J L P Gubrandy

ELECTROPHYSIOLOGICAL CHARACTERIZATION OF STRIATED MUSCLE CELLS INNERVATED BY CILIARY NEURONS IN TISSUE CULTURE

STELLINGEN

behorende bij het proefschrift:

Electrophysiological characterization of striated muscle cells innervated by ciliary neurons in tissue culture

I

De gevonden afwezigheid van "hot spots" in niet-geïnnerveerde spiervezels in weefselkweek toont de noodzaak aan speciale fenomenen, die optreden in weefselkweek, te verifiëren onder de in eigen laboratorium gebruikte kweekcondities.

Dit proefschrift: Hoofdstuk 4.

II

De door Engelhart et al. toegepaste selectie voor bruikbare spiervezels (1-2 mm lengte) is niet in overeenstemming met de door hen toegepaste lange kabeltheorie.

Engelhart et al.: Brain Res., 110, 170-174, 1976.

III

Powell en Fambrough berekenen de spatiële dempingsconstante λ van spiervezels in weefselkweek onder andere aan de hand van de "off-transient response". Analyse met behulp van de gewichtsfactoren in het door hen gegeven voorbeeld (fig. 8) demonstreert de onbetrouwbaarheid van de methode.

Powell en Fambrough: J. Cell. Physiol., 82, 21-38, 1973.

IV

Meervoudige innervatie van dwarsgestreepte spieren treedt in weefselkweek veelvuldig op.

Dit proefschrift: Hoofdstuk 3 en 5.

V

Universele toepassing van het door Röckemann ontwikkelde systeem om de stroomsterkte in kleine bloedvaten te meten is twijfelachtig, daar geen rekening gehouden wordt met het optreden van omkeer van stromingsrichting in de vaten.

Röckemann en Plesse: Bibl. Anat., 11, 50-54, 1973.
Delingat: Leitz Mitteilungen, 6, 249-257, 1976.

VI

Het niveau van intracellulaire enzymen in het plasma na een hartinfarct kan niet gebruikt worden om de grootte van het hartinfarct in een vroeg stadium met enige nauwkeurigheid vast te stellen.

Witteveen et al.: Br. Heart J., 37, 795-803, 1975.

VII

De tredmolen belastingstest als evaluatie voor conditie schept slechts de mogelijkheid om uitspraken te doen over conditie voor bepaalde sporten.

Roberts en Alspough: Med. Sci. Sports, 4, 6-10, 1972.

VIII

Fietsergometer-belastingstests waarbij constante pedaalfrequentie vereist is, zoals bijvoorbeeld noodzakelijk met een fietsergometer volgens het principe van Von Döbeln, moeten bij hogere belastingen veeleer gedaan worden bij 80 toeren per minuut dan bij de gebruikelijke 50 of 60 toeren per minuut.

Binkhorst en Vos: Geneeskunde en Sport, 7, 27-34, 1974.

IX

Trendanalyse van de 10 km tijdens een grote schaatsvierkamp leidt tot een betere voorspelling van het eindresultaat dan het hanteren van een zogenaamd vlak schema.

X

Het is lovenswaardig dat gewaarschuwd wordt tegen het onoordeelkundig gebruik van spiritus bij de aanmaak van barbecues. Het zou beter zijn wanneer een goed alternatief geboden werd.

D.W. Slaaf

11 oktober 1977

ELECTROPHYSIOLOGICAL CHARACTERIZATION OF STRIATED MUSCLE CELLS INNERVATED BY CILIARY NEURONS IN TISSUE CULTURE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE UTRECHT, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. A. VERHOEFF, VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP DINSDAG 11 OKTOBER 1977 DES NAMIDDAGS TE 4.15 UUR

DOOR

DIRK WILLEM SLAAF

GEBOREN OP 15 SEPTEMBER 1945 TE ROTTERDAM

Druk: Krips Repro – Meppel

PROMOTOR: PROF. DR. D. DE WIED

DIT PROEFSCHRIFT KWAM TOT STAND IN HET MEDISCH BIOLOGISCH LABORATORIUM TNO TE RIJSWIJK
ONDER SUPERVISIE VAN DR. W. F. STEVENS EN DR. E. MEETER

Aan mijn ouders

Jeannette

Eelco

Saskia

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Chapter 1

1. Introduction

The neuromuscular junction is the site at which the voluntary nervous system controls its target, the skeletal muscle. Injury, disease and intoxication can impair the function of the neuromuscular junction and in this way have grave consequences for the organism as a whole. The acute effects of organophosphorus compounds on the neuromuscular transmission have been studied for many years in the Medical Biological Laboratory TNO. A few years ago, however, it was decided to investigate also the effects of injury and chemical compounds on the development and the function of motoneurons, muscle cells and their junctions in more chronic studies. An increase in fundamental knowledge of the process of innervation and of the chronic effects of denervation was considered essential for improving the insight in the processes causing neuromuscular disorders.

