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Electrophysiological and Pharmacological Properties of Skeletal Muscle in Culture

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Tissue culture is the maintenance or growth of biological tissues in an artificial medium. Techniques in use include: (*a*) organ culture, which uses a complete organ in isolation from the body; (*b*) explant culture, in which a small portion of tissue is excised and maintained as a discrete piece; (*c*) cell culture, which involves dissociation of the explant to a suspension of separate cells; and (*d*) continuous cell lines, which can be maintained in culture conditions by continuous multiplication.

Tissue culture has several advantages over conventional *in vivo* or *in vitro* experimental systems. Blood flow, hormones, nerve trophic factors, and other body influences no longer affect the functions of the tissue; the cultures are in a controlled environment which is easy to alter for experimental purposes. The amount of tissue disintegration can be selected by the choice of technique, for example, by using explants or cell lines. Monolayer cultures of dissociated cells are usually more accessible than tissues *in situ*, thus enabling cells to be studied throughout development and allowing prolonged experiments. In addition, human tissue from biopsies or embryos can be readily studied using culture techniques.

Many cell types have been grown in tissue culture, but skeletal muscle appears to be particularly suitable. It remains remarkably similar in physiology and biochemistry to skeletal muscle *in situ* (1). Skeletal muscle from various species, including chick, rat, mouse, and human, has been grown as explants, cell cultures, or cell lines (2–7).

Skeletal muscle in culture is especially useful in pharmacological studies, because it provides a simple system of only muscle fibers without nerves. The effects of neurons on muscle function can, however, be studied in culture (8, 9).

Early studies on skeletal muscle in culture used organ culture or explants of muscle from embryos or adult animals. Often such tissue suffered from "dedifferentiation"—loss of characteristic structures of the tissue and reappearence of cytological features typical of an earlier state of differentiation. Advances were made rapidly after studies that led to a method of growing consistent cell cultures of skeletal muscle fibers (2, 10).

For cell cultures, tissue is ideally obtained from embryos or neonates. Muscle is dissected and broken down to a suspension of individual cells, either mechanically or by using enzymes such as trypsin or collagenase. The cell suspension contains myoblasts, the mononuclear precursors of muscle fibers, and connective tissue cells, the fibroblasts.

Cultures can easily be grown in plastic petri dishes that have been coated with collagen; collagen appears to be a necessary substrate during the early stages of muscle differentiation (10). The cells are maintained at 37° in a standard tissue culture medium supplemented with serum and embryo extract.

The sequence of events during differentiation of skeletal muscle in culture has been studied in some detail. After trypsin dissociation, the cultures consist of spherical mononuclear cells. These cells settle onto the surface of the culture dish, and different types can be distinguished after a few hours. Some cells appear extremely flattened and have an irregular multipolar shape; these cells are usually regarded as fibroblasts. Other cells have a marked bipolar

shape with a prominent central nucleus and scanty cytoplasm; these cells, thought to be myogenic in origin, are termed "myoblasts" (11).

The mononuclear cell population goes through a period of replication. After a time, some cells stop dividing, line up in parallel arrays, and fuse to form multinucleated cells called "myotubes." This fusion, taking 4–8 hr, occurs sooner in cultures with a higher initial cell density (12). The multinuclear myotubes are often highly branched (especially chick muscle), and the cell nuclei are in a central position. These myotube cells continue to grow and develop in culture, forming mature cross-striated muscle fibers with nuclei aligned along the cell membrane.

The fibroblasts continually divide in culture and eventually can overgrow the muscle fibers, thus making experiments difficult. After the formation of myotubes, the cultures can be treated with a DNA synthesis inhibitor, such as cytarabine (cytosine arabinoside), to remove the actively replicating fibroblasts. Since DNA synthesis in muscle ceases after cell fusion, this treatment does not hinder the development of the muscle fibers (13, 14).

The studies on skeletal muscle in culture that are dealt with in detail in this review are grouped for convenience into three separate areas: electrophysiological properties, development of chemosensitivity, and pharmacological properties. Nevertheless, there is considerable overlap among these sections.

ELECTROPHYSIOLOGY

Passive Electrophysiological Properties—In directing the techniques of conventional electrophysiology toward recording from cells in culture, a few refinements are necessary. While normal electronic amplification and glass microelectrodes of resistance 5–50 megohms may be used with ease, the nature of the test material requires a microscope, preferably of the inverted type, with phase contrast or Nomarski interference optics. Furthermore, the scale of the work dictates that the utmost stability in micromanipulators and worktop be sought.

Embryonic chick skeletal muscle fibers explanted into tissue culture retained a resting membrane potential between −60 and −75 mv (15), although Harris *et al.* (5) noted a drop from −63.8 (mean) to a mean of −51.0 mv over a 10-day culture period. No difference was noted between explants from chick anterior latissimus dorsi (slow muscle) and posterior latissimus dorsi (fast muscle) (16).

In muscle fibers developed from the fusion of disaggregated primary myoblasts obtained from chick embryos or neonatal rodents, the resting membrane potential changed from a value around −10 mv before fusion and immediately afterward, rising steadily as fiber length and diameter increased to about −50 or −60 mv (4, 6, 14, 17–20). Thereafter, little change in the resting membrane potential was observed. Higher values, between −70 and −95 mv, were reported by some workers but were less common (21–23).

The increase in membrane potential was correlated with numbers of nuclei in the fiber (4), with fiber thickness (14), and with fiber length (6, 18). Fibers of 1000 μm in length usually have attained their maximum resting membrane potential, although the morphology of multiply branched myotubes in culture presents formidable difficulties in measuring fiber length. However, Hooisma *et al.* (23) were unable to note any correlation between the resting membrane potential and the fiber age or size in their cultures.

