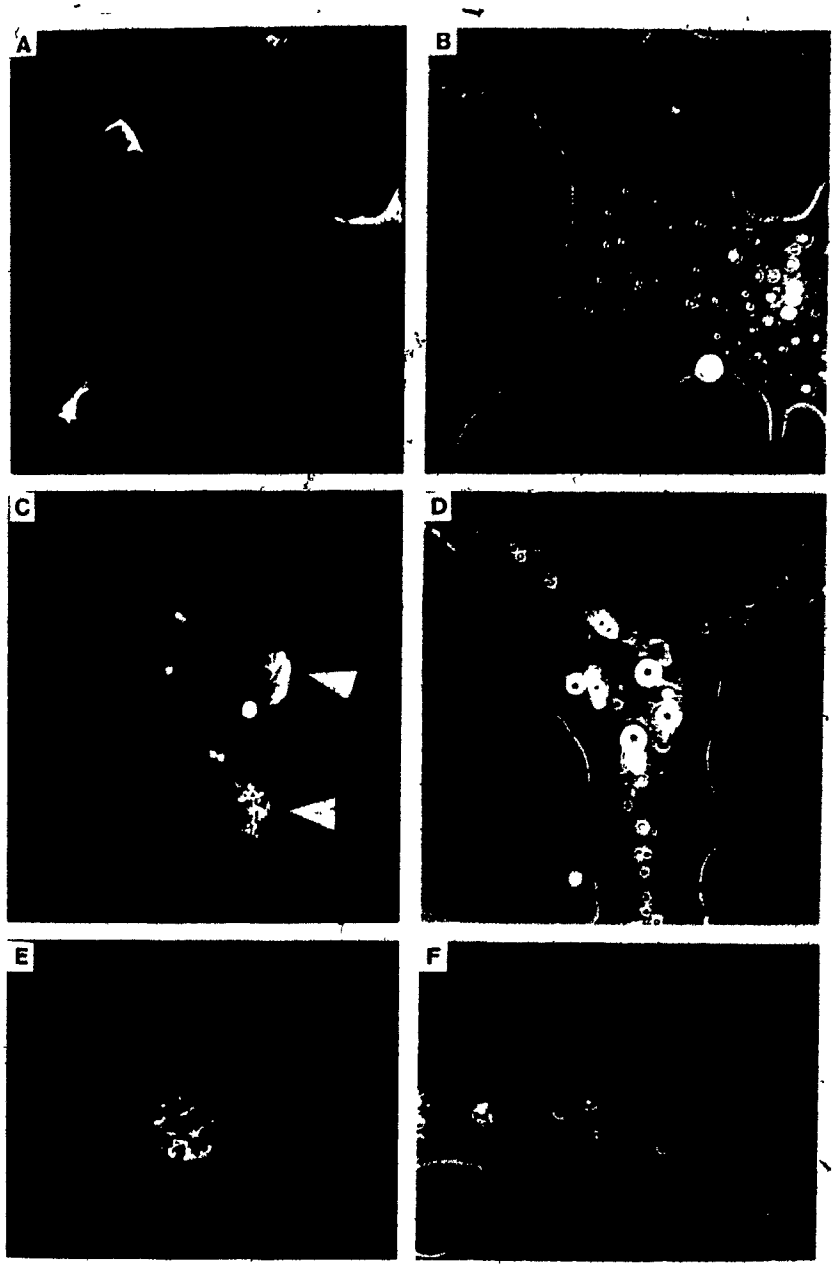
A
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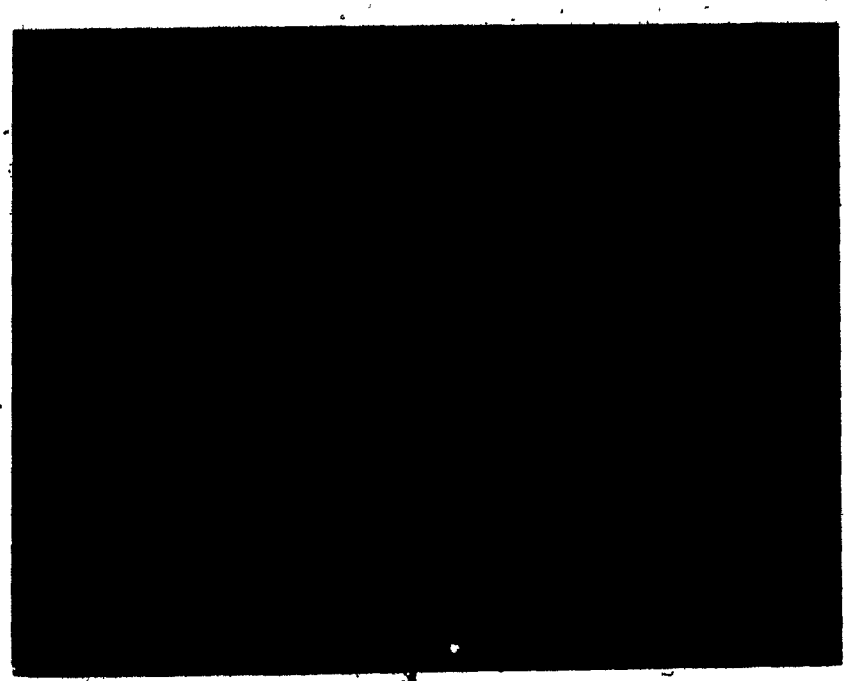
Plate 4

Colour micrographs of cultured muscle cells stained with rhodamine-labelled toxin. 3-day-old cultures fixed in ethanol, mounted in glycerol, and examined with transmitted dark-field illumination (see Methods). A: From a culture without neural tissue. The central patch of stain is on the free surface of the cell and consists of an aggregation of small fluorescent spots. Two other patches, not in the plane of focus, are on the collagen surface of the cell. B: From a mixed culture containing both nerve and muscle cells. The narrow bands of fluorescent stain are on the collagen surface of two muscle cells. Such bands of stain are typically associated with the path of nerve contact (not seen). Note the absence of stain elsewhere on these cells. The small bright refractile spots (A,B) were not seen with incident-light fluorescence.

(A)



(B)

20 um

Plate 5

Patterns of fluorescent stain on identified, innervated muscle cells. 3-day-old cultures were stained with rhodamine-toxin and examined alive. All of the nerve-contacted muscle cells in this plate had twitched spontaneously prior to being stained. Each pair of micrographs shows the same field viewed with fluorescence and phase contrast optics. Note that in all cases the stain is restricted to regions of nerve-muscle contact.

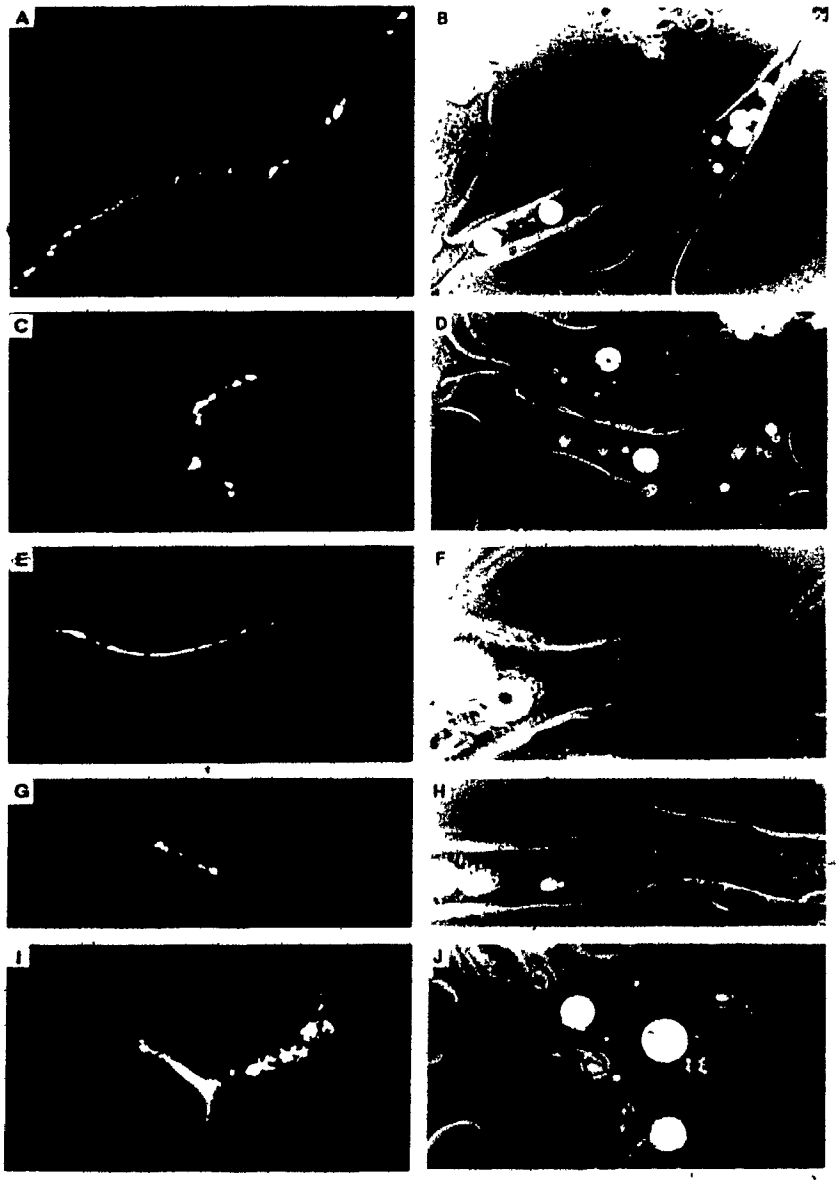
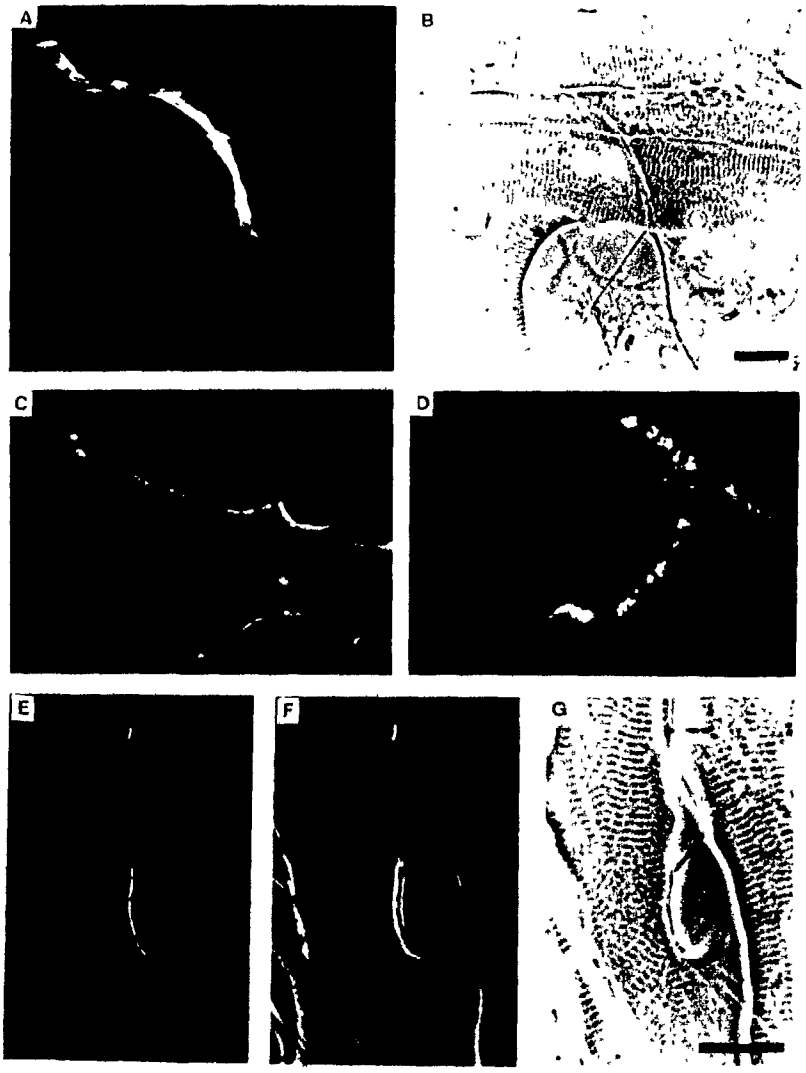

A
B
C
D
E
F
G
H
I
J

Plate 6

Patterns of fluorescent stain on nerve-contacted muscle cells. Cultures were stained with rhodamine-labelled toxin. A, B: Fluorescence and phase contrast views of the same field in a 4-day-old living culture. The stain follows the path of nerve-muscle contact and extends across the entire breadth of three successive cells. C,D: Fluorescent staining in a 2-day-old culture, fixed in ethanol and mounted in glycerol. Note the long lengths of stain along edge of cell (C) and across breadth of cells (D). Paths of nerve-muscle contact were not visible in these examples. Scale as in B. E,F,G: A field in a 5-day-old living culture viewed with fluorescence (E) and phase contrast (G) optics. In F both the fluorescence and phase contrast images in E and G are superimposed to show that the fluorescent stain is entirely restricted to the site of nerve-muscle contact.


A
B
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F
G

Plate 7

Patterns of fluorescent stain on nerve-contacted muscle cells in cultures to which neural tube cells were added on day 3. Cultures were stained with rhodamine-toxin and examined alive. Each pair of micrographs shows the same field viewed with fluorescence and phase contrast optics. A, B: A field in a culture stained one day after adding neural tube cells. Note the intense staining associated with one of the nerve contacts and the scattered small spots of fluorescent stain elsewhere on the cell. C,D: A field in a culture two days after adding neural tube cells. The stain is restricted to the path of nerve-muscle contact. E,F: From a culture stained one day after adding neural tube cells. The same nerve process has contacted several muscle cells and there is considerable variation in the amount of stain associated with the sites of contact. Note also the widespread scattering of spots of fluorescent stain on the cell in the upper left hand quadrant. In A and E cells have been outlined and nerve processes are indicated by dotted lines.

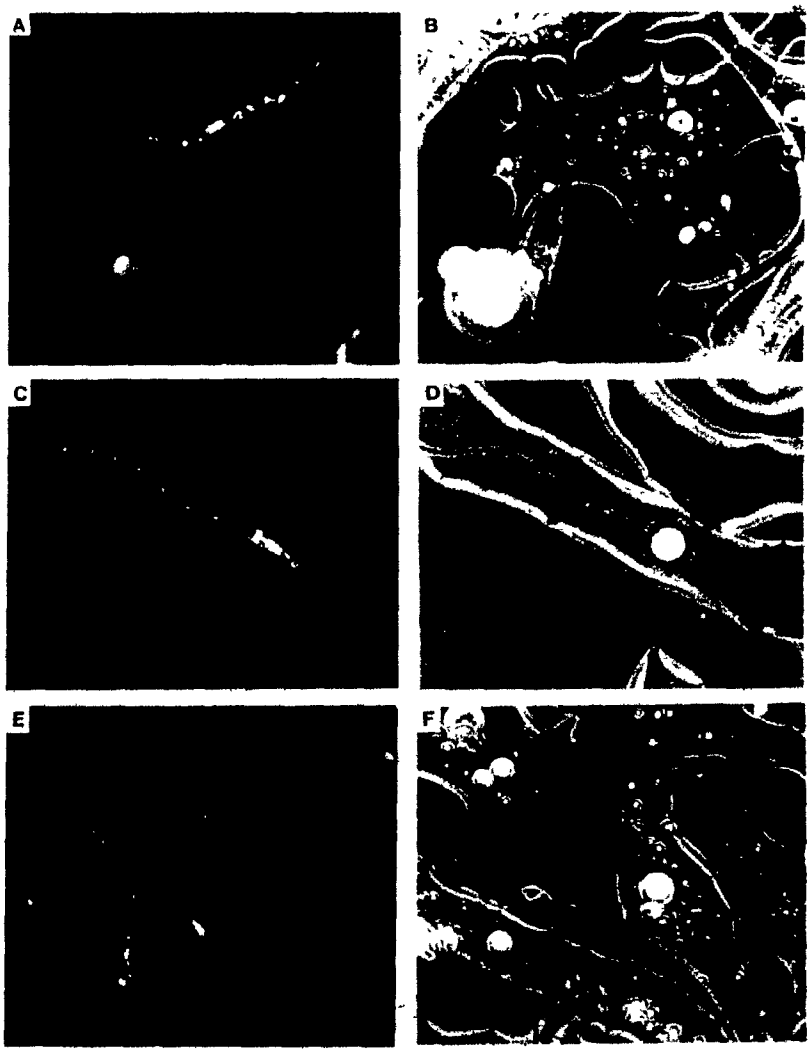
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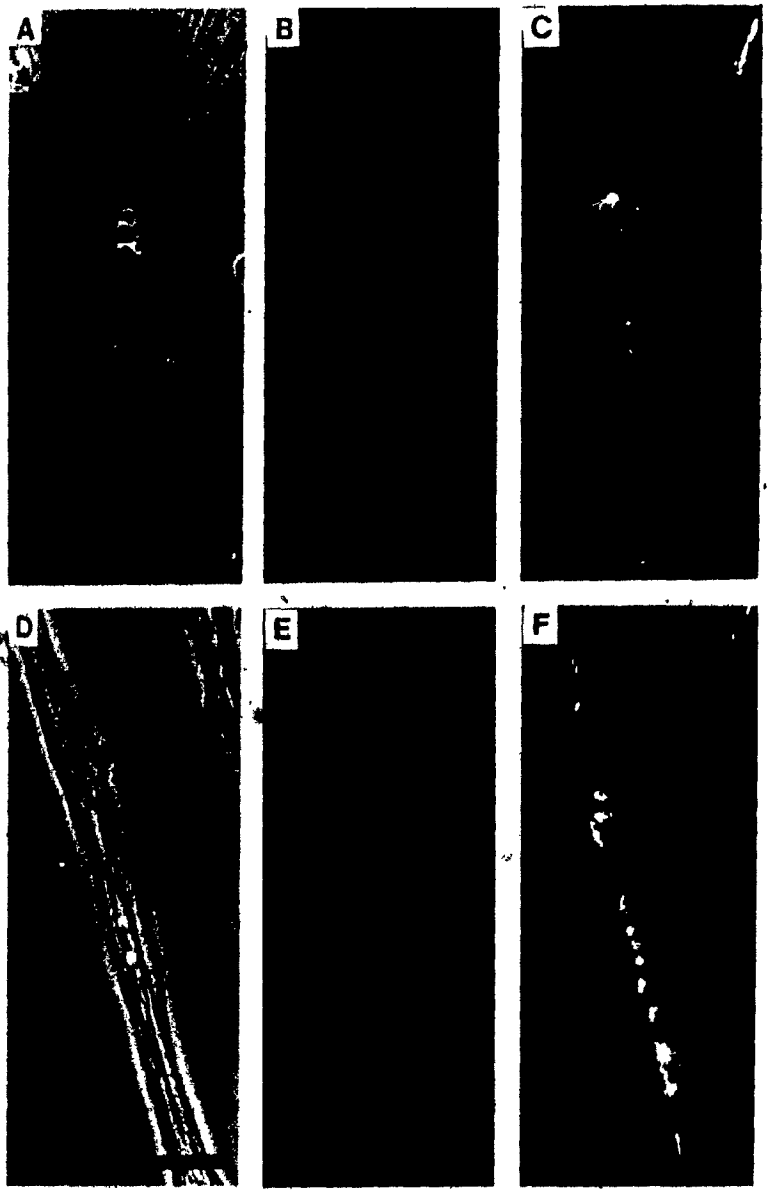
Plate 8