The investigations described in this thesis have been designed to study the formation of the neuromuscular junction in tissue culture. In the late nineteen sixties a few research groups had shown that in tissue culture neuromuscular junctions can be formed which are in many ways similar to those encountered in vivo (chapter 2). Tissue culture offers the advantage that the observer is able to follow the development of the contact between nerve axons and muscle cells throughout various stages. In tissue culture most cells are readily visualized in a two-dimensional system and specific membrane parameters can be estimated at any time. The formation of the junctions can be influenced by pharmacological and biochemical agents at known concentrations in a reasonably well-defined medium in the absence of homeostatic processes. A disadvantage is of course the absence of the normal cytoarchitecture and the usual cellular interactions.

In 1971, a program in cellular neurobiology was started in the Medical Biological Laboratory TNO by Dr. W.F. Stevens and Dr. E. Meeter

with the aim of studying the properties of neurons in tissue culture and the effects of neurohormones and chemical compounds on these properties. Two sources of neurons were used: the spinal ganglion and the ciliary ganglion. The spinal ganglion was chosen because a neurohormone ('nerve growth factor') had already been identified, that influences the growth of the neurons in this preparation. The ciliary ganglion was chosen because it contains exclusively cholinergic neurons and might be suitable for the detection of a cholinergic growth factor. In the meantime Fischbach published his exciting intracullular recordings showing that the neuromuscular junctions in tissue culture are electrophysiologically functional. In our laboratory research was undertaken to see whether ciliary ganglia or ciliary ganglion neurons were able to innervate skeletal muscle cells in tissue culture. In a parallel investigation, J. Hooisma started a study of the innervation process in mixed cultures of muscle cells together with dissociated cells or tissue slices obtained from the spinal cord. Advantages of the ciliary ganglion are that it contains only two classes of neurons instead of the unknown number of different neurons present in other neuronal tissues. Furthermore, due to the work of Pilar and co-workers (chapter 3) much is known about the ciliary ganglion in vivo.

In the department of neuropharmacology of the Medical Biological Laboratory TNO experience was available on the neuromuscular junction in vivo and in isolated preparations (Drs. E. Meeter and O.L. Wolthuis) and on the acetylcholine metabolism in central synapses (Dr. R.L. Polak). In other departments expert advice was available on biochemistry (Dr. F. Berends) and cellbiology (Drs. H. van Someren and A. Westerveld). Experience in intracellular electrophysiological techniques was brought in by the author, who had previously studied the passive membrane properties of human epidermis cells with Dr. J. Siegenbeek van Heukelom at the State University of Utrecht.

For the intracellular recording from the cells in tissue culture, it was necessary to construct a vibration-free table, make satisfacto-

ry microelectrodes and design a piezoelectric electrode driving device with which the cells could be impaled in a reproducible way. For the study of the acetylcholine sensitivity of the muscle cells the method of acetylcholine micro-iontophoresis had to be adapted to cells in tissue culture. It appeared that single ciliary ganglion neurons were difficult to cultivate and therefore the explanted ciliary ganglion or fragments of this ganglion were generally used as the source of the neuronal cells.