With respect to membrane potential, the development of muscle in culture resembled closely the development followed by embryonic muscle *in situ* (24); the highest membrane potentials recorded were similar to the values reported for isolated chicken muscle (25) and rodent muscle (26–28). Human muscle, developing in culture from fetal myoblasts, showed a similar increase in membrane potential[1]. The progressive increase in the resting membrane potential was apparently achieved by changes in the ratio of the permeabilities of the principal cations, sodium and potassium. Intracellular potassium remained constant at about 140 m*M* throughout muscle development in culture. In chick muscle, intracellular sodium fell from 56 to 22 m*M* over a period when the resting membrane potential rose from −10 to −21 mv (14). Catterall (29) estimated the internal sodium concentration of 5–9-day chick myotubes to be 13 m*M*; Ritchie and Fambrough (20) reported the same value for rat myotubes, although no change during development was observed.

Both chick and rat myoblasts and myotubes of low resting membrane potential are relatively insensitive to alterations in external potassium. As the resting membrane potential increases, however, the sensitivity to changes in $[K]_0$ increases until there is a virtual linear relationship between log $[K]_0$ and membrane potential (14, 20, 30). The curves obtained with rat myotubes closely followed the theoretical line derived from the Goldman equation and derived values for the internal ion concentrations (20). This correlation was interpreted as an increase in membrane potassium permeability during development, while sodium permeability remained low. However, since the resting membrane potential never attained the level of the potassium equilibrium potential, it must be assumed that sodium permeability remains a significant factor in determining the final resting membrane potential.

Chloride distribution across the cell membrane is such that the Nernst equilibrium potential (E_{Cl}) is the same as the resting membrane potential[2]. Chloride permeability, however, appears to be low, since changes in external chloride concentration caused no alteration in membrane potential or input resistance (20). The ionic composition of the cell is maintained by a ouabain-sensitive ion pump. Electrogenic pumping is not a significant contribution to the maintenance of membrane potential, at least in chick myotubes, although it can be identified during recovery from prolonged exposure to low temperature or acetylcholine. Presumably, the increased sodium within the myotube acts as the stimulus to such electrogenic pumping.

The input resistance of muscle fibers in culture was shown to fall from relatively high values of 30–40 megohms to about 1 megohm or less as the cells increased in size (5, 18). This change in input resistance was not apparently caused by changes in the specific resistance of the membrane, which remained constant. Fischbach *et al.* (18) were

[1] A. L. Harvey and W. F. Dryden, unpublished observations.
[2] W. F. Dryden, unpublished observations.

Table I—Physical Constants of Muscle Fibers in Culture

	Specific Resistance of Membrane (R_m), ohm cm^2	Resistance of Cytoplasm (R_i), ohm cm	Membrane Time Constant (T_m), msec	Membrane Capacitance (C_m), μF/cm^2	Length Constant (λ), μm
		Steady-State Analysis			
Chick (Ref. 5)	720	—	5	—	—
Mouse (Ref. 6)	694 ± 75	109 ± 19	5.4 ± 0.9	8.4 ± 1.2	609 ± 50
Rat (5 day) (Ref. 20)	1000 ± 94	—	3.1 ± 0.1	3.0 ± 0.3	—
		Transient Analysis			
Chick (Ref. 18)	2639 ± 1166	220 ± 190	9.95 ± 5.45	3.9 ± 1.45	639 ± 299
Mouse (Ref. 6)	2089 ± 343	132 ± 41	6.9 ± 1.6	4.6 ± 2	912 ± 158

unable to demonstrate any correlation between the specific resistance of the myotube membrane and the resting membrane potential. The values obtained for the membrane constants depended on the method of analysis after passing a current of known intensity through the membrane and recording the change in electrotonic potential.

Powell and Fambrough (6), using cultures of mouse muscle where the myotubes were relatively unbranched, preferred to base their calculations on the steady state of the electrotonic potential rather than on an analysis of transient potentials, since the inconstancy of potassium conductance and possible low resistance coupling between myotubes and other cells introduced indeterminate variables. The values reported (Table I) for mouse and chick showed no significant difference, whereas there was an appreciable difference in the results obtained by each method. The values showed similarities to values quoted for adult muscle preparations, but they appeared to occupy a position intermediate between the values quoted for innervated and denervated muscle, suggesting that they can be further modified after the establishment of neural control at innervation.

In contrast to muscle fibers developing from primary myoblasts, the myogenic cell line L6 shows little developmental change in passive electrical properties during the formation of myotubes. Line L6 was first reported by Richter and Yaffe (31), and a number of reports have appeared on its electrophysiological properties. The individual myoblasts, prior to fusion, showed an unusually high resting membrane potential of between −60 and −80 mv (32–34). After fusion, no significant change in the resting membrane potential was detectable. A linear relationship existed between membrane potential and the logarithm of the external potassium concentration, suggesting that the membranes of both myoblasts and myotubes were permeable to K^+ ions. Little Na^+ or Cl^- permeability, however, was measurable.

The specific membrane resistance and capacitance for L6 myoblasts were 8 kilohms cm^2 and 1 μF/cm^2; for myotubes, the values were 12 kilohms cm^2 and 5 μF/cm^2. These values differed significantly from those reported in Table I and probably reflected the altered ion permeability found in this cell line. Myoblasts did not show delayed rectification, which was demonstrable in myotubes. Clearly, therefore, a change in the nature of ionic channels in the membrane did occur at fusion of the L6 myoblast.