Reversible inhibition of fluorescent staining by cholinergic agents. A,B,C: A field in a 2-day-old mixed culture which was exposed to rhodamine-toxin in the presence of curare (10^{-4}gm/ml) and then to fluorescein-toxin after washing out the curare. The phase contrast view (A) shows a nerve process contacting a muscle cell. When examined for fluorescence virtually no rhodamine staining (B) was observed but the fluorescein staining (C) was bright. D,E,F: A field in 3-day-old mixed culture grown in the presence of carbachol (10^{-5}gm/ml). The culture was exposed to fluorescein-toxin in the presence of carbachol and then to rhodamine-toxin after washing out the carbachol. The phase contrast view (D) shows a nerve process in contact with two muscle cells. When examined for fluorescence virtually no fluorescein staining (E) was observed but the rhodamine staining (F) was bright. Cultures were fixed in ethanol and mounted in alkaline glycerol (see Methods). After such treatment it was usually impossible to visualize the path of nerve-muscle contacts (A,D).


A
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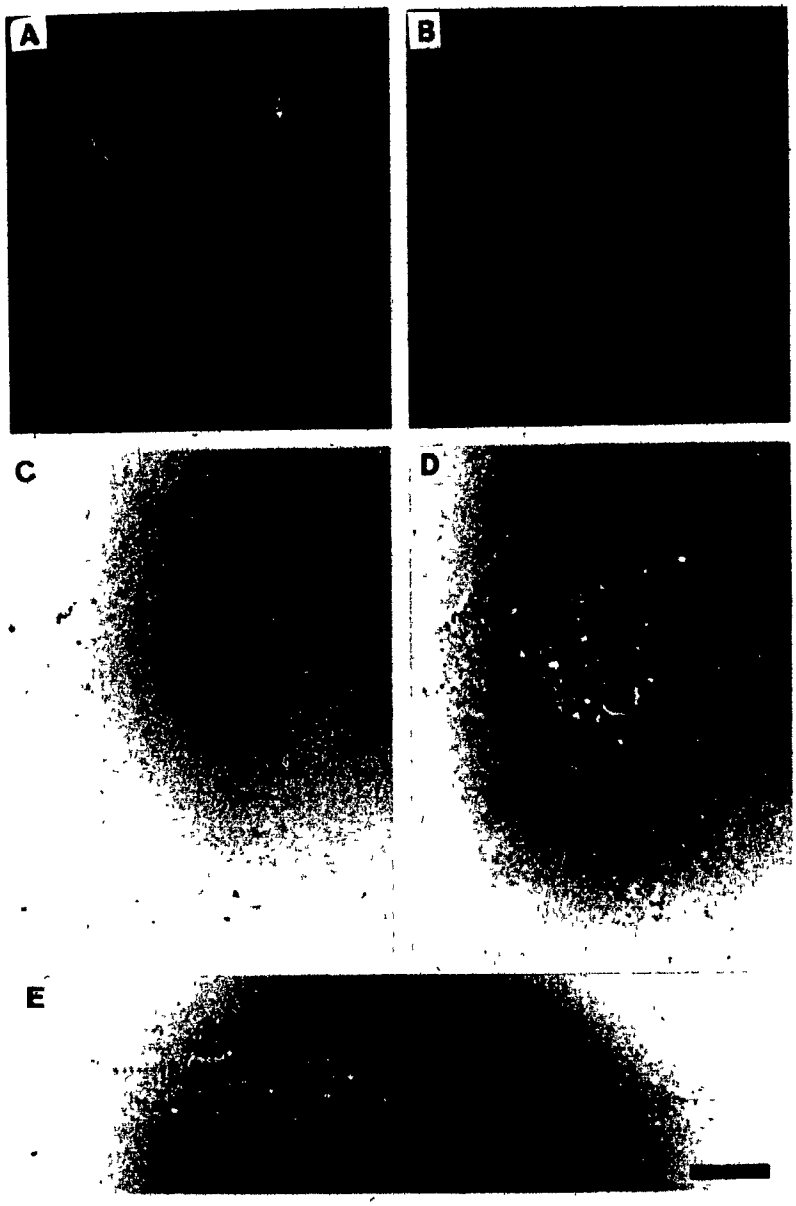
C
D
E
F

Plate 9

A,B: Fluorescence and phase contrast views of a field in a muscle culture which was fixed for 1 hr. in 4% paraformaldehyde before being stained with rhodamine-toxin. Note the typical patches of stain. C,D,E: Radioautographs from a 2-day-old muscle culture exposed to ^{125}I-labelled toxin. Brightfield (C) and phase contrast (D) views of the same field show a scattering of grains over the muscle cell as well as a patch of high grain density. Another field (E) viewed with phase contrast optics. Scale 20 um.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CHAPTER 4

NERVE-INDUCED AND SPONTANEOUS REDISTRIBUTION OF ACETYLCHOLINE RECEPTORS ON CULTURED MUSCLE CELLS

SUMMARY

1. Three-day-old cultures of myotomal muscle, obtained from embryos of Xenopus laevis, were stained with fluorescent conjugates of α-bungarotoxin and maintained in native toxin in order to ensure that ACh receptors subsequently inserted into the sarcolemma would not be stained. Neural tube cells were then added to the cultures.
2. When cultures were examined 1-3 days later fluorescent stain was found to be associated with sites of nerve-muscle contact. In some cases the stain along the path of contact extended for greater distances than the patches of stain seen on non-contacted muscle cells.
3. The development of new areas of fluorescent stain at sites of nerve-muscle contact was confirmed by making successive observations on the same muscle cell over a period of a day.
4. Similar experiments on muscle cells not contacted by nerve revealed the formation of new receptor patches, usually in areas of cell growth.
5. The majority of fluorescent patches on non-contacted muscle cells did not undergo changes in size or shape over the course of 1-2 days. However some examples of enlargement, shrinkage and disappearance were observed.
6. On the basis of these findings it is concluded that ACh receptors aggregate within the sarcolemma, spontaneously as well as in response to innervation. In the latter case extrajunctional receptors accumulate at the site of nerve contact thereby contributing to the development of a high receptor density in the subneural muscle

membrane. This process of receptor redistribution occurs in the absence of synaptic or contractile activity.

7. Possible mechanisms involved in the redistribution of ACh receptors are discussed in relation to those which appear to modulate ligand-induced changes in the distribution of lectin and immunoglobulin receptors.

INTRODUCTION

In the preceding paper it was concluded that innervation causes ACh receptors to accumulate in the subneural membrane of cultured amphibian muscle cells (Anderson, Cohen & Zorychta, 1976). This accumulation could arise in a number of different ways. Newly synthesized receptors might be preferentially inserted into the subneural membrane, receptors there might be protected against degradation, or else receptors present elsewhere in the sarcolemma might change their position and aggregate in the subneural membrane. We have attempted to test the latter alternative by examining the effect of innervation on the distribution of those ACh receptors already present on the muscle cells before contact is made. For this purpose muscle cultures were stained with fluorescent conjugates of α-bungarotoxin prior to the addition of neural tissue and then maintained in native toxin to ensure that receptors subsequently inserted into the membrane would not be stained. After a further 1-3 days of development the cultures were examined with fluorescence microscopy. In some experiments changes in the pattern of fluorescent stain were also followed on individual cells The results indicate that receptors originally present elsewhere on the muscle cell becomes localized at sites of nerve-muscle contact.

Similar experiments indicate that a process of receptor redistribution is also responsible for the formation of ACh receptor patches on non-innervated muscle cells.

A brief account of this work has been reported (Anderson & Cohen, 1976).

METHODS

The procedures used in this study were similar to those described previously (Anderson et al., 1976). Briefly, Xenopus laevis embryos were skinned and treated with collagenase in order to isolate myotomal muscle and neural tubes. The muscle was then dissociated into single cells and plated as a monolayer, whereas neural tubes were stored at 4-10°C for subsequent use. During the first three days the muscle cultures were carefully inspected with phase contrast microscopy and those with any signs of neural contamination were discarded. After being screened in this way the cultures were stained by incubation for 20-30 min with a sterilized (see below) fluorescent conjugate of α-bungarotoxin (10^{-5} g/ml) and then rinsed with several changes of 60% (v/v) L-15. Thereafter the cultures were kept in maintenance medium containing 5×10^{-6} g/ml native α-bungarotoxin. Previously isolated neural tubes were brought to room temperature, dissociated and added to the stained cultures. One to three days later the cultures were examined alive for fluorescent staining or were fixed and stored in cold 95% ethanol (-16°C) so that they could be conveniently examined later. In some experiments on living cultures, two or three successive observations were made on identified muscle cells over an interval of 1-2 days.

In experiments where only one observation was to be made the aim was to determine whether the pattern of fluorescent stain on nerve-contacted cells could be accounted for simply in terms of the nerve having made contact with a pre-stained patch. Since patches on muscle cells were never longer than 40 um, and usually less than 20 um

(Anderson et al., 1976), particular attention was paid to examples where the stain extended for greater distances than these. When such examples occurred in a single plane of focus they were photographed and subsequently measured. This procedure necessarily underestimates the number of examples of long lengths of "synaptic" staining because it does not include cases where the stain was associated with a changing plane of focus.

Native α-bungarotoxin and its fluorescent conjugates were sterilized by filtration. Fluoropore filters (Millipore: 0.22 um pore size), pretreated with about 2 ml serum, were found to be most effective in that adsorption of toxin was minimal. This procedure allowed over 90% recovery of ^{125}I-labelled α-bungarotoxin (10 ml, 10^{-6} g/ml).

RESULTS

Fluorescent staining on pre-stained muscle cells

Cultures without neural tube cells

Seven cultures were stained and maintained thereafter in native toxin without adding neural tube cells. The intensity of the fluorescence appeared to decrease progressively with time after staining. However the patterns were similar to those seen in freshly stained cultures and consisted of the characteristic patches previously described (Anderson et al., 1976). The patches were most commonly situated at or near the ends of muscle cells and their processes, on the side of the cell facing the collagen substrate. They also occurred in more central regions of muscle cells, on the collagen surface, on the free surface and less frequently on the edge. The patches were almost always less than 25 um in their longest dimension. Of the several hundred cells in each of the seven cultures examined here only twenty patches were more than 25 um in length and all were less than 40 um (Table 1). In addition, patches located in central regions of cells never extended from one edge of the cell to the other.

The results thus indicate that the fluorescent stain remains visible for up to three days, that the presence of native toxin during this period does not lead to any obvious changes in the patterns of stain, and that the cultures did not have any neural contamination which had gone unnoticed during the earlier inspection with phase contrast optics (see Methods).

Table 1. Long lengths of fluorescent stain in cultures with and without neural tube cells. The length of a stained area was measured as the longest straight line between its borders. The values obtained with and without neural tube cells are significantly different; $P < 0.05$ in column 3 and $P < 0.01$ in columns 4 and 5.

Days after staining	Number of cultures	Number of Examples: Stain extends more than 25um	Stain extends more than 40um	Stain extends entirely across central region of cell
	Cultures without neural tube cells			
1	4	7	0	0
2	2	7	0	0
3	1	6	0	0
Mean ± S.E.M.		2.9 ± 1.2	0	0
	Cultures with neural tube cells			
1	9	74	32	24
2	6	61	33	27
3	3	16	6	4
Mean ± S.E.M.		8.4 ± 1.4	3.9 ± 0.8	3.1 ± 0.8

Cultures with neural tube cells

Some thirty cultures were stained before the addition of neural tube cells and were examined 1-3 days later. As was the case for cultures without neural tube cells the intensity of the fluorescence appeared to decrease progressively during this 3-day period. Nevertheless the patterns of stain were similar to those seen in freshly stained cultures (Anderson et al., 1976). Muscle cells not contacted by nerve had the characteristic patches, whereas on many of the contacted cells the stain was associated with the path of the nerve and appeared as continuous or interrupted narrow bands and lines (Pls. 1-3). Most of these "synaptic" forms of stain extended for distances of less than 25 um. Some however were longer and, unlike patches on non-contacted cells, they sometimes extended for more than 40 um (Pls. 2, 3), or ran entirely across the central regions of cells (Pl. 1). As indicated in Table 1, counts were made of these examples in eighteen cultures and their numbers were small but significant. Such examples are most revealing insofar as they cannot be explained simply in terms of the nerve having contacted pre-existing patches of stain. Instead, they indicate that at least some previously-stained receptors changed their location and accumulated at sites of nerve-muscle contact.

As in freshly stained cultures an additional distinctive form of staining which occurred on nerve-contacted cells consisted of a scattering of small spots approximately 1 um in diameter. In some cases they extended over a greater area of the cell than the largest patches seen in muscle cultures (Pl. 2E, F). Such examples thus provide additional evidence that ACh receptors changed their position in the membrane.

Control Experiments

In the above experiments it is assumed that the fluorescent staining was due entirely to survival of toxin-receptor complexes formed during the short exposure to fluorescent toxin 1-3 days earlier. This would seem to be a reasonable assumption since the staining is due entirely to specific toxin binding (Anderson & Cohen, 1974; Anderson et al., 1976) and other studies indicate that radioactively-labelled toxin dissociates from receptors with a half-time of several days (Berg & Hall, 1975b, Chang & Huang, 1975). In any event, since cultures were maintained in a relatively high concentration (5×10^{-6} g/ml) of native α-bungarotoxin, trace amounts of fluorescent stain in the culture medium should have had little chance of binding to receptors inserted into the sarcolemma after the addition of neural tissue. Determinations made with ^{125}I-labelled toxin indicated that the total amount of labelled toxin remaining in solution as well as in association with cells was less than 0.02% of the native toxin subsequently added to the culture chamber. As described below additional tests were carried out in order to check that the native toxin remained effective throughout the culture period and that non-specific uptake of toxin did not contribute to any of the staining.

Effectiveness of native toxin after 1-3 days in culture

Three types of experiment were carried out in this regard.

(1) The relative potency of the toxin in culture medium was tested by determining how quickly the medium abolished the contraction response of stage 47-48 tadpole tails to 10^{-4} g/ml carbachol. The times-to-

block for the medium from each of six cultures were always less than 10 min and this was the case even when the toxin had been in culture for 3 days. These results indicate that the effective toxin concentration was at least 2×10^{-6} g/ml (see Anderson & Cohen, 1974) so that there could have been no more than about a two-fold loss of potency.

(2) Culture medium from six other cultures was tested for its ability to block fluorescent staining of Xenopus tadpole tails. The tails were first exposed to the culture medium for 20-30 mins and then to fluorescent toxin (10^{-5} g/ml) for a further 30 min. In no case was fluorescent staining ever observed.

(3) Two cultures, originally stained with rhodamine-toxin and allowed to develop for a further two days in native toxin, were exposed at the end of this period to the fluorescein-toxin conjugate (10^{-5} g/ml for 30 min). In neither culture was any fluorescein staining observed.