Active Electrophysiological Properties—Active changes in membrane potential of muscle fibers in culture have been observed. In general, they can be classified as either action potentials or as oscillations of membrane potential, often associated with twitching or fibrillation of the fibers. The latter activity was first detected by Li *et al.* (15), who noted that a discharge of an action potential ensued in fibers developing from explants when the oscillations in membrane potential caused it to fall below a threshold of about −50 mv. The origins of these fibrillation potentials were suggested to be metabolic (35).

Although action potentials do occur spontaneously in muscle in culture (6, 18), they are seen most frequently after extraneous stimulation. A much slower depolarization, described as a plateau, sometimes with a train of spike discharges superimposed, may also be observed (15, 22). Mainly, the response of a cell to a rapid depolarization from around −70 to below −50 mv is the discharge of an action potential with overshoot. Where the resting potential of the myotubes is less than −50 mv, it is usually necessary first to hyperpolarize the cell by passing an inward current (5, 6, 18).

Unlike the passive properties, the active responses of myotubes of both primary cultures and myogenic clones differ little. Catterall and Nirenberg (36) demonstrated that in both primary chick myotubes and a cloned line of muscle, the sodium channels which open transiently during the action potential, can be activated by veratridine. This activation was antagonized by tetrodotoxin. The fast spike potentials seen in discharging cultured muscle cells can be abolished by removing external sodium or by adding tetrodotoxin to the medium (5, 22, 37). Therefore, the fast spike component is caused by an inward sodium current and resembles that found in normal vertebrate muscle and nerve. The threshold potential necessary to initiate the fast sodium spike was about −45 mv (37).

A study of the currents flowing during voltage clamp suggested that the depolarization necessary to evoke the sodium spike was greater than that necessary to initiate a sodium current. This condition was also true of the peak or reversal potential at which the sodium current ceased to be inward. These differences were ascribed to the cable properties of myotubes, which permitted membrane currents to flow in restricted areas of the membrane during voltage clamp but necessitated a greater depolarization of the membrane for a propagated action potential in an unclamped fiber. To overcome such problems, the cable properties of myotubes of indeterminate length were eliminated by exposing the cultures of 10^{-8} *M* colchicine after 3–4 days of development (38). The destruction of microtubules that this procedure caused resulted in the formation of "myosacs," nearly spherical structures rather than the normal cylindrical shape. Contractile elements were present in such structures, and membrane potentials (−48 mv), action potentials, depolarization in response to acetylcholine, and contractions induced by various agents suggested that the properties of myosacs were comparable

with those of myotubes. An extensive t-tubular system was considered as a possible cause of slight deviations from the predicted response during the initial stages of constant current hyperpolarization. The cytoplasm of these cells was at the isopotential with respect to ground, so good membrane clamping could be achieved. Under these conditions, threshold and peak potentials for both current depolarization and current flow during clamping were in close agreement.

The slow potential was more difficult to characterize. It could be absent, present alone, or present in combination with a fast tetrodotoxin-sensitive spike. Where it occurred, the slow potential was insensitive to tetrodotoxin (37, 39), its amplitude and duration were increased by elevating the concentration of calcium in the bathing medium (40), and it could be abolished by the addition of 2 m*M* manganese chloride (39) or lanthanum chloride (41) to the bath. Removal of the external sodium from the medium also abolished the slow potential (33). Barium was apparently able to substitute for calcium in the bathing solution. The conclusion was that the slow potential was attributable to an inward current of both sodium and calcium. The time course of the tetrodotoxin-resistant slow potential resembled that found in neonatal muscle and denervated muscle (42); the slow channel may be a feature of incomplete development, which is eliminated once full tropic control is established after innervation.

A third type of depolarizing response was noted by Fukuda *et al.* (37, 43), when the membrane potential of the cells was hyperpolarized by passing an appropriate current through electrodes. After the fast spike and the slow potential had been elicited by a short depolarizing pulse superimposed on the hyperpolarizing current, a prolonged depolarization was seen and lasted for several seconds. The amount of depolarization was increased when the external chloride was reduced in concentration, and substitution of potassium chloride in the current passing electrode with the more impermeant potassium acetate resulted in a decrease in amplitude and duration. It appeared, therefore, that the prolonged depolarization of the fibers was attributable to an outward flow of chloride ions supplied to the cytoplasm from the hyperpolarizing electrode, which followed a post spike increase in chloride conductance.

In myosacs (44), prolonged depolarizations of up to 10 sec were associated with a late inward current, attributed to an outflow of chloride ions. This late current and the late chloride spike previously reported were attributed to the same mechanism, which operated in response to changes in membrane potential; chloride redistributed itself during a change in conductance, which was at a maximum around −50 mv. Under normal conditions, such a change in chloride conductance would not be manifested, since active fluctuations and action potentials have a much more rapid time course.

DEVELOPMENT OF CHEMOSENSITIVITY

Acetylcholine Receptor—Much of the relevance of skeletal muscle as a pharmacological preparation hinges on the presence and properties of specialized structures associated with chemosensitivity in the intact fiber, the acetylcholine receptor, and acetylcholinesterase. A detailed review of the developmental biology of membrane cholinoceptors is contained in a recent series of publications (30, 45, 46).