These experiments thus demonstrate that the native toxin remained effective throughout the period that it was present in culture.

Contribution of non-specific uptake to fluorescent staining

Two types of experiment were carried out to test this point. In one set three cultures were maintained from the start in the presence of 10^{-5} g/ml carbachol. After 3 days they were exposed for 30 min to either rhodamine-toxin (10^{-5} g/ml) or fluorescein-toxin (10^{-5} g/ml) in the presence of carbachol. Following this, they were rinsed in order to wash out the carbachol and fluorescent toxin, and were exposed for a further 30 min to the alternate dye-toxin conjugate. Finally they were rinsed again, medium containing 5×10^{-6} g/ml native toxin was

added to the culture chamber, and neural tube cells were plated. When examined 2 days later only fluorescent staining with the second dye-toxin conjugate was seen. No staining was ever observed with the dye-toxin conjugate which was used in the presence of carbachol (Pl. 3, E-G).

In an analogous test three cultures which had been grown in the absence of carbachol were exposed first to 10^{-5} g/ml rhodamine-toxin for 30 min, rinsed, and then exposed to 10^{-5} g/ml fluorescein-toxin, also for 30 min. After rinsing again, native toxin was added to the cultures and then neural tube cells were plated. On examination one day later the usual patterns of staining were observed, but in this case only with rhodamine optics.

These observations thus indicate that non-specific uptake did not contribute to the fluorescent stain which was subsequently observed. Nor did the traces of fluorescent toxin which remained in the culture medium contribute. Instead all of the stain must have been due to the specific binding which occurred during the initial brief exposure to the fluorescent toxin.

Development of new regions of stain on identified pre-stained muscle cells

In order to directly demonstrate changes in the distribution of fluorescent stain experiments were carried out in which successive observations were made on individual muscle cells. Since the intensity of the fluorescence generally decreases with time after staining, as well as with repeated excitation, these observations were limited to 2 or 3 photographic exposures over a period of up to 2 days. As in previous experiments, cultures were maintained in native toxin (5×10^{-6} g/ml) after being stained.

Muscle cells contacted by nerve

In three cultures, nine cells were followed which had already been contacted by nerve at the time of the first observation. In three of these cells upon initial observation there was no stain along the path of nerve contact and patches of stain were visible elsewhere. No significant changes in this pattern were observed 18-24 hours later. The other six cells did have stain along the path of nerve-muscle contact at the time of the first observation. In one of these cells no apparent change was observed 20 hr later; however, in the other five cells additional stain appeared along the path of nerve-muscle contact. Pl. 4 illustrates the most striking example. A and C show phase-contrast and fluorescence views of a field 22 hr after staining and adding neural tissue to the culture. A nerve process is already in contact with a muscle cell and there are several small patches of staining along the path of contact. Elsewhere on the cell are four larger patches as well as a few small spots of stain. B and D show the same field 18 hr later. The nerve has grown and branched, and some of the muscle processes have become narrower and thicker. The large fluorescent patches which were initially present are now very faint or entirely absent. On the other hand, fluorescent stain along the path of nerve-muscle contact has become considerably more extensive. In addition numerous small spots of fluorescent stain have also appeared in the central region of the cell.

Pl. 5 indicates another example of accumulation of stain at the site

of nerve-muscle contact. In this case the first observation (A and C) was made 21 hr after staining and adding neural tube cells. A nerve process can be seen which runs along the edge of a muscle cell and then crosses to the other side. There is little visible stain except for a short length along the edge of the muscle cell in the region of nerve contact. The second observation (B and D), 18 hr later, shows that the nerve process is now thicker and appears to have been joined by others. The muscle cell outline has also changed slightly. Of most significance, the length of stain along the path of nerve-muscle contact has greatly increased.

These observations thus indicate that pre-stained receptors, which originally existed elsewhere on the muscle cell, were induced by the nerve to aggregate at the site of contact.

While following changes in the pattern of fluorescent staining on nerve-contacted cells, one field was found to undergo a quite different change. In the first observation (Pl. 6 A and C), a growing nerve process can be seen approaching a pair of muscle cells which have patches of fluorescent stain on the cell surface in contact with the collagen substrate. In the second observation (Pl. 6 B and D), 21 hr later, the nerve has disappeared but the pattern of staining has changed dramatically. Both cells have lost their original patches of stain and have developed instead a widespread stippling of small fluorescent spots. Since such patterns of stain are never observed in cultures without neural tissue it is likely that this change in staining pattern was brought about by transient nerve-muscle contact.

Muscle cells not contacted by nerve

During the course of the above experiments a further interesting

change was observed, as revealed in Pl. 4. At the top of the field can be seen part of a muscle cell which at the time of the first observation had a single bright fluorescent patch (A and C). During the next 18 hr the cell grew and developed a new patch of fluorescent stain in the region of growth (B and D). This observation suggested that receptor redistribution might also occur spontaneously and similar experiments were therefore carried out in muscle cultures.

Pl. 7 illustrates one such example where two bright patches were observed at the time of the first observation. Twenty-four hours later the original patches were less intense but two new patches had formed. Both of these new patches were located on cell processes which had developed during the interval between observations. Altogether, while following a total of 30 patches, 13 new patches were observed and, of these, 11 were associated with regions of cell extension. In all cases the new patches were smaller than those already present on the cell. These results indicate that the formation of patches on non-innervated cells also involves a process whereby receptors change their position and packing density within the sarcolemma.

A variety of other spontaneous changes were also observed on non-innervated cells. Of the 30 patches which were examined 25 were still visible 18-24 hr after the initial observation. In all, 15 remained the same size and shape, 5 appeared smaller, and 5 underwent some extension. Examples of the latter two categories are shown in Pl. 8. In this instance the first observation, which was made shortly after staining, revealed two adjacent patches at the end of a cell on the surface which was contacting the collagen substrate (A and C). The second observation, 21 hr later, revealed that the cell had grown in the region near the

patches. A major portion of one patch was no longer visible but the neighboring patch had apparently elongated in the direction of cell growth (B and D). In two other examples patch elongation also occurred in a region of cell growth.

Six of eight patches were still visible when a third observation was made two days after the first. Of these, three underwent no change in size or shape, one elongated, and another became smaller between the first two observations but then remained the same shape. Finally, one patch first became smaller, but then underwent elongation.

Together these results indicate that receptor patches on non-innervated cells form by a process of receptor aggregation within the sarcolemma. Once formed they tend to remain unchanged in size and shape for a 1-2 day period, but may also decrease in size or undergo enlargement.

DISCUSSION

The present experiments have demonstrated the appearance of new areas of fluorescent stain on cultured muscle cells, one or more days after they were exposed to fluorescent α-bungarotoxin. Since dye-toxin conjugates, like the native toxin, bind specifically to ACh receptors, new areas of stain reflect new accumulations of ACh receptors. Residual traces of fluorescent toxin in the medium could not have stained receptors subsequently inserted into the sarcolemma because, after exposure to the fluorescent toxin, cultures were maintained in a high concentration of native toxin. Internally controlled experiments, in which both fluorescein- and rhodamine-labelled toxin were used, further indicated that non-specific uptake of fluorescent toxin did not contribute to any of the patterns of fluorescent staining. In particular, if non-specific uptake did occur it did not result in the staining of any intracellular receptors which were later inserted into the membrane. In this vein it is interesting to note that a recent study by Devreotes & Fambrough (1975) on cultured chick myotubes has indicated that receptors which are destined to be inserted into the membrane cannot be labelled even by overnight exposure to α-bungarotoxin. These considerations therefore lead to the conclusion that new areas of fluorescent stain arose because previously stained receptors changed their location in the sarcolemma.

Neural regulation of ACh receptors

The formation of new regions of fluorescent staining along paths of nerve-muscle contact indicates that a process of receptor redistribution contributes to the development of a high ACh receptor density in the

subneural membrane of cultured myotomal cells. The same process presumably operates during the establishment and growth of synapses in vivo and would be expected to lower the density of receptors in adjacent muscle membrane. This might explain the decrease in extrajunctional chemosensitivity which has been observed in some studies prior to the resumption of neuromuscular transmission during reinnervation (Miledi, 1960; Bennet, Pettigrew & Taylor, 1973; Letinsky, 1975) as well as during recovery from treatment with botulinum toxin (Bray & Harris, 1975). The incorporation of extrajunctional receptors into the subsynaptic membrane may also continue in normal adult muscle and participate in a slow turnover of junctional receptors. This idea is consistent with the finding that denervation leads to a progressive decline in the number of junctional receptors thus indicating that the intact nerve terminal is necessary for their continued maintenance (Frank, Gautvik & Sommerschild, 1975).

Whereas contractile activity is unnecessary for receptor redistribution it is well established that it can affect the density of extrajunctional receptors. For example, direct stimulation has been shown to markedly reduce the extrajunctional chemosensitivity of denervated adult muscle (Jones & Vrbova, 1970, 1974; Lomo & Rosenthal, 1972; Drachman & Witzke, 1972; Purves & Sakman, 1974; Lomo & Westgaard, 1975). Conversely, procedures which block neuromuscular transmission lead to the appearance of extrajunctional receptors in otherwise normally-innervated adult muscle (Berg & Hall, 1975; Chang, Chuang & Huang, 1975; Lavoie, Collier & Tenenhouse, 1976; Pestronk, Drachman & Griffin, 1976). Other studies have indicated that in denervated and cultured muscle extrajunctional receptors are continuously being synthesized (Brockes & Hall, 1975a; Devreotes & Fambrough, 1976) and degraded (Berg & Hall, 1974, 1975b; Chang & Huang, 1975;

Devreotes & Fambrough, 1975). Contractile activity which markedly reduces extrajunctional chemosensitivity does not appear to increase the rate of receptor degradation and it has therefore been suggested that activity regulates the density of extrajunctional receptors by limiting their rate of synthesis (Hogan, Marshall & Hall, 1976).

It is evident therefore that innervation has at least two distinct actions which regulate ACh receptors. Independently of muscle activity it causes a redistribution of receptors within the sarcolemma leading to their accumulation in the subsynaptic membrane. In addition, by virtue of the contractile activity it generates in muscle cells innervation also modifies receptor metabolism, apparently reducing the rate of receptor synthesis. Both of these actions of the nerve will lead to a decrease in the number of extrajunctional receptors. Their relative importance in this context is obviously a quantitative question whose answer will depend on many parameters such as the rate of growth of the subsynaptic membrane and the amount of muscle activity. Clearly these factors will vary at different stages of development. It also remains to be determined whether innervation regulates the distribution of ACh receptors by other actions as well.

Mechanisms involved in receptor redistribution

ACh receptors can exist in at least two phases within the surface membrane of cultured myotomal muscle cells. One of these is a relatively dispersed phase which is not visible after fluorescent staining but can be detected by radioautography (Anderson et al., 1976). The other is a more densely packed phase which can be visualized either by radioautography or fluorescent staining. The resolution afforded by fluorescent staining has allowed at least three topographical sub-classes of the more densely

packed phase to be recognized. They include (a) large complex patches which are usually 5-20 um in their longest dimension, (b) much smaller patches or spots approximately 1 um diameter or less, and (c) the subneural muscle membrane of innervated cells.

The present study has demonstrated that these regions of high receptor density can form on cultured muscle cells by a process of redistribution. The only well established mechanism whereby integral glycoproteins have been shown to change their position in the plasma membrane is that of lateral movement within the lipid bilayer (for reviews see Singer & Nicolson, 1972; Nicolson, 1974; Raff & dePetris, 1974). The formation of new patches of ACh receptors and the incorporation of receptors into the subneural membrane can thus be explained most readily by a process whereby individual receptor units move and aggregate within the sarcolemma. Conversely, both the decrease in size of the large receptor patches on non-innervated muscle cells, and their disappearance following innervation, may involve a process of dissolution into the dispersed receptor phase; however the techniques used in this study cannot exclude alternatives such as removal and degradation within the muscle cell (see Devreotes & Fambrough, 1975).

Evidence for more complex routes of receptor movement is provided by the transient appearance of small receptor clusters over large areas of the sarcolemma on nerve-contacted cells. Such a scattering of receptor clusters is not observed either on non-contacted muscle cells, or on contacted cells which have extensive subneural staining (Anderson et al., 1976). They are however common on both freshly stained and pre-stained muscle cells within 1-2 days of adding neural tube cells to 2- and 3-day-old muscle cultures. While it is therefore clear that they constitute a new arrangement of the receptor population which was present before nerve contact, it is

less obvious whether they develop through a process of aggregation from the dispersed receptor phase, by the breaking up of larger patches, or both. Similarly their disappearance may involve incorporation into the developing subsynaptic membrane, dissolution into the disperse receptor phase, or degradation. However, it is interesting to note that both the receptor patches on non-innervated cells (see Anderson et al., 1976, Pl. 3C) and the immature subneural membrane (Pls. 2C, 4C) are often composed of small receptor clusters. It is therefore possible that the receptor clusters themselves move as a unit within the sarcolemma. By examining the changes in the location of fluorescent stain on a larger sample of individual muscle cells, and at shorter intervals between observations, it may be possible to resolve whether this is in fact the case.

In recent years it has become apparent that the organization of the plasma membrane is governed by several interacting processes. In many cases integral glycoproteins move laterally through the membrane in a manner analogous to free diffusion (Frye & Edidin, 1970; Edidin & Fambrough, 1973; Poo & Cone, 1974; Liebman & Entine, 1974; Edidin, Zagyansky & Lardner, 1976). In contrast, in the plasmalemma of red blood cells the mobility of the principle glycoprotein appears to be restricted by association with the intracellular protein, spectrin (Nicolson & Painter, 1973; Nicolson, 1973). In the lymphocyte still more complex processes modulating the distribution of surface receptors have been revealed. For example, the binding of immunoglobulin to a fraction of the receptors on the cell surface leads to aggregation of the uniformly distributed receptors into small clusters, and their subsequent collection into a larger aggregate at one pole of the cell

(Taylor, Duffus, Raff & dePetris, 1971; Raff & dePetris, 1974). While the first step in this process can be accounted for simply by adhesive interactions provided by the multivalent ligand, the later movement of the small receptor clusters to one pole of the cell is a more complex process dependent upon energy metabolism. Also, when lymphocytes interact with the lectin concanavalin A, covalently attached to either latex beads or blood platelets, the local binding to a small faction of the lectin receptors has been found to restrict the mobility of immunoglobulin receptors over the entire cell surface (Rutishauser, Yahara & Edelman, 1974; Yahara & Edelman, 1975). This too is difficult to interpret on the basis of adhesive interactions between individual receptor units. Instead both of these phenomena imply the existence of an intracellular mechanism which is capable of producing either co-ordinated movement or immobilization of surface receptors in response to local interactions with macromolecules in the plasmalemma. Observations that drugs which are known to disrupt cytoplasmic structures such as microfilaments and microtubules also affect ligand-induced changes in receptor mobility, have led to the suggestion (Edelman, Yahara & Wang, 1973) that these organelles are involved in a complex cytoplasmic structure which regulates the mobility of some surface receptors.

There appear to be several similarities between the ligand-induced clustering of surface receptors and the spontaneous aggregation of ACh receptors on cultured muscle cells. For example, in either situation there is an aggregation within the membrane of a fraction of the previously dispersed receptors. Also, aggregates of receptors in both cases can exist either as small clusters or larger compound structures. While we have not observed the formation of small ACh receptor clusters

to be a preliminary event in the development of large patches, as occurs during ligand-induced receptor aggregation, large ACh receptor patches often consist of a collection of smaller units. As in the case of ligand-induced aggregates, the existence of these compound structures is difficult to understand simply in terms of direct interactions between individual receptors, and seems to require some form of long-range interaction between separate receptor clusters. Taken together these similarities suggest that cytoplasmic structures may participate in regulating the distribution of ACh receptors. They further suggest that cellular mechanisms which mediate the aggregation of integral membrane glycoproteins can act both in response to external ligands and also independently. If one presumes that such mechanisms are available for modulating the distribution of ACh receptors on non-innervated muscle cells, it is reasonable that they should also participate in the redistribution of receptors induced by innervation. Should this be the case a developing nerve process might, by a local interaction with the sarcolemma, provide a locus for an aggregation of ACh receptors mediated by cytoplasmic structures in the muscle cell.

While a more complex mechanism is suggested by the above considerations it is not yet possible to exclude some simpler alternatives. For example, direct adhesive interactions may occur between individual receptor units such that receptor patches form in response to an increasing receptor density in the sarcolemma, in a manner analogous to crystal formation from a supersaturated solution. As described above, however, the complex organization of many receptor patches suggests that this alternative is implausible. It is also possible that complementary ligands associated with nerve processes might undergo direct adhesive interactions with ACh

receptors in the sarcolemma. Such interactions could trap mobile receptor units and thereby lead to their accumulation in the subneural membrane. This mechanism, however, also appears to be inadequate for explaining the observation that fluorescent stain at sites of nerve-muscle contact commonly extended beyond the breadth of the nerve process (Pls. 3E, G; 4B, D).