The presence of sensitivity to acetylcholine in muscle in culture was confirmed by Dryden (47) by direct application of acetylcholine from a micropipet to individual fibers in culture. The response was simple contractions of the fiber, but this response was dependent on the possession by the fiber of an intact contractile mechanism. Noncontractile cells and myoblasts cannot be expected to respond mechanically, and intracellular electrophysiological methods are required to investigate fully the properties of the acetylcholine receptor in cultured muscle. The receptor was found in myoblasts insofar as an electrophysiological response could be evoked from myoblasts, especially more developed ones (4, 14), but not in myoblasts of the L6 clone (48). Instead, a slow hyperpolarization of some L6 myoblasts from −25 to −60 mv was observed after acetylcholine iontophoresis. This response was not susceptible to α-neurotoxin or tubocurarine blockade and was not comparable with the rapid hyperpolarization to the reversal potential seen in primary chick myoblasts of low membrane potential exposed to acetylcholine (14). It probably represents an interaction with a membrane component other than the conventionally recognized nicotinic receptor and serves to distinguish clone L6 myoblasts from primary cells.

The rapid rate of acetylcholine receptor formation that occurs at fusion is not apparently dependent on fusion itself, since it also occurs when fusion is prevented by lowering the calcium content of the bathing medium. Indeed, fusion appears to be incidental to the acquisition of differentiated characteristics, since myofilaments, acetylcholinesterase activity, and adenylate cyclase activity all were greatly increased at the time of normal fusion (4, 49). A functioning receptor was not apparently required for fusion of myotubes, since this process proceeded in the presence of tubocurarine, acetylcholine (4), or α-bungarotoxin (50).

The use of ^{125}I-labeled α-bungarotoxin has greatly aided research into receptor distribution and incorporation in view of the virtual irreversibility of the reaction between the two. Autoradiographic investigation of cells and fibers thus labeled confirmed the presence of receptors on the occasional myoblast; on young myotubes, the label appeared to be uniformly distributed over the surface (51, 52), corresponding to a receptor density between 1500 and 2000/μm^2 (53). The uniformity of receptor distribution was a feature only of young myotubes; as development proceeded, clusters of greater receptor density were noted with increasing frequency. The average cluster was 125 μm^2 in area and ovoid and occurred predominantly at the fiber margin. No correlation between the occurrence of clusters and cytological features of the fibers could be found (52, 53).

Electrophysiological confirmation of nonuniform sensitivity along the muscle fibers was provided by a number of reports. Initially, fibers when formed exhibited uniform sensitivity to the transmitter along their length (5, 14, 54). The iontophoretic sensitivities were between 4 and 400 mv/nC. Close application of acetylcholine from an iontophoretic pipet revealed variations in sensitivity ranging from 10 to 1000 mv/nC (55). The areas of greatest sensitivity coincided with the areas of greatest density of

^{125}I-labeled α-bungarotoxin. Furthermore, the areas of greatest sensitivity were thought to be associated with prominent muscle nuclei, although no great significance has been attached to this observation. Such "hot spots" of acetylcholine sensitivity also were noted in myosacs (38). Fetal skeletal muscle prior to innervation, denervated muscle, and adult muscle maintained in organ culture were all sensitive to acetylcholine over the whole muscle surface (56–58), although the sensitivity was not necessarily uniform. The original end-plate membrane retained a much higher receptor density than surrounding areas (59, 60). The muscle in cell culture, therefore, had properties similar to other muscle preparations devoid of nervous control.

The accumulation of receptors, at least at points of innervation in nerve–clonal muscle cultures, was not inhibited by blockade of muscle activity by long-term depolarization in a high K^+ growth medium (61) or by exposure to cobra α-neurotoxin (62). However, the overall sensitivity of muscle fibers was lower if they were repeatedly stimulated by field current depolarization during development (63). The mechanism relating electrical properties of the membrane and receptor incorporation remains to be demonstrated.

The dynamic aspects of receptor biosynthesis and degradation have been studied. Work with fluorescent labeled antimuscle antibody revealed a fair degree of mobility in the surface molecules of membranes of cells in culture (64). It was supposed that the acetylcholine receptor in the absence of innervation was no exception and that a fluid to rigid transformation occurred at synaptogenesis to provide the lattice of receptors found in normal adult end-plates (30).

The binding of ^{125}I-α-bungarotoxin to myotubes was biphasic with a rapid and a slow component (53). The slow component was attributed to the appearance of new receptors, since it could be abolished by inhibitors of RNA synthesis such as cycloheximide and dactinomycin. There was, however, a delay in the reduction in the appearance of new receptors after inhibitor treatment, which suggested that there existed within the myotube a pool of newly synthesized receptors available for incorporation into the membrane. The rate of new receptor incorporation was 90 receptors/μm^2/hr, although the increase in density was only 35/μm^2/hr. This difference was explained by the fact that the surface area in the developing myotubes was also increasing by about 4–5%/hr.

Further work (65) revealed a proteolytic degradation of receptors which required energy and was not affected by prior binding of ^{125}I-α-bungarotoxin. The degradation of the receptors followed first-order kinetics and was, therefore, presumably random. The half-life of receptors in both chick and rat myotubes was about 22 hr. Proteolysis was apparently a cytoplasmic event requiring prior internalization of the receptor. Whether this sequence of events occurs in innervated muscle, which is susceptible to the trophic regulation of the neuron, is a question of considerable interest.

The interactions of receptors with both agonist and antagonist drugs are considered in the following section, but the ionic parameters associated with the activation of the receptor in cultured muscle are discussed here. In myotubes, including clone L6 myotubes (48), exposure of the cell to cholinomimetic agonists resulted in a rapid depolarization of the cell membrane as in "normal" muscle. The action of carbachol was associated with a substantial increase in the rate of sodium entry into a fiber (29). The time course of this entry was not constant throughout equilibration in the presence of carbachol but fell after an initial rapid influx. The fall in influx was attributed to desensitization of the receptors by the agonist. By reducing the temperature to 2°, the rapid initial sodium influx could be studied. The maximum influx (V_{max}) under these conditions was 2×10^7 min^{-1} ions/ionophore. This value compared well with the value of $4–12 \times 10^8$ min^{-1} at 35° computed from the membrane noise measurements of Sachs and Lecar (66) in cultured muscle. Their value for the elementary conductance of a single channel was 0.8×10^{-10} mho which was close to values obtained in frog sartorius muscle (67, 68).

When activated by a cholinomimetic agonist drug, the acetylcholine receptor in cultured muscle fibers apparently opens a sodium permeable ionophore in a manner similar, if not identical, to that taking place in the normal muscle end-plate. Within the ion channel, there is apparently a sodium binding site or energy barrier, possibly analogous to those proposed for the transient tetrodotoxin-sensitive sodium channels of nerve (69).

More recent work (70) on acetylcholine current noise in cultured muscle cells that had fused but had been prevented from elongating (myosacs or "myoballs") showed reasonable agreement with Sachs and Lecar (single-channel conductance = 0.3×10^{-10} mho) and provided evidence that the conformational freedom of the acetylcholine channel is limited by the fluidity of membrane lipids. Since these membrane lipids undergo a phase transition to a more rigid state at about 20° in homeothermic animals, the degree of channel opening is restricted below this temperature. The channel is not apparently specific for sodium, since potassium permeability too is increased on activation (and also trivalent ions, if present) but chloride remains impermeant (71). The behavior of the two permeant ions is interesting in that potential changes attributable to potassium can be predicted from the Goldman equation, *i.e.*, ion flow is through channels of fixed conductance and dependent on ion concentration on each side of the membrane. Sodium-dependent potential changes are described by:

$$E_r = E_K + \frac{(\Delta g_{Na}/\Delta g_K)E_{Na}}{1 + \Delta g_{Na}/\Delta g_K} \qquad \text{(Eq. 1)}$$

where the conductance of the channels is variable. The precise mechanism of ion transfer through the ionophore is clearly complex and awaits further work.

The reversal potential of the activated receptor was described as from 0 to +10 mv for chick embryo muscle explants (5), between −20 and +10 mv (55) and −7 mv (14) in dissociated chick embryo muscle cultures, from +5 to +10 mv in dissociated rat muscle cultures (4), and −2.5 mv in clone L6 myotubes (48). Ritchie and Fambrough (71) reported a mean reversal potential of −2.6 mv, which remained constant despite the increase in the resting membrane potential (30). This result was obtained using rat tissue, which the same authors had shown to retain an unchanged internal ion composition during this period (20). Where the internal sodium concentration fell, a change in reversal potential was expected. The reversal

potential of L6 myoblasts was the same at 22° as at 35° (48). Reduction in $[K]_0$ or $[Na]_0$ caused the reversal potential to be more negative, reversing at between −30 and −40 mv in sodium-free solution. Chloride movement was not apparently involved in determining this value, so suspicion fell on Ca^{2+} as a possible participant in the receptor activated current. No experimental evidence was available, however, to substantiate this view.

Acetylcholinesterase—The enzyme responsible for the hydrolysis of acetylcholine is found in close association with the cholinoceptor in intact muscle, and similar activity was found in muscle in culture (72, 73). Choline acetyltransferase activity was predictably absent in cultures of muscle cells (74). Although acetylcholinesterase was present in myoblasts, a substantial increase in enzymic activity occurred at the time of fusion (75–77). This association of activity and myoblast fusion was also noted for a number of other enzymes (78). The cholinesterase was specific for acetylcholine, since butyrylcholinesterase activity remained at a low level throughout the culture period (76, 77). However, a number of different isozymic forms were present, comparable with those found in embryonic muscle *in situ*.

Harvey and Dryden (77) identified soluble and membrane-bound fractions of total activity and showed that the soluble activity reached a plateau after 4 days of culture while the membrane-bound fraction continued to increase. Little of the enzyme activity was found at the cell surface, most being associated with intracellular membranes (79). The distribution of the enzyme in the myotube showed developmental changes reminiscent of those noted with the cholinoceptor. Enzyme could be histochemically demonstrated in a uniform distribution in the cytoplasm of young myotubes; but, as the cultures aged, the enzyme was apparently localized into areas of greater activity than the surrounding cytoplasm. No correlation between the distribution of receptors and enzyme activity has been shown, and the factors that influence this development remain a matter of speculation.

The sequence of enzyme synthesis in 14-day myotubes was studied by Wilson and Walker (80) after inhibition of existing enzyme with isoflurophate (diisopropyl fluorophosphate). Within 4 hr, 78% of the original activity was restored. The newly synthesized enzyme was of low molecular weight and was not stained by traditional cytochemical methods. The low molecular weight isozymes were readily released from the cell and could be detected in the medium after 4 hr. About this time, the first cytochemically identifiable enzyme appeared around the myotube nuclei. This enzyme was associated with the polymerization of the low molecular weight isozymes and their incorporation into membranous structures. The process continued with a reduction in the proportion of the low molecular weight form present.

The physiological function of acetylcholinesterase in cultured muscle is not obvious. Pharmacological studies, discussed in the following section, suggest that the developmental process in cultures of muscle alone is incomplete.