Regardless of the mechanisms which regulate their distribution it would not be surprising if an early step in synapse formation does involve a direct neural interaction with ACh receptors. It has been known for some time that experimental conditions which cause mature muscle cells to develop extrajunctional receptors also make them receptive to further innervation. These findings have led to the suggestion that ACh receptors may be a prerequisite for the establishment of synaptic contact between muscle and nerve (Katz & Miledi, 1964; Fex, Sonesson, Thesleff & Zelena, 1966). If some ligand associated with cholinergic neurons were specific for ACh receptors, and varied in other neurons according to transmitter, it would provide a molecular basis for the appropriate matching between transmitter and receptor at synapses.

EXPLANATION OF PLATES

Except where stated otherwise the experimental procedure consisted of staining 3-day-old muscle cultures with rhodamine-labelled toxin, rinsing them with toxin-free medium, and then adding native toxin to the culture. Neural tube cells were then plated (Pls. 1-6) or not (Pls. 7-8). Each pair of micrographs shows the same field viewed with fluorescence optics and phase contrast optics. In Pls. 4-8 muscle cells have been outlined and nerve processes are shown as dotted lines.

Plate 1

Fluorescent stain along paths of nerve contact extending across the central regions of muscle cells. Two days (A, B) and one day (C-F) after staining and adding neural tube cells. The cultures were examined alive. Scale 20 um.

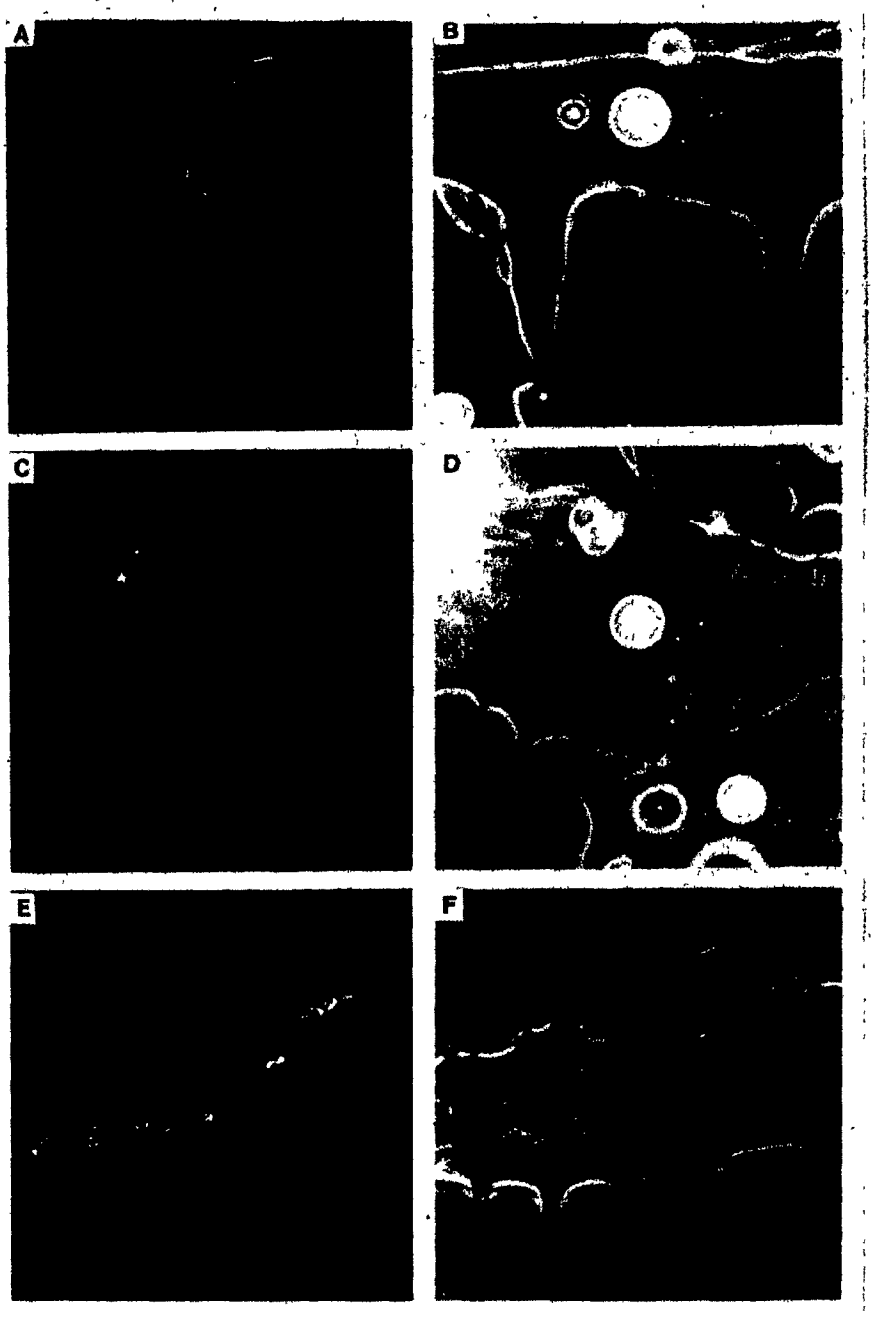

A
B
C
D
E
F

Plate 2

Variety of patterns of fluorescent stain on nerve-contacted cells, one day after staining and adding neural tube cells. Cultures were fixed in ethanol and mounted in glycerol. Scale 20 um.

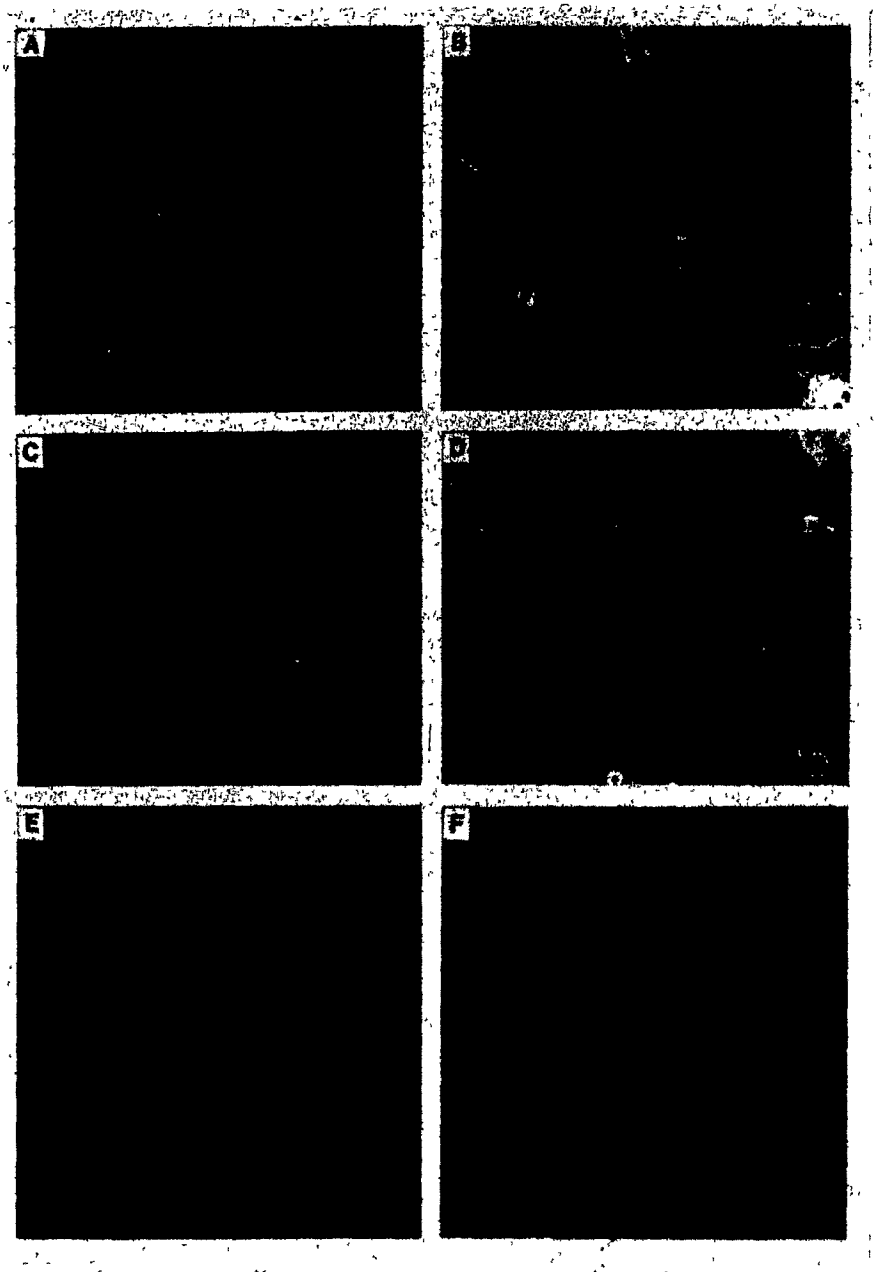

A
B
C
D
E
F

Plate 3

A-D: Long lengths of fluorescent stain associated with paths of nerve-muscle contact. Cultures were examined alive two days after staining and adding neural tube cells. E-G: Reversible inhibition of fluorescent staining by carbachol. In this experiment a culture of muscle cells was maintained in the presence of carbachol (10^{-5} g/ml). On day 3 it was exposed to rhodamine-toxin with carbachol present, rinsed and then exposed to fluorescein-toxin in the absence of the drug. After rinsing again, native toxin was added and neural tube cells were plated. The culture was examined alive two days later. The example shows bright fluorescein staining (E) along the path of nerve-muscle contact (G) and a corresponding absence of rhodamine staining (F). Thus carbachol reversibly inhibited staining. The bright areas in the upper right hand corners of E and F are associated with the cellular debris seen in G. Scale 20 um.

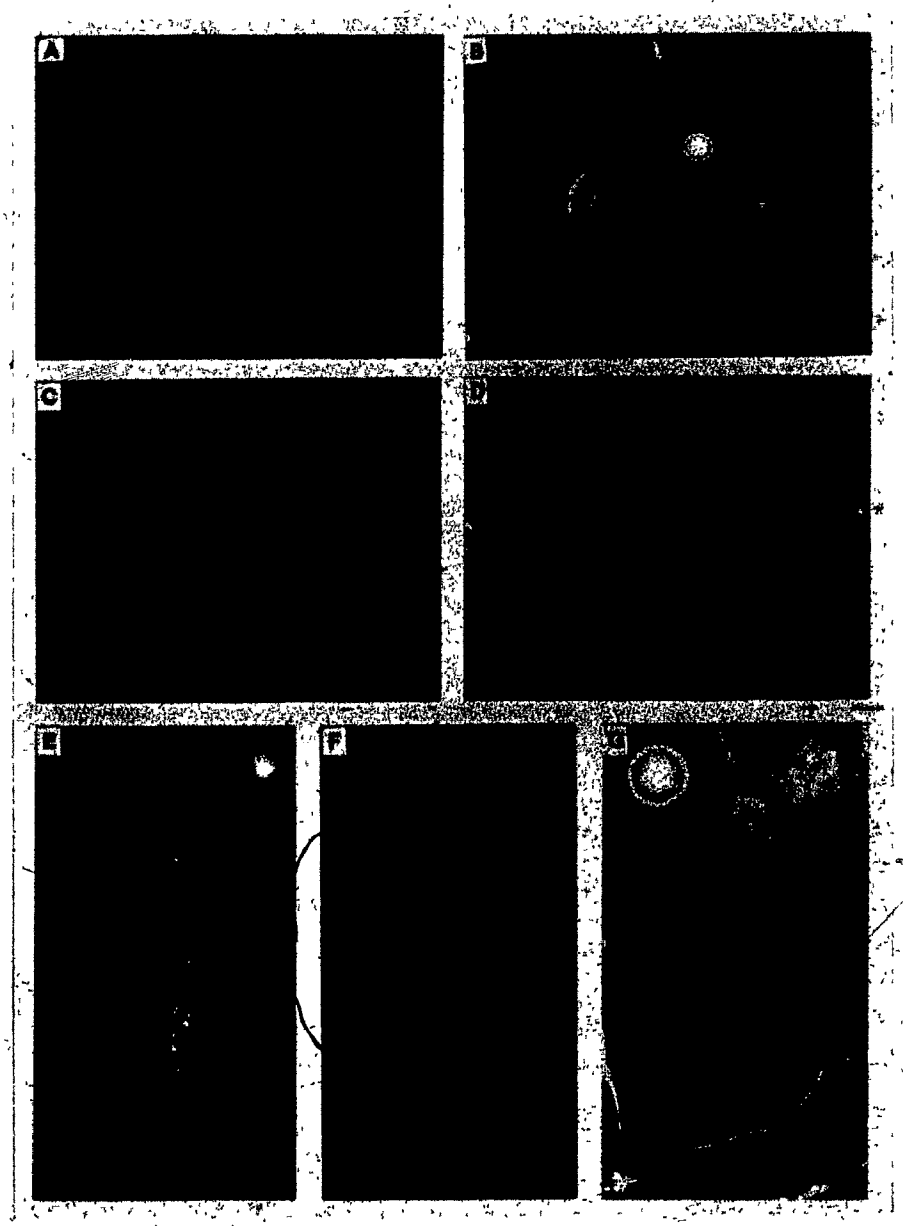

A
B
C
D
E
F
G

Plate 4

Changes in distribution of fluorescent stain on a nerve-contacted muscle cell. A,C: 22 hr after staining and adding neural tube cells. B, D: The same field 18 hr later. There is now more stain along the path of nerve-muscle contact, and the patches which were originally present elsewhere on the cell have either become much fainter or disappeared entirely. In addition many small spots of fluorescent stain have appeared. Note also that the cell in the upper left quadrant grew and developed a new patch of stain in the region of cell extension. Scale 30 um.

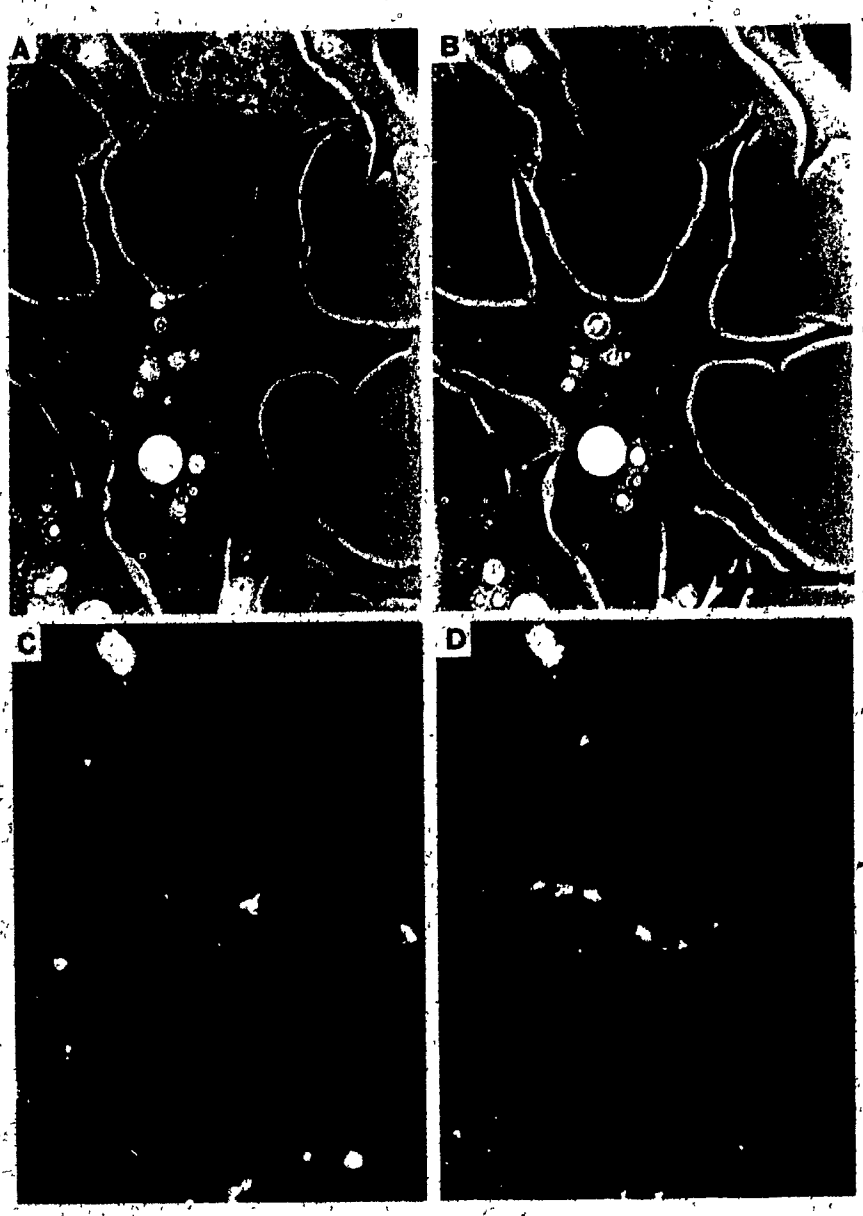

A
B
C
D

Plate 5

Changes in the distribution of fluorescent stain on a nerve-contacted muscle cell. A, C: 21 hr after staining and adding neural tube cells. B,D: The same field 18 hr later. Note the new nerve processes along the edge of the cell in the right hand portion of the field, and the increased extent of the staining at the site of nerve-muscle contact. Scale 30 um.