PHARMACOLOGICAL PROPERTIES

Receptor Specificity—The close similarity of some physiological properties of skeletal muscle in culture to muscle *in vivo* led to the assumption that the pharmacological characteristics would also be similar. Indeed, the response of cultured muscle to acetylcholine was blocked by tubocurarine (4, 47, 54). In the last few years, more detailed studies have shown the faith of muscle physiologists to be justified.

Obata (81), Steinbach (48), and Harvey and Dryden (82) considered the possibility that the development of muscle in the artificial environment of culture may cause the appearance of unusual receptor types. Obata tested cell cultures prepared from the diaphragms of 1-day-old rats. Depolarization responses were obtained with acetylcholine added iontophoretically or directly to the bath, but levarterenol, γ-aminobutyric acid, and glutamic acid had no effect on membrane potential. Steinbach applied methacholine, choline, and levarterenol iontophoretically to L6 myotubes without success. Harvey and Dryden tested a series of compounds (epinephrine, levarterenol, histamine, 5-hydroxytryptamine, dopamine, and γ-aminobutyric acid) for agonist and antagonist activities on cultured chick embryonic muscle. None of these substances produced a significant change in membrane potential. However, epinephrine (but not levarterenol) and 5-hydroxytryptamine reduced responses to the test agonist, carbachol; the mechanism of this depressant activity is unknown and requires further investigation.

Three muscarinic receptor stimulants, methacholine, bethanechol, and pilocarpine, were tested on cultures of chick embryo leg muscle (14, 80). Since no response could be obtained, it is likely that skeletal muscle in culture does not have muscarinic receptor sites.

All nicotinic cholinoceptor stimulant drugs that have been tested on skeletal muscle in culture produced a response. The drugs used included acetylcholine, carbachol (83), decamethonium, tetramethylammonium, nicotine, succinylcholine, and dimethylphenylpiperazinium (82).

From the results of such studies, it is concluded that skeletal muscle in culture responds to agonists that normally activate nicotinic cholinoceptors at the neuromuscular junction but not to agonists that do not normally act on skeletal muscle.

The reversal potential of the acetylcholine response in cultured muscle is between +10 and −20 mv. Feltz and Mallart (84) reported that the reversal potential in junctional areas of frog skeletal muscle was −16 mv, whereas it was −42 mv in extrajunctional areas. This finding suggested that the receptors in muscle in culture behave in a manner more closely resembling junctional rather than extrajunctional cholinoceptors.

The specificity of receptors on cultured skeletal muscle was examined using antagonist drugs. Muscarinic receptor antagonists in high concentrations blocked the response to acetylcholine of normal rat and frog skeletal muscle preparations (85, 86). Similar results were found with cultured muscle. A low concentration of atropine was without effect on responses of cultured skeletal muscle to acetylcholine (81), but responses were inhibited by concentrations of 10^{-4}–10^{-2} *M* atropine (5, 29, 55, 82) and hyoscine (82). Similarly, high concentrations of atropine inhibited end-plate potentials in combined chick muscle–ciliary ganglia cultures (23). Acetylcholine responses of primary mouse cultures and of rat cell line cultures were blocked by both tubocurarine and atropine (40).

All of these studies indicated that the concentrations of atropine have to be 100–1000 times greater than the effective concentrations of tubocurarine. A similar ratio was seen in other skeletal muscle preparations (85, 86).

The reduction of acetylcholine-induced responses by the nicotinic antagonist tubocurarine was demonstrated in several culture systems (13, 47, 48, 53, 81, 83). The concentration–effect curves to carbachol were shifted in a parallel fashion to the right by increasing concentrations of tubocurarine. Similar results were obtained with other nicotinic antagonists, *e.g.*, gallamine (82) and chandonium iodide (87). Affinity constants were calculated to be around 10^7 for tubocurarine, about $2–5 \times 10^6$ for gallamine, and between 10^7 and 10^8 for chandonium iodide. From the half-blocking concentrations of tubocurarine on miniature end-plate potentials in combined spinal cord–rat muscle cell line cultures, the affinity constant would be 0.25×10^7 (88). These values were of the same order as reported from experiments on other more conventional skeletal muscle preparations.

Acetylcholine-induced depolarization can be antagonized by neurotoxins from snake venoms. Such toxins were reported to be highly specific for nicotinic receptors in skeletal muscle preparations (89). Cobra neurotoxin blocked responses in a rat cell line (90) and in explants of chick embryo skeletal muscle (5); α-bungarotoxin blocked responses in primary cultures of rat (53, 81) and chick muscle (55, 91, 92). Dose–response curves to carbachol in the presence of increasing concentrations of α-bungarotoxin showed a reduction in maximal response, as would be expected with a noncompetitive antagonist. Blockade with α-bungarotoxin was essentially irreversible (53, 91). Moreover, β-type bungarotoxins that are selective prejunctional antagonists (93) were without effect on skeletal muscle fibers in nerve-free cultures (92).

The results of experiments with antagonists further substantiated the postulate that cultured skeletal muscle fibers possess one main receptor type that resembles the nicotinic cholinoceptor. There is evidence that the receptor in denervated skeletal muscle has different affinities for antagonists from those of the receptor at the normal motor end-plate (85, 94). While it was suggested (40) that the receptors on cultured muscle resembled more closely those of denervated muscle, available evidence does not support this proposal.

The nicotinic cholinoceptor was suggested to be a protein with an easily reducible disulfide bond near the acetylcholine binding site, and reduction of the disulfide bond was thought to alter the conformation of the receptor protein and its specificity (95). Treatment of electroplax with the disulfide bond reducing agent, 1,4-dithiothreitol, decreased the effects of carbachol and acetylcholine and converted the action of hexamethonium from that of an antagonist to that of an agonist; these changes could be reversed by oxidation (96, 97). Similar results were obtained with several skeletal muscle preparations including frog rectus abdominis (98) and sartorius muscles (99–101), denervated rat skeletal muscle (102), chick biventer cervicis muscle (103), and leech dorsal muscle (104). Despite some species variation (101, 104), 1,4-dithiothreitol appeared to affect the properties of the nicotinic receptor in skeletal muscle.

Harvey and Dryden (105) examined the effects of 1,4-dithiothreitol treatment on chick embryo skeletal muscle grown in cell culture and compared the behavior of the receptors present in cultured muscle to that of the receptors in adult muscle. The receptors on cultured chick embryonic skeletal muscle underwent the characteristic changes in properties following disulfide bond reduction. The concentrations of 1,4-dithiothreitol and exposure times required to produce these effects were similar to the conditions employed in other studies. The changes after 1,4-dithiothreitol (decrease in response to carbachol and conversion of the action of hexamethonium from antagonist to agonist) were as readily reversed by oxidation in cultured muscle as in other systems (96, 97, 103). Membrane depolarization by K^+ ions was not affected by 1,4-dithiothreitol treatment, suggesting that the action of 1,4-dithiothreitol is related to the receptor itself rather than to a nonspecific membrane effect. The actions of 1,4-dithiothreitol were also distinguished from receptor desensitization effects. Harvey and Dryden (105) concluded that the receptors in cultured aneural muscle and in normal muscle were affected similarly by disulfide bond reduction.

Effects of Anticholinesterase Drugs—At the adult neuromuscular junction, the transmitter action of acetylcholine is terminated by hydrolysis by the enzyme acetylcholinesterase. The activity of this enzyme can be affected by anticholinesterase drugs (106). The actions of acetylcholine are generally potentiated and prolonged after administration of an anticholinesterase. The actions of anticholinesterases have been tested on responses to acetylcholine applied directly to cultured muscle cells and on cholinergic end-plate potentials found in combined cultures of nerve and muscle. Physostigmine or neostigmine in concentrations between 0.1 and 100 μg/ml did not affect the size or time course of end-plate potentials in cultures of chick embryo muscle and spinal cord (13, 21). Physostigmine (5–20 μg/ml) did not increase the duration of end-plate potentials in combined cultures of chick embryo muscle and ciliary ganglia; however, 20 μg of physostigmine/ml decreased the amplitude of the end-plate potentials (23). Edrophonium (3.8×10^{-6}–10^{-4} M) did not increase amplitude, time to peak, or half decay time of miniature end-plate potentials recorded in cultures of the muscle cell line L6 and fetal rat spinal cord (88).

Physostigmine and edrophonium did not potentiate the response of cultured chick embryo muscle to iontophoretically applied acetylcholine, and high concentrations of both anticholinesterase drugs inhibited the response to acetylcholine (5, 79). The response of cultured chick embryo muscle to bath application of 10^{-6} M acetylcholine was not increased in the presence of 0.5 μg of neostigmine or physostigmine/ml; the responses to 10^{-3} M acetylcholine and carbachol were inhibited slightly (83). Higher concentrations of anticholinesterase produced depolarization and reduced subsequent acetylcholine responses.

An extension of this study showed that concentration–effect curves to acetylcholine were not altered by 10^{-8}–10^{-7} M physostigmine or neostigmine or by pretreatment of the cultures with 5.4×10^{-6} M of the irreversible anticholinesterase isoflurophate (107).

Although anticholinesterase treatment potentiates the action of acetylcholine in normal muscle, it has little effect on chronically denervated muscle (57). In this respect,

muscle in culture is similar to denervated muscle. A recent electron microscope study of the subcellular distribution of cholinesterase in cultured muscle indicated that little enzyme is on the surface membrane, most being located in the cytoplasm, over myofilaments, or associated with internal membranes (79). The localization of acetylcholinesterase on the external surface of the muscle and the assumption of its physiological function probably occur later in development and are regulated by a "trophic" influence from nerves. Presumably, the lack of effect of anticholinesterases on end-plate potentials in combined nerve and muscle cultures is due to the immaturity of the functional contacts.

Direct effects of the anticholinesterases on membrane potential have also been tested. At concentrations above 10^{-7} *M*, both neostigmine and physostigmine produced a concentration-dependent depolarization of myotubes (107). The depolarization induced by neostigmine, but not that by physostigmine, was inhibited by tubocurarine (107). Isoflurophate at 5.4×10^{-6} *M* had no effect on membrane potential but reduced responses to both acetylcholine and carbachol (107). It was postulated that the antagonism by isoflurophate and the inhibitory actions of high concentrations of other anticholinesterases on acetylcholine responses in culture (5, 79, 83) may be caused by receptor desensitization induced by the anticholinesterase.

Owing to its lack of physiologically functional acetylcholinesterase, nerve-free muscle cultures were the preparation of choice in a study of the site of action of nicotinic agonists (108). It had been postulated that certain agonists other than acetylcholine act at the neuromuscular junction by releasing endogenous acetylcholine (109). This postulate was based on the findings that triethylcholine, tetraethylammonium, and hexamethonium, drugs assumed to block choline and cholinomimetic uptake by the nerve terminal, inhibited responses to carbachol, nicotine, tetramethylammonium, decamethonium, and dimethylphenylpiperazinium but not to acetylcholine (109, 110). However, these effects may result from a combination of two postjunctional actions of these drugs: a curare-like blocking action and an anticholinesterase action (111).