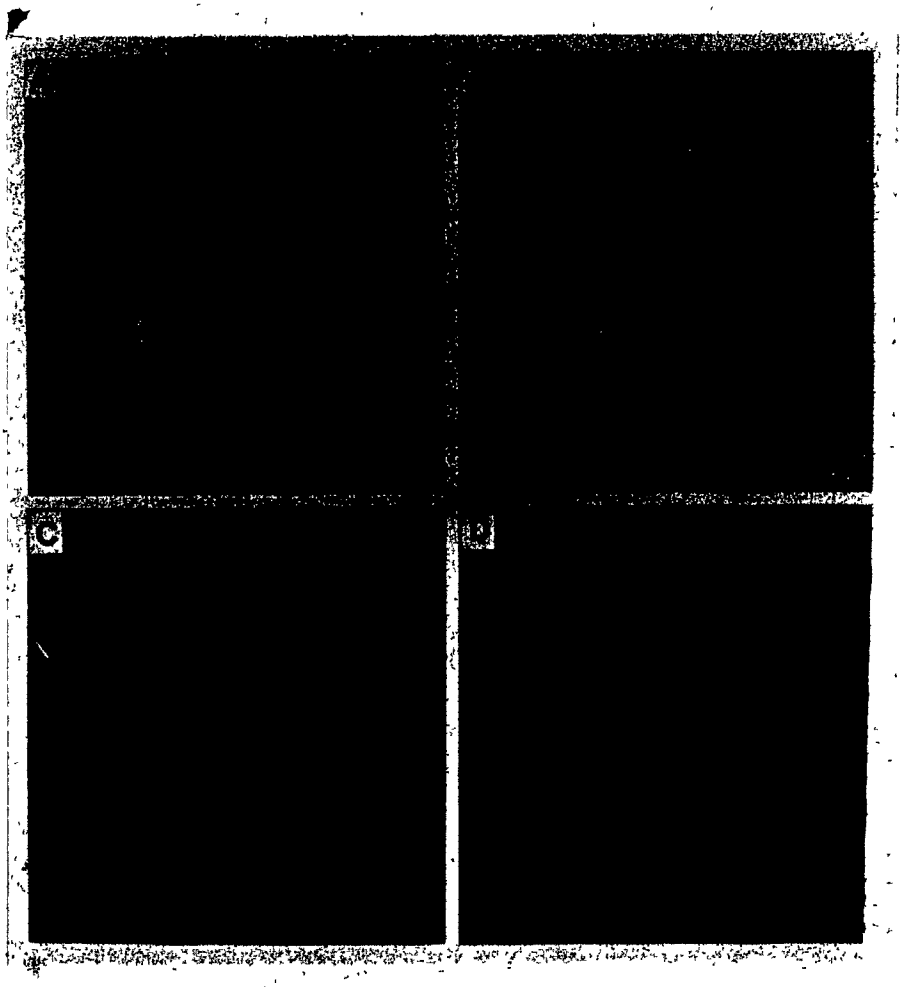

C
D

Plate 6

Changes in the distribution of fluorescent stain after transient nerve contact. A, C: 22 hr after staining and adding neural tube cells. Note that the growth cone of the nerve process in the lower right quadrant has contacted one of the muscle cells. B,D: The same field 21 hr later. The nerve process has disappeared and the patterns of fluorescent stain on both muscle cells have changed. Two particles in the lower right quadrant (B) were autofluorescent (D).
Scale 20 um.

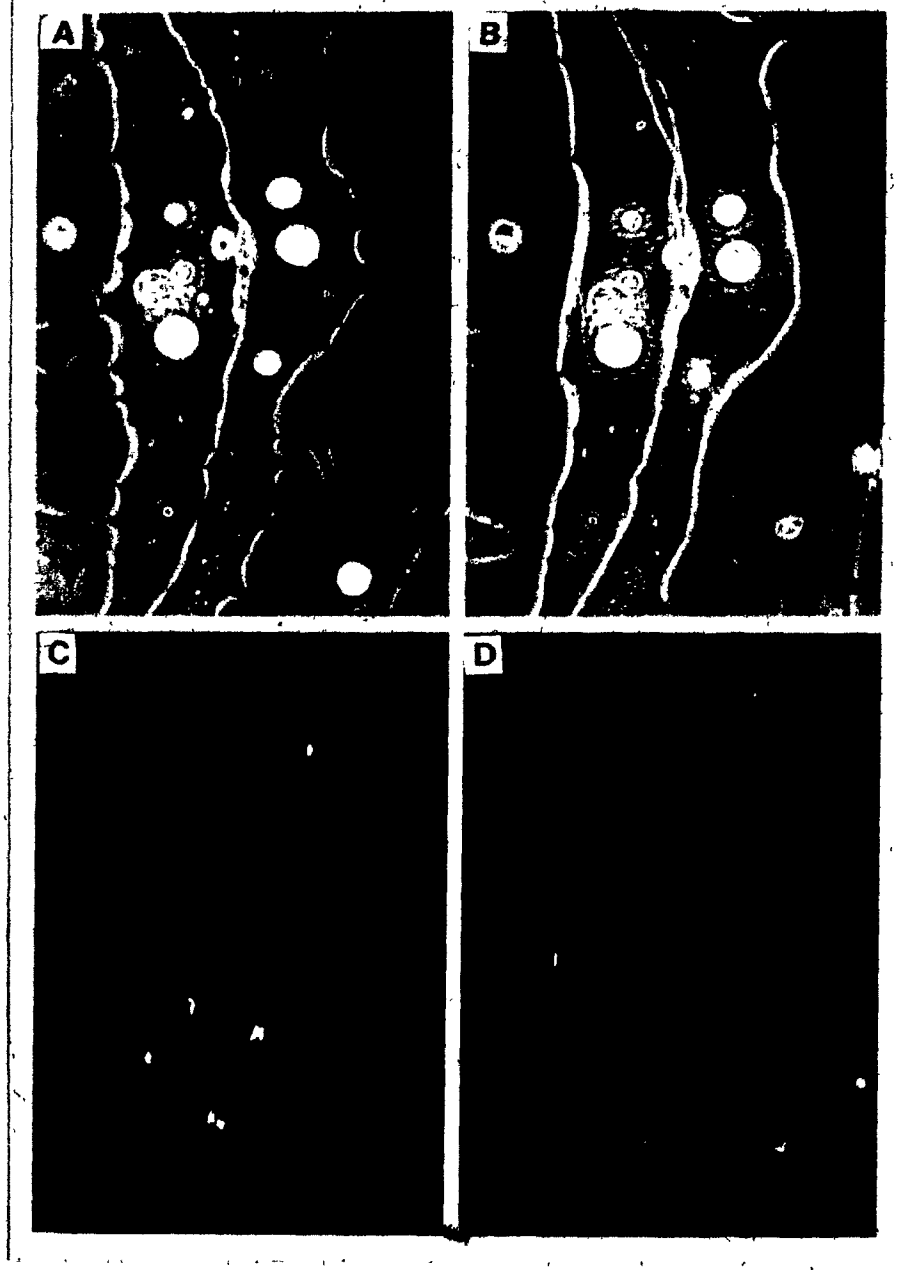

A
B
C
D

Plate 7

Changes in the distribution of fluorescent stain on non-contacted muscle cells. A, C: 3 hr after staining. B, D: The same field 24 hr later. The original patches of stain are still present but two additional patches have appeared on new cell projections. Scale 30 um.

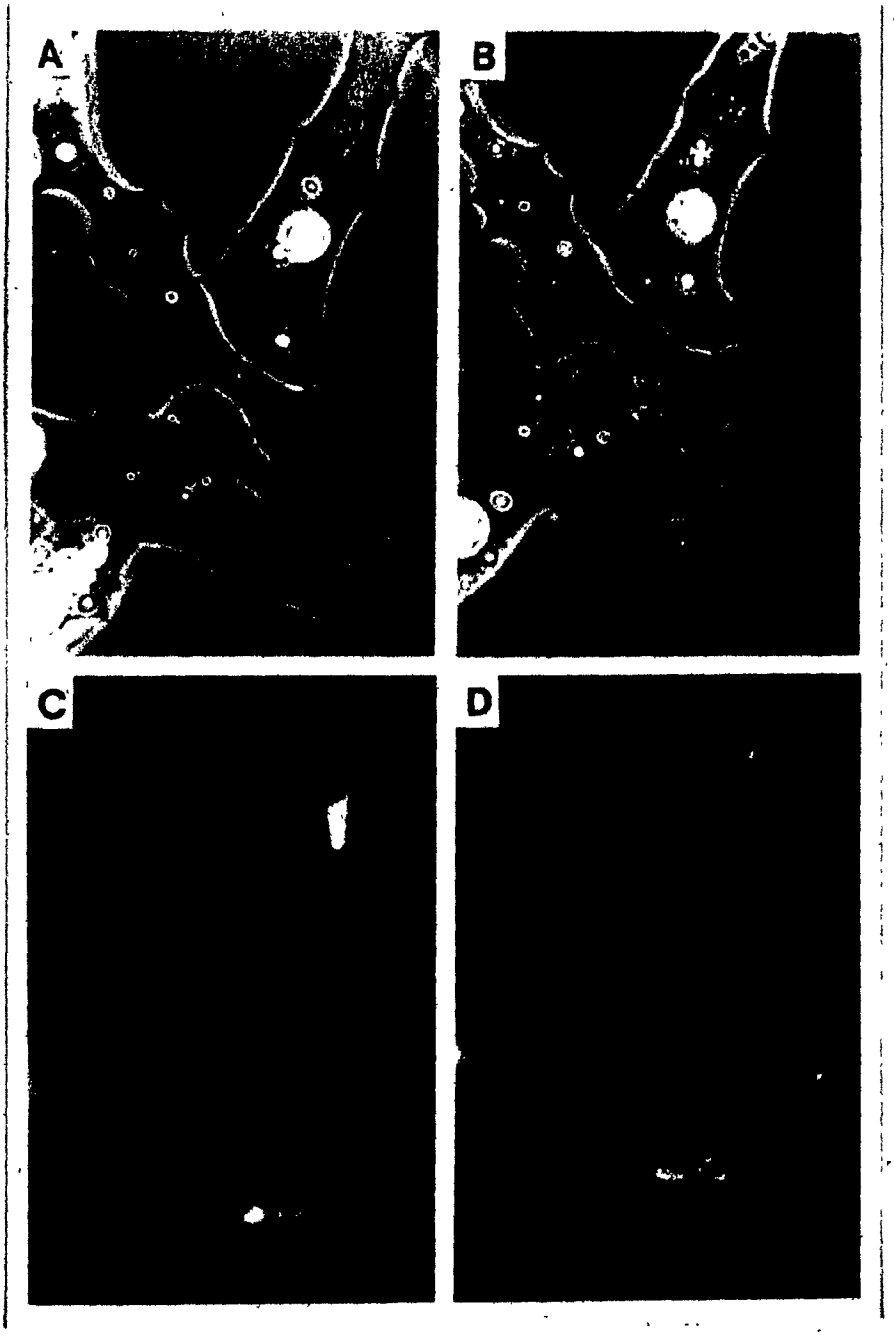
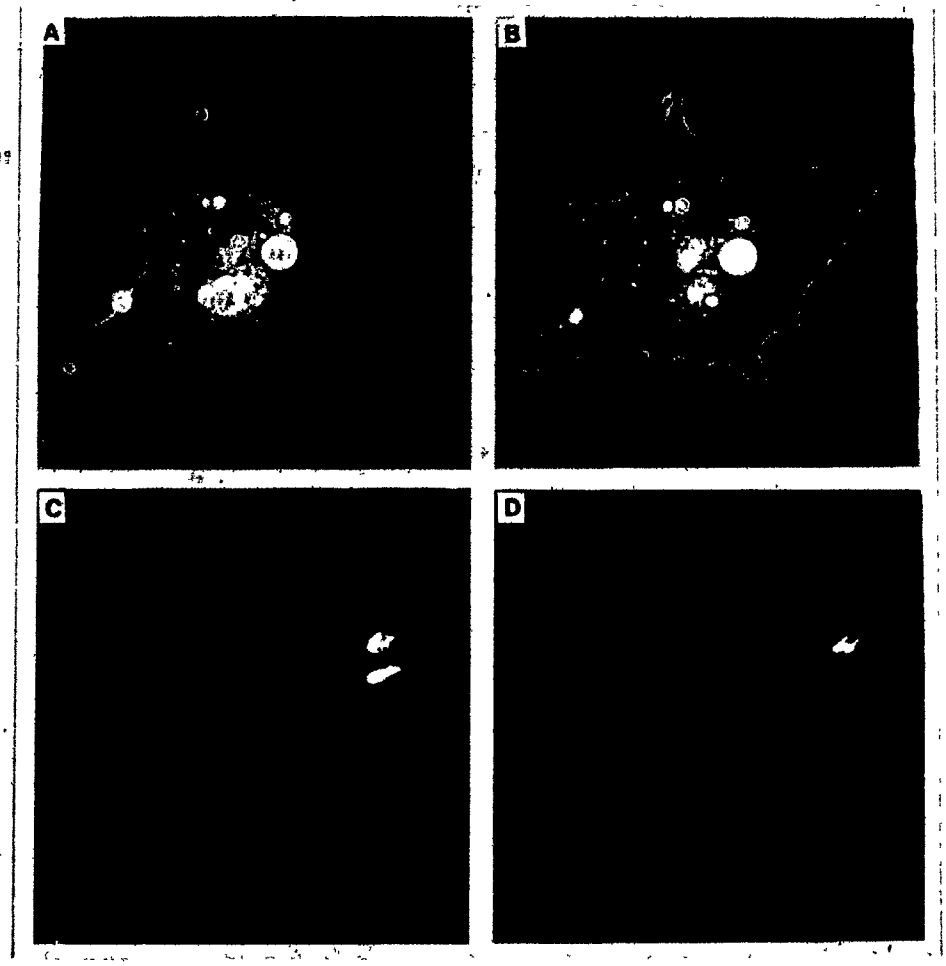

A
B
C
D

Plate 8

Changes in the pattern of fluorescent staining on a non-contacted muscle cell. A, C: 2 hr after staining. B, D: The same field 21 hr later. One of the original patches has elongated whereas the other has almost disappeared. Note also that the cell grew in the region of the patches.

Scale 30 um.


A
B
C
D

CHAPTER 5

CONCLUSIONS

Conclusions

The present study has demonstrated that dramatic changes can occur in the distribution of ACh receptors on cultured embryonic muscle through a rearrangement of receptor units within the sarcolemma. A direct demonstration of this process was possible because fluorescent staining allowed the spatial distribution of the receptor population which was present in the sarcolemma before nerve contact to be observed as a function of time. The success of these experiments can also be attributed to the rapid development of the amphibian embryos that were used to prepare the cultures. Thus the rate of receptor turnover (see Berg & Hall, 1974; 1975b; Devreotes & Fambrough, 1975), which was presumably responsible for the fading of the fluorescent stain observed after pulse-labelling ACh receptors in the sarcolemma, was slow compared to the nerve-induced receptor redistribution. Had the rate of redistribution been slow, as may well be the case in more slowly developing species, the fraction of the receptor population which was still labelled by the time the subneural membrane had formed might have been too low to detect. For similar reasons it may be difficult in future studies to detect any incorporation of extrajunctional receptors into the subsynaptic membrane which may occur in normally innervated adult muscle. In fact, the available evidence indicates that the post-synaptic structural elements of vertebrate skeletal muscle cells are extremely stable metabolically, at least in the adult. Thus the junctional folds, acetylcholinesterase and ACh receptors all develop in response to innervation, but are very resistant to change following removal of the neural influence in the adult. Nevertheless, on

prolonged denervation there does appear to be a gradual loss of both esterase activity (Filogamo & Gabella, 1966) and the number of ACh receptors (Frank et al., 1975). Obviously some neural influence is involved in maintaining the integrity of these synaptic structures. By far the most likely candidate for such a role would appear to be the inductive mechanism which was responsible for their initial formation. There is evidence, for example, that mammalian end plates undergo both a slow growth and regression throughout life (see Barker & Ip, 1966; Tuffery, 1971) which suggests that developmental processes do continue in the adult, but at a very much reduced rate compared to that observed during embryonic development. Unfortunately, the slow rate of these biological processes in the adult may make a direct demonstration of their existence virtually impossible.

While the present experiments have been possible because the nerve-induced aggregation of receptors during synaptogenesis requires only 1-2 days at the most, the decline in the number of junctional receptors in denervated adult muscle, which may approximate the normal replenishment of junctional receptors, is exceedingly slow with a half-time in the order of five weeks (Frank et al., 1975). This difference in rate also indicates that, at least in the adult, the aggregation of receptors in the subsynaptic membrane is not a readily reversible process, and suggests that additional mechanisms may be involved in anchoring receptors within the subneural membrane after their initial aggregation. There is, for example, evidence that junctional and extrajunctional ACh receptors in adult muscle are slightly different chemically, and can be separated by by isoelectric-focussing (Brockes & Hall, 1975b). Although speculative,

it is reasonable to presume that such a chemical modification may be involved in a process whereby ACh receptors become anchored in the membrane at the site of innervation.

Just as there may be conceptually different mechanisms involved in producing and maintaining the localization of receptors in the subneural membrane, there may be more than one regulatory control over the synthesis of ACh receptors. The control of extrajunctional chemosensitivity has long been thought to be related to either the level of usage, or the more-obscure 'trophic' effect of innervation. Experiments in adult muscle have indicated that theeffect of usage is probably mediated directly by an effect of muscle contraction on receptor synthesis (see for example Hogan et al., 1976). However, it has also been found that denervation has a greater effect than inactivity per se in causing the appearance of extrajunctional receptors (Lavoie et al., 1976; Pestronk et al., 1976). This difference is likely to be due to the removal of an inductive effect of the nerve contact. On the basis of the present experiments innervation, independently of activity, can remove extrajunctional receptors by converting them into junctional receptors. This inductive process could thus contribute to a reduction of extrajunctional chemosensitivity in conditions of rapid synaptic growth, such as might occur during reinnervation. It might even contribute partially to the maintenance of a low extrajunctional chemosensitivity in normally innervated adult muscle by participating in a slow replenishment of junctional receptors lost to turnover. The quantitative difference between the effects of muscle inactivity and denervation can thus be explained in one of two ways. Either the more rapid accumulation of extrajunctional receptors following denervation reflects an on-going rate of receptor synthesis which would in normal circumstances

have replenished junctional receptors lost to turnover, or else innervation must have an additional 'inductive' influence which limits the synthesis of new receptors.

The latter possibility is supported by the available evidence which suggests that the receptors appearing after denervation are the result of new gene activity. For example, drugs which block the synthesis of either new protein or messenger RNA prevent the development of extrajunctional receptors caused by denervation (Harris & Miledi, 1966; Fambrough, 1970; Grampp et al., 1972; Kimura & Kimura, 1973; Chang & Tung, 1974; Sakman, 1975). In contrast, once receptors have begun to form, the continued appearance of new receptors is still blocked by protein-synthesis inhibitors, but only minimally affected by drugs which block RNA-synthesis (Grampp et al., 1972; see also Hartzell & Fambrough, 1973). The most reasonable interpretation of this difference is that denervation initiates a new synthesis of receptors by first causing the synthesis of new messenger RNA. The later ineffectiveness of the RNA-synthesis inhibitor probably reflects its inability to block on-going protein synthesis which was initiated before the drug treatment. Artificially induced muscle contraction likewise can either prevent the development of extrajunctional receptors in denervated muscle, or cause their density to decline after they have already developed (Jones & Vrbova, 1970, 1974; Drachman & Witzke, 1972; Lomo & Rosenthal, 1972; Purves & Sakman, 1974; Lomo & Westgaard, 1975), and therefore seems to resemble the effect of drugs which inhibit protein-synthesis. However since RNA-synthesis inhibitors prevent the appearance of extrajunctional receptors only when they are given before denervation, it is likely that the rapid accumulation of receptors following denervation results almost entirely from new genetic

activity, brought about in part by the removal of an inductive effect of the nerve which would otherwise limit the rate of receptor synthesis.

The present study has demonstrated one mechanism by which the inductive influence of innervation can determine the distribution of ACh receptors on muscle cells. The process of receptor redistribution is clearly directed at cellular mechanisms which determine the arrangement of 'structural' proteins within the sarcolemma. Together the controls which regulate receptor synthesis and subsequent localization on the cell surface can be viewed teleologically as a means for ensuring that muscle fibres retain synaptic contact only with motor neurons which are both 'trophically' competent and appropriately connected with 'higher' centres which direct motor activity. It is known for example that during normal development there is an initial superabundance of motor neurons, most of which are eventually lost (Hughes, 1961; Prestige, 1967; Prestige & Wilson, 1972; Reier & Hughes, 1972). Likewise there is evidence that mammalian muscle fibres are initially innervated by several nerve processes (Redfern, 1970; Bagust, Lewis & Westerman, 1973). This redundancy in the early formation of motor neurons and nerve-muscle junctions suggests that there may be a form of 'natural selection' at work which determines that only those neurons which establish appropriate connexions will retain synaptic contact with muscle, or even survive in the adult. Competition over the cellular means for the localization of ACh receptors might well be important in determining which synaptic contacts survive into adulthood. It is known that a widespread distribution of extrajunctional ACh receptors seems to be required for innervation to take place (see Katz & Miledi, 1964b; Fex et al., 1966). It thus appears reasonable that, by incorporating

the bulk of the receptors in its developing subneural membrane, and supressing receptor synthesis, the most appropriate nerve contact might block the development of competing junctions on the same muscle fibre. In the adult the new development of extrajunctional receptors following nerve damage or insufficient activity might permit another nerve contact to develop and replace the no-longer competent nerve ending. Thus the mechanisms which regulate the metabolism and distribution of transmitter receptors may also provide a means for adjusting the pattern of innervation to changes in both the number of functional units and the pattern of usage. While this may be of lesser importance at the level of nerve-muscle connexions which do not appear to undergo dramatic plastic changes, similar mechanisms operating between neurons in the central nervous system may modulate a variety of changes in which the patterns of connectivity are determined by the usage of specific neural pathways.

The finding of the present study, that innervation leads to a rearrangement of integral proteins within the sarcolemma, has an additional relevance for the general problem of cell differentiation. It has been suggested that 'surface modulation' may be a fundamental mechanism which mediates inductive interactions between many types of cells (see Edelman, 1976). This possibility has been raised by the observation that other cell types, principally lymphocytes, are stimulated to proliferate by interaction with multivalent ligands which bind to receptors in the plasmalemma, and sometimes cause them to aggregate (see reviews, Raff & de Petris, 1974; Nicolson, 1974). An important difficulty for this intriguing hypothesis has been the fact that these

agents can induce cell proliferation even without causing the aggregation of their receptors. Furthermore, the modulation of surface structure has not previously been found to occur as a necessary part of any natural biological process. The present study, however, has demonstrated that the aggregation of ACh receptors in the sarcolemma is a normal feature of synaptogenesis, and provides the high density of receptors necessary for the function of the synapse. Since elaborate mechanisms are clearly available for controlling the distribution and mobility of substances in the surface membrane, they are likely to be involved in other inductive interactions which influence cell behavior. Future studies of the molecular mechanisms which underlie receptor redistribution should therefore continue to provide information relevant to the fundamental problems of induction and differentiation.

BIBLIOGRAPHY

Albuquerque, E. X., Barnard, E. A., Porter, C. W. & Warnick, J. E. (1974). The density of acetylcholine receptors and their sensitivity in the post-synaptic membrane of muscle end-plates. Proc. Natl. Acad. Sci. 71, 2818-2822.

Albuquerque, E. X. & McIsaac, R. J. (1970). Fast and slow mammalian muscle after denervation. Exp. Neurol. 26, 183-202.

Albuquerque, E. X., Schuh, F. T. & Kaufman, F. C. (1971). Early membrane depolarization of the fast mammalian muscle after denervation. Pflugers Arch. 328, 36-50.

Albuquerque, E. X., Warnick, J. E., Tasse, J. R., & Sansone, F. M. (1972). Effects of vinblastine and colchicine on neural regulation of fast and slow muscles of the rat. Exp. Neurol. 37, 607-634.

Anderson, M. J. & Cohen, M. W. (1973). Staining of cholinergic receptors at vertebrate neuromuscular junctions with α-bungarotoxin conjugated to fluorescent dye. Canada Physiology. 4, 23.

Anderson, M. J. & Cohen, M. W. (1974). Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. J. Physiol. 237, 385-400.

Anderson, M. J. & Cohen, M.W. (1975). Fluorescent staining of acetylcholine receptors in living muscle cells. J. Physiol. 252, 63P-64P.

Anderson, M. J. & Cohen, M. W. (1976). Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. J. Physiol. (Submitted).

Anderson, M. J., Cohen, M. W. & Zorychta, E. (1976). Effects of innervation on the distribution of acetylcholine receptors on cultured amphibian muscle cells. J. Physiol. (submitted)

Anderson, M. J., Cohen, M. W. & Zorychta, E. (1976). Distribution of acetylcholine receptors on muscle cells cultured with and without nerve. Proc. Can. Fed. Biol. Soc. 19, 91.

Anderson, K. E. & Edstrom, A. (1973). Effects of nerve blocking agents on fast axonal transport of proteins in frog sciatic nerves in vitro. Brain Res. 50, 125-134.

Andersson-Cedergren, E. (1959). Ultrastructure of motor end plate and sarcoplasmic componants of mouse skeletal muscle fibre. J. Ultrastruct. Res. Suppl. 1, pp. 1-191.

Axelsson, J., & Thesleff, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. J. Physiol. 147, 178-193.

Bagust, J. & Lewis, D. M. & Westerman, R. A. (1973). Polyneuronal innervation of kitten skeletal muscle. J. Physiol. 229, 241-255.

Barker, D. & Ip, M.C. (1966). Sprouting and degeneration of mammalian axons in normal and de-afferentated skeletal muscle. Proc. Roy Soc. B. 163, 538-554.

Barnard, E. A., Wieckowski, J., & Chiu, T. H. (1971). Cholenergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions. Nature 234, 207-209.

Barnett, R. J., (1962). The fine structural localization of acetylcholinesterase at the myoneural junction. J. Cell Biol. 12, 247-262.

Bauer, W. C., Blumberg, J. M. & Zachs, S. I. (1962). Short and long-term ultrastructural changes in denervated mouse motor end plates, in Proc. IV Int. cong. Neuropathol. pp. 16-18, Stuttgart.

Bennett, M. R., Pettigrew, A. A. & Taylor, R. S. (1973). The formation of synapses in reinnervated and cross-reinnervated adult avian muscle. J. Physiol. 230, 331-357.

Berg, D. K., Kelly, R. B., Sargent, P. B., Williamson, P. & Hall, Z. W. (1972). Binding of α-bungarotoxin to acetylcholine receptors in mammalian muscle. Proc. natl. Acad. Sci. 69, 147-151.

Berg, D. K. & Hall, Z. (1974). Fate of α-bungarotoxin bound to acetylcholine receptors of normal and denervated muscle. Science 184, 473-475.

Berg, D. K. & Hall, Z. W. (1975a). Increased extrajunctional acetylcholine sensitivity produced by chronic post-synaptic neuromuscular blockade. J. Physiol. 244, 659-676.

Berg, D. K. & Hall, Z. W. (1975b). Loss of α-bungarotoxin from junctional and extrajunctional acetylcholine receptors in rat diaphragm in vivo and in organ culture. J. Physiol. 252, 771-789.

Bernard, C. (1856). Analyse physiologique des proprietes des systemes musculaires et nervaux au moyer du curare. C. R. Lebh. Seanc. Acad. Sci. 43, 825-829.

Bernard, C. (1857). Lecons sur les effects des substances toxiques et medicammenteuses. J. B. Balliere et Fils. Paris.

Birks, R., Katz, B. & Miledi, R. (1960). Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. J. Physiol. 150, 145-168.

Bornstein, M. B., Iwanami, H., Lehrer, A. M. & Breitbart, L. (1968). Observations on the appearance of neuromuscular relationships in cultured mouse tissues. Z. Zellforsch. 92, 197-206.

Bosmann, H. B. (1972). Identification and biochemical characteristics of a cholinergic receptor of guinea pig cerebral cortex. J. Biol. Chem. 247, 130-145.

Bourgeois, J. P., Tsuji, S., Boquet, P., Pillot, J., Ryter, A. & Changeux, J. P. (1971). Localization of the cholinergic receptor protein by immuno-fluorescence in eel electroplax. Febs. Lett. 16, 92-94.

Bray, J. J. & Harris, A. J. (1975). Dissociation between nerve-muscle transmission and nerve trophic effects on rat diaphragm using type D botulinum toxin. J. Physiol. 253, 53-77.

Brockes, J. P. & Hall, Z. W. (1975a). Synthesis of acetylcholine receptor by denervated rat diaphragm muscle. Proc. natl. Acad. Sci. 72, 1368-1372.

Brockes, J. P. & Hall, Z. W. (1975b). Acetylcholine receptors in normal and denervated rat diaphragm muscle. II. Comparison of junctional and extrajunctional receptors. Biochem. 14, 2100-2106.

Buchthal, F. & Lindhard, J. (1937) Direct application of acetylcholine to motor end plates of voluntary muscle fibres. J. Physiol. 90, 82P-83P.

Cartaud, J., Benedetti, E. L., Cohen, J. B., Meunier, J. C., & Changeux, J. P. (1973). Presence of a lattice structure in membrance fragments rich in nicotinic receptor protein from the electric organ of Torpedo Marmorata. Febs Lett. 33, 109-113.

Chang, C. C., Chen, T. F. & Chuang, S. T. (1973). N, O-di and N,N,O-tri (^{3}H) acetyl α-bungarotoxin as specific labelling agents of cholinergic receptors. Brit. J. Pharmacol. 47, 147-160.

Chang, C. C., Chen, T. F. & Lee, C. Y. (1973). Studies of the presynaptic effect of β-bungarotoxin on neuromuscular transmission. J. Pharmacol. exp. ther. 184, 339-345.

Chang, C. C., Chuang, S. T. & Huang, M. C. (1975). Effect of chronic treatment with various neuromuscular blocking agents on the number and distribution of acetylcholine receptors in the rat diaphragm. J. Physiol. 250, 161-173.

Chang, C. C. & Huang, M. C. (1975). Turnover of junctional and extrajunctional acetylcholine receptors of rat diaphragm. Nature 253, 643-644.

Chang, C. C. & Lee, C. Y. (1963) Isolation of neurotoxins from the venom of Bungarus multicinctus and their modes of neuromuscular blocking action. Arch. int. Pharmacodyn. 144, 241-257.

Chang, C. C. & Tung, L. H. (1974). Inhibition by actinomycin D of the generation of acetylcholine receptors induced by denervation in skeletal muscle. Eur. J. Pharmacol. 26, 386-388.

Changeux, J. P., Benedetti, L., Bourgeois, J. P. Brisson, A., Cartaud, J., Devaux, P., Grunhagen, H., Moreau, M., Popot, J. L., Sobel, A. & Weber, M. (1976). Some structural properties of the cholinergic receptor protein in its membrane environment relevant to its function as a pharmacological receptor. Cold Spring Harbor Symp. Quant. Biol. XL (in press).

Changeux, J. P., Kasai, M., Huchet, M. & Meunier, J. C. (1970). Extraction a partir du tissu electrique de gyamnote d'une proteine presentant plusiers proprietes characteristiques du recepteur physiologiques de l'acetylcholine. C. r. hebd. seanc. Acad. Sci. 270, 2864-2867.

Changeux, J. P., Kasai, M. & Lee, C. Y. (1970). Use of a snake venom toxin to characterise the cholinergic receptor protein. Proc. natl. Acad. Sci. 67, 1241-1247.

Changeux, J. P., Meunier, J. C. & Huchet, M. (1971). Studies on the cholinergic receptor protein of Electrophorus electricus. Mol. Pharmacol. 7, 538-553.

Cohen, M. W. (1972). The development of neuromuscular connexions in the presence of d-tubocurarine. Brain Res. 41, 457-463.

Cohen, S. A. & Fischbach, G. D. (1973). Regulation of muscle acetylcholine sensitivity by muscle activity in cell cultures. Science 181, 76-78.

Couteaux, R. (1947). Contribution a l'etude de la synapse myoneurale. Revue can. biol. 6, 563-711.

Couteaux, R. (1955). Localization of cholinesterases at neuromuscular junctions. Intrn. Rev. Cytol. 4, 335-375.

Couteaux, R. (1958). Morphological and cytochemical observations on the post-synoptic membrane at motor end plates and ganglionic synapses. Expl. Cell Res. 3, 347-384.

Couteaux, R. (1963). The differentiation of synaptic areas. Proc. R. Soc. B. 158, 457-480.

Couteaux, R. & Taxi, J. (1952). Recherches histochimiques sur la distribution des activites cholinesterasiques au niveau de la synapse myo-neurale. Arch. Anat. Microsc. 41, 352-392.

Crain, S. M. & Peterson, E.R. (1971). Development of paired explants of fetal spinal cord and adult skeletal muscle during chronic exposure to curare and hemicholinium. In Vitro 6, 373.

Dale, H. H. (1914). The action of certain esters and ethers of choline, and their relation to muscarine. J. Pharmac. exp. ther. 6, 147-190.

Daniels, M. P. & Vogel, Z. (1975). Immunoperoxidase staining of α-bungarotoxin binding sites in muscle endplates shows distribution of acetylcholine receptors. Nature 254, 339-341.

del Castillo, J. & Katz, B. (1955). On the localization of acetylcholine receptors. J. Physiol. 128, 157-181.

Devreotes, P. N. & Fambrough, D. M. (1975). Acetylcholine receptor turnover in membranes of developing muscle fibres. J. Cell Biol. 65, 335-358.

Devreotes, P. N. & Fambrough, D. M. (1976). Synthesis of the acetylcholine receptor by cultured chick myotubes and denervated mouse extensor digitorum longus muscles. Proc. natl. Acad. Sci. 73, 161-164.

Diamond, T. & Miledi, R. (1962). A study of foetal and new-born rat muscle fibres. J. Physiol. 162, 393-408.