When tested in cultured skeletal muscle at concentrations used previously to eliminate neural acetylcholine release (109, 110, 112), hemicholinium, triethylcholine, tetraethylammonium, and hexamethonium blocked responses to all agonists, and acetylcholine responses were affected as much as those of other cholinomimetics (108). This finding confirms previous work (111, 113) and lends support to the belief that the lack of effect of these blocking drugs on acetylcholine responses in whole muscle was because of their anticholinesterase activity. The results obtained with cultured muscle support the classical theory that nicotinic agonists act directly on receptors on the muscle itself and not by releasing endogenous acetylcholine.

Receptor Desensitization—In many skeletal muscle preparations, cholinomimetic-induced depolarization is not maintained despite a constant drug concentration. This phenomenon, termed "desensitization" (114), is equivalent to a progressive decrease of the response during prolonged contact with cholinomimetics. Desensitization in skeletal muscle has several characteristic features: it is specific for cholinoceptors, it increases with agonist concentration and contact time, and it is reversible.

In explant cultures of chick embryo muscle, the response to iontophoretic pulses of acetylcholine declined if the frequency of stimulation was above 6/min (5). Since the responses did not recover fully after rest, the decline in sensitivity may have represented desensitization or a gradual deterioration of the preparation. Desensitization was, however, evident in rat muscle cultures when acetylcholine pulses were given during a maintained acetylcholine-induced depolarization (81). A similar phenomenon occurred with repeated or prolonged application of acetylcholine or carbachol to L6 myotubes (48). The responses to the brief acetylcholine pulses recovered to control level after the desensitizing exposure was stopped.

The response of cultured chick embryo muscle to prolonged exposure to acetylcholine and carbachol was not maintained, and the membrane gradually repolarized (83). This fading response was suggested to reflect desensitization, and evidence was presented that the desensitization affected cholinoceptors specifically and depended on the concentration of the agonist. Cross-desensitization between acetylcholine and carbachol was also reported. Desensitization in cultured chick embryo muscle was reversible, although the recovery rate was much slower than the recovery rate of other muscle preparations (83). Recovery of cultured muscle appeared to be a two-component process, with an initial phase of rapid recovery followed by a very slow recovery rate (115).

Ritchie and Fambrough (20) found a marked difference in the response of rat and chick myotubes to prolonged application of acetylcholine. Chick myotubes repolarized rapidly in the presence of 10^{-5} *M* acetylcholine, but the depolarization of rat myotubes could be maintained for as long as 100 min. The reason for this difference is unknown.

Desensitization was reported to be more pronounced in older cultures (115). Catterall (29) thought that desensitization increased with the morphological maturity of the myotubes, being more rapid in myotubes possessing cross-striations and hypolemmal nuclei. However, it is possible that the increased rate of desensitization reflects the increased resting membrane potential of the more developed cells (116).

Different agonists have differing abilities to produce desensitization in cultured chick embryo muscle. Concentrations of drugs that produced the same depolarization had markedly different rates of repolarization (117). Succinylcholine produced rapid desensitization, while nicotine caused slow desensitization; acetylcholine and carbachol were intermediate in activity.

Certain drugs increased the rate of repolarization in the presence of a cholinomimetic. Isoflurophate (107), atropine, and hyoscine (115) produced this effect, which is equivalent to an increase in rate of desensitization. Isoflurophate increased the rate of decay of the end-plate current of frog sciatic nerve–sartorius muscle preparations (118). Isoflurophate was thought to be affecting the receptor–ionic conductance modulator complex. Atropine shortened the time that an acetylcholine molecule opened the ionic channels in the muscle membrane (119). This action seems to indicate an enhancement by atropine of desensitization by acetylcholine. The results obtained with

cultured muscle suggest that these drugs (isoflurophate, atropine, and hyoscine) may operate similar mechanisms in cultured muscle fibers.

Desensitization protected cholinoceptors of frog sartorius muscle preparations from binding of snake venom neurotoxins (120, 121). In a similar study with cultured chick embryo muscle, α-bungarotoxin was ineffective in blocking agonist responses after receptor desensitization (122). These results provide further evidence that the nicotinic receptors present in cultured skeletal muscle are similar in properties to the receptors of normal muscle. The finding that desensitization prevents α-bungarotoxin from blocking receptors suggests that desensitization involves a change in the conformation of the receptor so that the toxin-binding sites are no longer operational.

Recent studies showed that binding of labeled snake venom toxins to cultured muscle was reduced in the presence of cholinomimetics (51, 90, 123). Desensitization, rather than physical occlusion, of receptor sites may account for some part of this protective effect.

CONCLUSION

Much has been written on the suitability of some cells in culture for pharmacological investigation. The evidence presented in this review supports the inclusion of skeletal muscle in this category. Most of the pharmacology so far investigated involved electrophysiological changes occurring at the sarcolemma. T-Tubule and sarcoplasmic reticulum sensitivities have not yet been investigated, nor have phenomena involving the contractile element itself. There are formidable technical problems in measuring tension generated by single muscle fibers, and little additional information would be added to existing information achieved using conventional methods.

Yet the increasing availability of sophisticated techniques involving ion-sensitive electrodes, fluorescent probes, and other physical and physicochemical tools will undoubtedly add cell culture to the list of preparations available for the study of all aspects of pharmacology in muscle. The development of membrane noise analysis together with the easy availability of receptor sites gives promise of answers to some fundamental questions concerning the reaction of a drug molecule with a single receptor.

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