Drachman, D. B. & Witzke, F. (1972). Trophic regulation of acetylcholine sensitivity of muscle: effect of electrical stimulation. Science, 176, 514-516.

Dreyer, F. & Peper, K. (1974). The acetylcholine sensitivity in the vicinity of the neuromuscular junction of the frog. Pflugers Arch. 348, 273-286.

Eccles, J. C. & Jaeger, J. C. (1958). The relationship between the mode of operation and the dimensions of the junctional regions at synapses and motor end-organs. Proc. Roy Soc. B. 148, 38-56.

Edelman, G. M. (1976). Surface modulation in cell recognition and cell growth Science. 192, 218-226.

Edelman, G. M., Yahara, I. & Wang, J. L. (1973). Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. Proc. natl. Acad. Sci. 70, 1442-1446.

Edidin, M. & Fambrough, D. (1973). Fluidity of the surface of cultured muscle fibres: rapid lateral diffusion of marked surface antigens. J. Cell Biol. 57, 27-37.

Edidin, M., Zagyansky, U. & Lardner, T.J. (1976). Measurement of membrane protein lateral diffusion in single cells. Science 191, 466-468.

Eldefrawi, M. E. & El [illegible]awi, A.T. (1973). Purification and molecular properties of the [illegible]tylcholine receptor from Torpedo electroplax. Archs. Biochem. Biophys. 159, 362-373.

Eldefrawi, M. E., Eldefrawi, A. T., Siefert, S. & O'Brien, R. D. (1972). Properties of lubrol-solubilized acetylcholine receptor from Torpedo electroplax. Archs. Biochm. Biophys. 150, 210-218.

Emmelin, N. & Malm, L. (1965). Development of supersensitivity as dependent on the length of degenerating nerve fibres. Quart. J. exp. Physiol. 50, 142-145.

Fambrough, D. M. (1970). Acetylcholine sensitivity of muscle fiber membranes: mechanism of regulation by motoneurons, Science. 168, 372-373.

Fambrough, D.M. & Hartzell, H. C. (1972). Acetylcholine receptors: number and distribution at neuromuscular junctions in rat diaphragm. Science 176, 189-191.

Fambrough, D., Hartzell, H.C., Rash, J. E. & Ritchie, A. K. (1974). Receptor properties of developing muscle. Ann. N.Y. Acad. Sci. 228, 47-61.

Fambrough, D. & Rash, J. E. (1971). Development of acetylcholine sensitivity during myogenesis. Devel. Biol. 26, 55-68.

Feltz, A. & Mallart, A. (1971). An analysis of acetylcholine responses of junctional and extrajunctional receptors of frog muscle fibres. J. Physiol. 218, 85-100.

Fertuck, H. C. & Salpeter, M. M. (1974). Localization of acetylcholine receptor by ^{125}I-labelled α-bungarotoxin binding at mouse motor end plates. Proc. natl. Acad. Sci. 71, 1376-1378.

Fertuck, H. C. & Salpeter, M. M. (1976). Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after ^{125}I-α-bungarotoxin binding at mouse neuromuscular junctions. J. Cell Biol. 69, 144-158.

Fex, S., Sonesson, B., Thesleff, S. & Zelena, J. (1966). Nerve implants in botulinum poisoned mammalian muscle. J. Physiol. 184, 872-882.

Filogamo, G. & Gabella, G. (1966). Cholinesterase behavior in denervated and reinnervated muscles. Acta Anat. 63, 199-214.

Filogamo, G. & Gabella, G. (1967). The development of neuromuscular correlations in vertebrates, Arch. Biol. 78, 9-60.

Fischbach, G. D. (1970). Synaptic potentials recorded in cell cultures of nerve and muscle. Science 169, 1331-1333.

Fischbach, G. D. (1972). Synapse formation between dissociated nerve and muscle cells in low density cell cultures. Develop. Biol. 28, 407-429.

Fischbach, G. D. & Cohen, S. A. (1973). The distribution of acetylcholine sensitivity over uninnervated muscle fibers grown in cell cultures, Develop. Biol. 31, 147-162.

Frank, E. Gautvik, K. & Sommerschild, H. (1975). Cholinergic receptors at denervated mammalian motor endplates. Acta Physiol. Scand. 95, 66-76.

Franklin, G. I. & Potter, L. T. (1972). Studies of the binding of α-bungarotoxin to membrane-bound and detergent-dispersed acetylcholine receptors from Torpedo electric tissue. FEBS Lett. 28, 101-106.

Frye, L. D. & Edidin, M. (1970). The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. J. Cell Sci. 7, 319-335.

Giacobini, G., Filogamo, G., Weber, M., Boquet, P. & Changeux, J. P. (1973). Effects of snake α-neurotoxin on the development of innervated skeletal muscles in chick embryo. Proc. natl. Acad. Sci. 70, 1708-1712.

Giller, E. L., Schrier, B. K., Schainberg, A., Fisk, H. R. & Nelson, P. G. (1973). Increased choline acetyltransferase activity in combined cultures of spinal cord and muscle cells from the mouse. Science 182, 588-589.

Ginetzinsky, A. G. & Shamarina, N. M. (1942). The tonomotor phenomenon in denervated muscle. Advanc. Bod. Biol. (U.S.S.R.) Usp. Sovrem. Biol. 17, 283-294.

Goldman, M. (1968). Fluorescent Antibody methods, pp 97-138, Academic Press, New York.

Goldstein, A., Aronowi, L. & Kalman, S. M. (1974). Principles of drug action: the basis of phamacology, J. Wiley & Sons, N. Y.

Grampp, W., Harris, J. B. & Thesleff, S. (1972). Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis. J. Physiol. 221, 743-754.

Greene, L. A., Sytkowski, A. J., Vogel, Z. & Nirenberg, M. W. (1973). -bungarotoxin used as a probe for acetylcholine receptors of cultured neurons. Nature 243, 163-166.

Guth, L. (1968). 'Trophic' influences of nerve on muscle. Physiol. Rev. 48, 645-687.

Harris, A. J. (1975). Inductive functions of the nervous system. Ann. Rev. Physiol. 36, 251-305.

Harris, A. J., Heinemann, S., Schubert, D. & Tarakis, H. (1971). Trophic interaction between cloned tissue culture lines of nerve and muscle. Nature 231, 296-301.

Harris, A. J., Kuffler, S. W. & Dennis, M. J. (1971). Differential chemosensitivity of synaptic and extrasynaptic areas on the neuronal surface membrane in parasympathetic neurons of the frog, tested by microapplication of acetylcholine. Proc. Roy Soc. B. 177, 541-553.

Harris, A. J. & Miledi, R. (1966). Prolonged survivial of isolated frog muscle and its sensitivity to acetylcholine. Nature 209, 716-717.

Harris, J. B. & Thesleff, S. (1972). Nerve stump length and membrane changes in denervated skeletal muscle. Nature New Biol. 236, 60-61.

Harrison, R. G. (1910). The outgrowth of the nerve fiber as a mode of protoplasmic movement. J. exp. Zool. 9, 787-846.

Hartzell, H. C. & Fambrough, D.M. (1972). Acetylcholine receptors: distribution and extra-junctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. J. Gen. Physiol. 60, 248-262.

Hartzell, H. C. & Fambrough, D. M. (1973). Acetylcholine receptor production and incorporation into membranes of developing muscle fibres. Develop. Biol. 30, 153-165.

Hazelbauer, J. &.Changeux, J. P. (1974). Reconstitution of a chemically excitable membrane. Proc. natl. Acad. Sci. 71, 1479-1483.

Heuser, J. E., Reese, T. S. & Landis, D.M.D. (1974). Functional changes in frog neuromuscular junctions studied with freeze-fracture. J. Neurocytol. 3, 108-131.

Hirano, H. (1967). Ultrastructural study on the morphogenesis of the neuromuscular junction in the skeletal muscle of the chick. Z. Zellforsch. 79, 198-208.

Hofmann, W.W. & Thesleff, S. (1972). Studies on the trophic influence of nerve on skeletal muscle, Eur. J. Phamacol. 20, 256-260.

Hogan, P. G., Marshall, J. M. & Hall, Z. W. (1976). Muscle activity decreases rate of degradation of α-bungarotoxin bound to extrajunctional acetylcholine receptors. Nature 261, 328-330.

Hubbard, J. I., Schmidt, R. F. & Yokota, T. (1965). The effect of acetylcholine upon mammalian motor nerve terminals. J. Physiol. 181, 810-829.

Hughes, A. (1961). Cell degeneration in the larval ventral horn of Xenopus laevis (Daudin). J. Embryol. exp. Morphol. 9, 269-284.

James, D. W. & Tresman, R. L. (1968a). De novo formation of neuromuscular junctions in tissue culture. Nature 220, 384-385.

James, D. W. & Tresman, R. L. (1968b). Synaptic profiles in the outgrowth from chick spinal cord in vitro. Z. Zellforsch. 101, 598-606.

James, D. W. & Tresman, R. L. (1969). An electronmicroscopic study of the de novo formation of neuromuscular junctions in tissue culture. Z. Zellforsch. 100, 126-140.

Jansen, J.K.S. & Van Essen, D. C. (1975). Re-innervation of rat skeletal muscle in the presence of α-bungarotoxin. J. Physiol. 250, 651-667.

Jones, R. & Vrbova, G. (1970). Effect of muscle activity on denervation hypersensitivity. J. Physiol. 210, 144p - 145p.

Jones, R. & Vrbova, G. (1974). Two factors responsible for the development of denervation hypersensitivity. J. Physiol. 236, 517-538.

Juntunen, J. & Teravainen, H. (1972). Structural development of myoneural junctions in the human embryo. Histochemie 32, 107-112.

Kano, M. & Shimada, Y. (1971a). Innervation and acetylcholine sensitivity of skeletal muscle cells differentiated in vitro from chick embryo. J. Cell Physiol. 78, 233-242.

Kano, M. & Shimada, Y. (1971b). Innervation of skeletal muscle cells differentiated in vitro from chick embryo. Brain Res. 27, 402-405.

Karlin, A. (1974). The acetylcholine receptor: progress report, Life Sci. 14, 1385-1415.

Karlsson, E., Heilbronn, E., & Widlund, L. (1972). Isolation of the nicotinic acetylcholine receptor by biospecific chromatography on insolubilized Naja naja neurotoxin. Febs Lett. 28, 107-111.

Karnovsky, M. J. (1964). The localization of cholinesterase activity in the rat cardiac muscle by electron microscopy. J. Cell Biol. 23, 217-232.

Katz, B. (1969). The release of neural transmitter substances. pp. 45-46, Liverpool University Press, Liverpool.

Katz, B. & Miledi, R. (1964a). Further observations on the distribution of acetylcholine-reactive sites in skeletal muscle. J. Physiol. 170, 379-388.

Katz, B. & Miledi, R. (1964b). The development of acetylcholine sensitivity in nerve-free segments of skeletal muscle. J. Physiol. 170, 389-396.

Kelly, A. M. & Zachs, S. I. (1969). The fine structure of motor endplate morphogenesis. J. Cell Biol. 42, 154-169.

Kimura, M. & Kimura, I. (1973). Increase of nascent protein synthesis in neuromuscular junctions of rat diaphragm induced by denervation. Nature New Biol. 241, 114-115.

Klett, R. P., Fulpius, B. U., Cooper, D., Smith, M., Reich, E. & Possani, L.D. (1973). The acetylcholine receptor. I. Purification and characterization of a macromolecule isolated from Electrophorus electricus. J. biol. Chem. 248, 6841-6853.

Kopriwa, B. M. & Leblond, C. P. (1962). Improvements in the coating technique for radioautography. J. Histochem. Cytochem. 10, 269-284.

Krnjevic, K., Mitchel,, J. F. & Szerb, J. C. (1963). Determination of iontophoretic release of acetylcholine from micropipettes. J. Physiol. 165, 421-436.

Kuffler, S. W. (1943). Specific excitability of the end-plate-region in normal and denervated muscle. J. Neurophysiol. 6, 99-110.

Kuffler, S. W. & Yoshikami, D. (1975). The distribution of acetylcholine sensitivity at the post-synaptic membrane of vertebrate skeletal twitch muscles: iontophoretic mapping in the micron range, J. Physiol. 244, 703-730.

Kuhne, W. (1887). Neue untersuchungen uber motorische nervenendung. Z. Biol. 23, 1-148.

Kullberg, R. W. (1974). Onset and development of synaptic activity at an amphibian neuromuscular junction. PhD Thesis, McGill Univ.

Landowne, D., Potter, L. T. & Terrar, D. A. (1975). Structure function relationships in excitable membranes. Annu. Rev. Physiol. 37, 485-508.

Langley, J. N. (1905). On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and curari. J. Physiol. 33, 374-413.

Langley, J. N. (1907). On the contraction of muscle, chiefly in relation to the presence of 'receptive' substances. Part I. J. Physiol. 36, 347-384.

Langley, J. N. & Dickenson, W. L. (1890). Pituri and Nicotin. J. Physiol. 11, 265-406.

Laskey, R. A. (1970). The use of antibiotics in the preparation of amphibian cell cultures from highly contaminated material. J. Cell Sci. 7, 653-659.

Lavoie, P. A., Collier, B. & Tenenhouse, A. (1976). Comparison of α-bungarotoxin binding to skeletal muscles after inactivity or denervation Nature. 260, 349-350.

Lee, C. Y. (1972). Chemistry and pharmacology of polypeptide toxins in snake venoms. Ann. Rev. Pharmacol. 12, 265-286.

Lee, C. Y. & Chang, C. C. (1966). Modes of actions of purified toxins from elapid venoms on neuromuscular transmission. Mem. Inst. Butantan. 33, 555-572.

Lee, C. Y., Chang, S. L., Kau, S. T. & Luh, S. H. (1972). Chromatographic separation of the venom of Bungarus multicinctus and characterization of its componants. J. Chromatogr. 72, 71-82.

Lee, C. Y. & Tseng. L. F. (1966). Distribution of Bungarus multicinctus venom following envenomation. Toxicon. 3, 281-290.

Lee, C. Y., Tseng. L. F. & Chiu, T. H. (1967). Influence of denervation on localization of neurotoxins from elapid venoms in rat diaphragm. Nature 215, 1177-1178.

Lehrer, G. M. & Ornstein, L. A. (1959). A diazo coupling method for the electron microscopic localization of cholinesterase. J. biophys. biochem. Cytol. 6, 399-406.

Lentz, T. L. (1969). Development of the neuromuscular junction. I. Cytological and cytochemical studies on the neuromuscular junction of differentiating muscle in the regenerating limb of the newt Triturus. J. Cell Biol. 42, 431-443.

Lester, H. A. (1970) Postsynaptic action of cobra toxin at the myoneural junction. Nature 227, 727-728.

Lester, H. A. (1972a). Blockade of acetylcholine receptors by cobra toxin: electrophysiological studies. Mol. Pharmacol. 8, 623-631.

Lester, H. A. (1972b). Vulnerability of desensitized or curare-treated acetylcholine receptors to irreversible blockade by cobra toxin. Mol. Pharmacol. 8, 632-644.

Letinsky, M. S. (1975). Acetylcholine sensitivity changes in tadpole tail muscle fibres innervated by developing motor neurons. J. Neurobiol. 6, 609-618.

Lewis, P. R. & Hughes, A.F.W. (1960). Patterns of myo-neural junctions and cholinesterase activity in the muscles of tadpoles of Xenopus laevis. Q. Jl microsc. Sci. 101, 55-67.

Libelius, R. (1974). Binding of ^{3}H-labelled cobra neurotoxin to cholinergic receptors in fast and slow mammalian muscles. J. Neural Transm. 35, 137-149.

Liebman, P. A. & Entine, G. (1974). Lateral diffusion of visual pigment in photoreceptor disk membranes, Science 185, 457-459.

Loewi, O. (1921). Uber humorale ubertragbarkeit der herznervenwirkung. Pflugers. Arch. ges. Physiol. 189, 239-242.

Loewi, O. & Navratil, E. (1926). Uber humorale ubertragbarkeit der herznervenwirkung. X. Mitteilung. Uber das schicksal des vagusstoff. Pflugers Arch. ges. Physiol. 189, 239-242.

Lazarides, E. (1976). Actin, α-actinin and tropomyosin interaction in the structural organization of actin filaments in nonmuscle cells. J. Cell Biol. 68, 202-219.

Lomo, T. (1974). Neurotrophic control of colchicine effects on muscle? Nature 249, 473-474.

Lomo, T. & Rosenthal, J. (1972). Control of ACh sensitivity by muscle activity in the rat. J. Physiol. 221, 493-513.

Lomo, T. & Westgaard, R. H. (1975). Further studies on the control of ACh. sensitivity by muscle activity in the rat, J. Physiol. 252, 603-626.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J (1951). Protein measurement with the folin phenol reagent. J. Biol Chem. 193, 265-275.

Luco, J. V. & Eyzaguirre, C. (1955). Fibrillation and hypersensitivity to ACh. in denervated muscle: effect of length of degenerating nerve fibres. J. Neurophysiol. 18, 65-73.

Marshall, J. D., Eveland, W.C. & Smith, C. W. (1958). Superiority of fluoroscein isothiocyanate for fluorescent application. Proc. Soc. Exp. Biol. Med. 98, 898-900.

McMahon, U. J., Spitzer, N. C. & Peper, K. (1972). Visual identification of nerve terminals in living isolated skeletal muscle. Proc. Roy Soc. B. 181, 421-430.

Mebs, D., Narita, K., Iwanaga, S., Samejima, Y. & Lee, C. Y. (1971). Amino acid sequence of α-bungarotoxin from the venom of Bungarus multicinctus. Biochem. Biophys. Res. Commun. 44, 711-716.

Meunier, J.C., Olsen, R.W., Menez, A., Fromageot, P., Boquet, P. & Changeux, J.P. (1972). Some physical properties of the cholinergic protein from Electrophorus electricus revealed by tritiated α-toxin from Naja nigricollis venom. Biochem. 11, 1200-1210.

Michaelson, D.M. & Raftery, M.A. (1974). Purified acetylcholine receptor: its reconstitution to a chemically excitable membrane. Proc. natl. Acad. Sci. 71, 4768-4772.

Michelson, M.J. & Ziemal, E.V. (1973). Acetylcholine: an approach to the molecular mechanism of action. Pergamon Press Ltd., Oxford.

Miledi, R. (1960a). The ACh sensitivity of frog muscle fibres after complete or partial denervation. J. Physiol. 151, 1-23.

Miledi, R. (1960b). Junctional and extrajunctional ACh receptors in skeletal muscle fibres. J. Physiol. 151, 24-30.

Miledi, R. (1960c). Properties of regenerating neuromuscular synapses in the frog. J. Physiol. 154, 190-205.

Miledi, R. (1962). Induced innervation of end-plate free muscle segments. Nature 193, 281-282.

Miledi, R. & Potter, L.T. (1971). Acetylcholine receptors in muscle fibres. Nature 233, 599-603.

Miledi, R., Molinoff, P. & Potter, L.T. (1971). Isolation of the cholinergic receptor protein of Torpedo electric tissue. Nature 229, 554-557.

Miledi, R. & Slater, C.R. (1968). Electrophysiology and electronmicroscopy of rat neuromuscular junctions after nerve degereration. Proc. R. Soc. B. 169, 289-306.

Miledi, R. & Zelena, J. (1966). Sensitivity to acetylcholine in rat slow muscle. Nature 210, 855-856.

Muntz, L. (1975). Myogenesis in the trunk and leg muscles during development of the tadpole of Xenopus laevis. J. Embryol. exp. Morph. 33, 757-774.

Murray, M.R. (1972). Skeletal muscle in culture. In: Structure and function of muscle. 2nd ed. G.H. Bourne ed. Academic Press, New York.

Nastuck, W.L. (1953). Membrane potential changes at a single muscle endplate produced by transitory application of acetylcholine with an electrically controlled microjet. Fedn. Proc. 12, 102.

Nelson, P.G. (1975). Nerve and muscle cells in culture. Physiol. Rev. 55, 1-61.

Nickel, E. & Waser, P.G. (1968). Electronmikroskopische untersuchungen am diaphragma der maus nach einseitiger phrenikotomie. Z. Zellforsch. 88, 278-296.

Nickel, E. & Potter, L.T. (1973). Ultrastructure of isolated membranes of Torpedo electric tissue. Brain Res. 57, 508-517.

Nicolson, G.L. (1973). Cis- and trans-membrane control of cell surface topography. J. Supramol. Struct. 1, 410-416.

Nicolson, G.L. (1974). The interactions of lectins with animal cell surfaces. Int. Rev. Cytol. 39, 89-190.

Nicolson, G.L. & Painter, R.G. (1973). Anionic sites of human erythrocyte membranes anti-spectrin induced transmembrane aggregation of the binding sites of positively charged colloidal particles. J. Cell Biol. 59, 395-406.

Nieuwkoop, P.D. & Faber, J. (1956). Normal table of Xenopus laevis (Daudin). 2nd ed. pp 162-188, North Holland, Amsterdam.

Olsen, R.W., Meunier, J.C. & Changeux, J.P. (1972). Progress in the purification of the cholinergic receptor protein from Electrophorus electricus by affinity chromatography. Febs Lett. 28, 96-100

Pappas, G.D., Peterson, E.R., Masurovsky, E.B. & Crain, S.M. (1971). Electron microscopy of the in vitro development of mammalian motor endplates. Ann. N.Y. Acad. Sci. 183, 33-45.

Patrick, J., Heinemann, S.F., Lindstrom, J., Schubert, D. & Steinbach, J.H. (1972). Appearance of acetylcholine receptors during differentiation of a myogenic cell line. Proc. natl. Acad. Sci. 69, 2762-2766.

Peper, K., Dreyer, F., Sandri, C., Akert, K. & Moor, H. (1974). Structure and ultrastructure of the frog motor endplate. Cell Tiss. Res. 149, 437-455.

Peper, K. & McMahon, U.J. (1972). Distribution of acetylcholine receptors in the vicinity of nerve terminals on skeletal muscle of the frog. Proc. R. Soc. B. 181, 431-440.

Pestronk, A., Drachman, D.B. & Griffin, J.W. (1976). Effect of muscle disuse on acetylcholine receptors. Nature 260, 352-353.

Peterson, E.R. & Crain, S.M. (1970). Innervation in culture of fetal rodent skeletal muscle by organotypic explants of spinal cord from different animals. Z. Zellforsch. 106, 1-21.

Pilar, G. & Landmessar, L. (1972). Axotomy mimmicked by localized colchicine application. Science 177, 1116-1118.

Poo, M. & Cone, R.A. (1974). Lateral diffusion of rhodopsin in the photo-receptor membrane. Nature 247, 438-441.

Porter, C.W., Chiu, T.H., Wieckowski, J. & Barnard, E.A. (1973). Types and locations of cholinergic receptor-like molecules in muscle fibres. Nature New Biol. 241, 3-7.

Prestige, M.C. (1967). The control of cell number in the lumbar ventral horns during the development of Xenopus laevis tadpoles. J. Embryol. exp. Morph. 18, 359-387.

Prestige, M.C. & Wilson, M.A. (1972). Loss of axons from ventral roots during development. Brain Res. 41, 467-470.

Purves, D. & Sackman, B. (1974). The effect of contractile activity on fibrillation and extrajunctional acetylcholine sensitivity in rat muscle maintained in organ culture. J. Physiol. 237, 157-182.

Raff, M. & de Petris, S. (1974). The lymphocyte plasma membrane. In: The immune system, ed. Sercarz, E.E., Williamson, A.R., Fox, F.F. pp 247-257, New York, Academic Press.

Raftery, M.A., Schmidt, J., Clark, D.G. & Wolcott, R.G. (1971). Demonstration of a specific α-bungarotoxin binding componant in Electrophorus electricus electroplax membranes. Biochem. Biophys. Res. Comm. 45, 1622-1629.

Raftery, M.A., Schmidt, J., Martinez-Carrion, T.M., Moody, T., Vandlen, R. & Duguid, J. (1973). Biochemical studies on Torpedo californica receptors. J. Supramol. Struct. 1, 360-375.

Raftery, M.A., Vandlen, R., Reed, K. & Lee, T. (1976). Structure and functional characteristics of an acetylcholine receptor. Cold Spring Harbor Symp. Quant. Biol. XL (in press)

Rang, H.P. (1975). Acetylcholine receptors, Quart. Rev. Biophys. 7, 283-399.

Rash, J.E. & Ellisman, M.H. (1974). Studies of excitable membranes I. Macromolecular specializations of the neuromuscular junction and the non-junctional sarcolemma. J. Cell Biol. 63, 567-578.

Redfern, P.A. (1970). Neuromuscular transmission in new-born rats. J. Physiol. 209, 701-709.

Redfern, P. & Thesleff, S. (1971). Action potential generation in denervated rat skeletal muscle. II. The action of tetrodotoxin. Acta Physiol. Scand. 82, 70-78.

Reger, J.F. (1959). Studies on the fine structure of normal and denervated neuromuscular junctions from mouse gastrocnemius. J. Ultrastruc. Res. 2, 268-282.

Robbins, N. & Yonezawa, T. (1971a). Developing neuromuscular junctions: first signs of chemical transmission during formation in tissue culture. Science 172, 395-397.

Robbins, N. & Yonezawa, T. (1971b). Physiological studies during the formation and development of rat neuromuscular junctions in tissue culture. J. gen. Physiol. 58, 467-481.

Reier, P.J. & Hughes, J. (1972). Evidence of spontaneous axon degeneration during peripheral nerve maturation. Am. J. Anat. 135, 147-152.

Roper, S. (1976). The acetylcholine sensitivity of the surface membrane of multiply-innervated parasympathetic ganglion cells in the mudpuppy before and after denervation. J. Physiol. 254, 455-474.

Rosenblith, J.Z., Ukena, T.E., Yin, H.H., Berlin, R.D. & Karnovsky, M.J. (1973). A comparative evaluation of the distribution of concanavalin A binding sites on the surfaces of normal, virally-transformed, and protease treated fibroblasts. Proc. natl. Acad. Sci. 70, 1625-1629.

Rutishauser, U., Yahara, I., Edelman, G.M. (1974). Morphology, mobility and surface behavior of lymphocytes bound to nylon fibres. Proc. natl. Acad. Sci. 71, 1149-1153.

Ryan, G.B., Borysenko, J.Z. & Karnovsky, M.J. (1974). Factors affecting the redistribution of surface-bound concanavalin A on human polymorphonuclear leucocytes. J. Cell Biol. 62, 351-365.

Sakman, B. (1975). Reappearance of extrajunctional acetylcholine sensitivity in denervated rat muscle after blockade with α-bungarotoxin. Nature 255, 415-416.

Salafsky, B., Bell, J. & Prewitt, M.A. (1968). Development of fibrillation potentials in denervated fast and slow skeletal muscle. Am. J. Physiol. 215, 637-643.

Schmidt, T.J. & Raftery, M.A. (1972). Use of affinity chromatography for acetylcholine receptor purification. Biochem. Biophys. Res. Comm. 49, 572-578.

Schmidt, J. & Raftery, M.A. (1973). Purification of acetylcholine receptors from Torpedo californica electroplax by affinity chromatography. Biochem. 12, 852-855.

Shimada, Y. & Fishman, D.A. (1973). Morphological and physiological evidence for the development of functional neuromuscular junctions in vitro. Devel. Biol. 31, 200-225.

Shimada, Y., Fishman, D.A. & Moscona, A.A. (1969a). The development of nerve-muscle junctions in monolayer cultures of embryonic spinal cord andskeletal muscle cells. J. Cell Biol. 43, 382-387.

Shimada, Y., Fishman, D.A. & Moscona, A.A. (1969b). Formation of neuromuscular junctions in embryonic cell cultures. Proc. natl. Acad. Sci. 62, 715-721.

Singer, S.J. & Nicolson, G.L. (1972). The fluid mosaic model of the structure of cell membranes. Science 175, 720-731.

Sonesson, B. & Thesleff, S. (1968). Cholinesterase activity after DFP application in botulinum poisoned, surgically denervated or normally innervated rat skeletal muscles. Life Sci. 7, 411-417.

Sytkowski, A.J., Vogel, Z. & Nirenberg, M.W. (1973). Development of acetylcholine receptor clusters on cultured muscle cells. Proc. natl. Acad. Sci. 70, 270-274.

Takeuchi, A. & Takeuchi, N. (1964). The effect on crayfish muscle of iontophoretically applied glutamate. J. Physiol. 170, 296-317.

Takeuchi, A. & Takeuchi, N. (1965). Localized action of gamma-amino-butyric acid on the crayfish muscle. J. Physiol. 177, 225-238.

Tamiya, N. & Arai, H. (1966). Studies on sea-snake venoms: crystallization of erabutoxins a and b from Laticauda semifasciata venom. Biochem. J. 99, 624-630.

Taylor, R.B., Duffus, W.P.H., Raff, M.C. & de Petris, S. (1971). Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. Nature New Biol. 233, 225-229.

Teravainen, H. (1968a). Carboxylic esterases in developing myoneural junctions of rat striated muscle. Histochemie 12, 307-315.

Teravainen, H. (1968b). Development of the myoneural junction in the rat. Z. Zellforsch. 87, 249-265.

Thesleff, S. (1960). Supersensitivity of skeletal muscle produced by botulinum toxin. J. Physiol. 151, 598-607.

Tonge, D.A. (1974). Chronic effects of botulinum toxin on neuromuscular transmission and sensitivity to acetlycholine in slow and fast skeletal muscle of the mouse, J. Physiol. 241, 127-139.

Tuffery, A.R. (1971). Growth and degeneration of motor end-plates in normal cat hind limb muscles, J. Anat. 110, 221-247.

Usherwood, P.N.R., Machili, D. & Leaf, G. (1968). L-glutamate in insect excitatory nerve-muscle synapses. Nature 219, 1169-1172.

Veneroni, G., & Murray, M.R. (1969). Formation de novo and development of neuromuscular junctions in vitro. J. Embryol. exptl. Morph. 21, 369-382.

Vogel, Z. & Daniels, M.P. (1976). Ultrastructure of acetylcholine receptor clusters on cultured muscle fibres. J. Cell Biol. 69, 501-506.

Vogel, Z., Sytkowski, A.J. & Nirenberg, M.W. (1972). Acetylcholine receptors of muscle grown in vitro. Proc. natl. Acad. Sci. 69, 3180-3185.

Yahara, I. & Edelman, G.M. (1975). Modulation of lymphocyte receptor mobility by locally bound concanavalin A. Proc. natl. Acad. Sci. 72, 1579-1583.

ABSTRACT

α-Bungarotoxin was labelled with fluorescent dyes and used as a stain for visualizing the distribution of acetylcholine receptors in vertebrate skeletal muscle fibres. Dye-toxin conjugates had the same pharmacological properties as native toxin, but their potencies were lower. Fluorescent staining was examined in teased muscle fibres. The stain was found to be confined to the neuromuscular junction and associated with the subsynaptic membrane. Staining intensity was reduced by curare and even more so by carbachol, but not by atropine or neostigmine. Pretreatment of muscles with unlabelled α-bungarotoxin entirely prevented staining. The staining at amphibian neuromuscular junctions was characterized by a pattern of intense transverse bands occurring at intervals of approximately 0.5-1 u, with fluorescence of lower intensity between them. Fluorescent staining was not detected on adjacent, extrasynaptic, muscle membrane. In side views the staining appeared as a fine line with small protuberances occurring at the same intervals as the intense bands seen face-on. These results indicate that acetylcholine receptors are associated with the entire subsynaptic membrane, including the membrane of the junctional folds and that their density changes abruptly at the border between synaptic and extrasynaptic muscle membrane.

Myotomal muscle cells from embryos of Xenopus laevis were cultured as a monolayer either alone or together with neural tube cells from the same embryos. Spontaneous twitching and contractions evoked by electrical stimulation of neuronal perikarya were observed only in nerve-contacted

muscle cells, and could be abolished by curare or α-bungarotoxin. Within 2 days in culture muscle cells not contacted by nerve developed one or more discrete patches of acetylcholine (ACh) receptors as revealed by staining with fluorescent conjugates of α-bungarotoxin. Similar patches were also seen when staining was carried out after paraformaldehyde fixation, suggesting that they were not induced by the dye-toxin conjugate. Radioautography after labelling with ^{125}I-α-bungarotoxin revealed patches with grain densities approximately 25-fold greater than over the remainder of the cell. Fluorescent stain on innervated cells was restricted to the path of nerve-muscle contact and sometimes extended for greater lengths than the largest patches seen on non-contacted muscle cells. Similar long bands of stain associated with nerve-muscle contacts were observed when cultures were grown in high concentrations of curare and carbachol which prevented spontaneous twitching. They were also seen in cultures in which the addition of neural tube cells was delayed for 2-3 days. It is concluded that innervation caused receptors to accumulate at sites of nerve-muscle contact and that this process can operate independently of muscle activity.

Three-day-old cultures of myotomal muscle, obtained from embryos of Xenopus laevis, were stained with fluorescent conjugates of α-bungarotoxin and maintained in native toxin in order to ensure that ACh receptors subsequently inserted into the sarcolemma would not be stained. Neural tube cells were then added to the cultures. When cultures were examined 1-3 days later fluorescent stain was found to be associated with sites

of nerve-muscle contact. In some cases the stain along the path of contact extended for greater distances than the patches of stain seen on non-contacted muscle cells. The development of new areas of fluorescent stain at sites of nerve-muscle contact was confirmed by making successive observations on the same muscle cell over a period of a day. Similar experiments on muscle cells not contacted by nerve revealed the formation of new receptor patches, usually in areas of cell growth. The majority of fluorescent patches on non-contacted muscle cells did not undergo changes in size or shape over the course of 1-2 days. However some examples of enlargement, shrinkage and disappearance were observed. On the basis of these findings it is concluded that ACh receptors aggregate within the sarcolemma, spontaneously as well as in response to innervation. In the latter case extrajunctional receptors accumulate at the site of nerve contact thereby contributing to the development of a high receptor density in the subneural muscle membrane. This process of receptor redistribution occurs in the absence of synaptic or contractile activity. Possible mechanisms involved in the redistribution of ACh receptors are discussed in relation to those which appear to modulate ligand-induced changes in the distribution of lectin and immunoglobulin receptors.