The innervation of chick skeletal muscle cells by chick ciliary ganglia in tissue culture was demonstrated for the first time in this laboratory and many properties of neuromuscular junctions have been studied in this preparation such as the nature of the end plate receptors and the generation of end plate potentials (epp's). By use of a blockade of the nerve impulse conduction with tetrodotoxin, it was possible to distinguish between epp's generated spontaneously at the junction and those evoked by action of the ciliary ganglion neurons. In addition, the effect of a change in the ratio of magnesium and calcium ions in the perfusion medium on the amplitude of the epp's was studied (chapter 3). The acetylcholine sensitivity of rather large areas of cell membrane of muscle fibers was determined by use of acetylcholine micro-iontophoresis, in order to localize areas of high sensitivity induced by innervation and to measure the acetylcholine sensitivity intrinsic to muscle membranes (chapter 4). Rabbit ciliary ganglia from new born rabbits have also been cultured together with chick muscle cells (chapter 5). In vivo the chick and the rabbit ciliary ganglia differ in the type of synapses they form with muscle cells. In the chick these junctions are much more readily blocked by d-tubocurarine than by atropine, whereas those in the rabbit are more readily blocked by atropine. The question arose as to what would happen if both ganglia innervate the same type of muscle cells in tissue culture. This experiment could help in determining which properties of the neuromuscular junctions are imposed by the neuronal cells and which by the innervated muscle cells. It was found that the muscle cells determine the nature of the acetylcholine receptors in the neuromuscular junction.

Chapter 2

2. Review of litterature on the formation of nerve-muscle contacts in tissue culture

2.1. History

Within the past decade there has been an increasing number of investigators that have demonstrated synaptogenesis in cultures of nerve and muscle cells. It is now accepted that neuromuscular junctions can be formed in vitro and that the junctions formed de novo are in many aspects remarkably similar to those formed in vivo.

Historically, the studies of Harrison (1907) with frog embryonic tissue probably provided the first demonstration of formation of neuromuscular junctions in vitro. 'The immediate object of the experiments was to obtain a method by which the end of a growing nerve could be brought under direct observation while alive, in order that a correct conception might be had, regarding what takes place as the fiber extends during embryonic development from the nerve center out to the periphery'. The results were fascinating. 'When portions of myotomes are left attached to a piece of the medullary cord the muscle fibers which develop will, after two or three days, exhibit frequent contractions'. The nerve endings moved so rapidly that it was difficult to draw the details accurately. 'Still more instructive are the cases in which the fiber is brought under observation before it has completed its growth. Then it is found that the end is very active and that its movement results in the drawing out and lengthening of the fiber to which it is attached. One fiber was observed to lengthen about 20 µm in 25 minutes, another over 25 µm in 50 minutes. The longest fibers observed were 0.2 mm in length'.

Szepsenwol (1941) found 'that under certain conditions explants of skeletal muscle of young chick and rat embryos may undergo normal differentiation and start to contract rythmically'. In 1946 the same author combined extracellular recording with microscopic observation, and reported: 'The distance between the electrodes and the explant,

together with their orientation, seems to be of importance in the amplitude of the spikes. These are higher when the electrodes are closer to the explant and are oriented to the direction of the muscle fibers'. Szepsenwol (1947) also applied electrical stimuli to the explants. 'The explants were stimulated by condenser discharge shocks of a time constant of 0.1 to 0.2 milliseconds, from a thyratron controlled apparatus'. 'The strength of the stimuli used in the present experiments varies from less than 1 to 70 volts, which is the maximum voltage provided by the apparatus'. Szepsenwol reached his conclusions about prolonged differentiation and excitability after observing the patterns of contraction of innervated and noninnervated muscle fibers and the response to pharmacological agents. However, in view of the fact that muscle in vitro frequently contracts spontaneously without any nerve present, his interpretations, based solely on visual observations of muscle contraction, needed confirmation by the recording of electrical potentials form synapses.

2.2. Culture systems

Only recently experiments have been carried out which conclusively demonstrate the formation of functioning synapses in tissue culture. This evidence has been obtained by combining histology, electron microscopy and electrophysiology. Four types of tissue culture techniques were used: 1. organ culture of whole cross sections of embryos; 2. organ culture of explants coupled in vitro; 3. cell cultures of muscle cells combined with explants of spinal cord or other neuronal explants, and 4. cell culture of muscle cells combined with neurons obtained by dissociation of nerve tissues.

2.2.1. Organ culture of whole cross sections of embryos

Harrison's (1907) original paper on the developing nerve fiber in cultured frog embryonic tissue and Szepsenwol's (1946, 1947) studies of chick embryo cross sections suggested the possibility that neuromuscular junctions might develop in culture. Corner (1964) and Corner and Crain (1965) explanted embryonic amphibian tissue (Xenopus

laevis and Rana pipiens) and noted responses of the muscles to electrical stimuli applied to a distant neural region. These authors concluded that significant neuromuscular relationships had formed in culture. When cross sections of foetal rat, mice or chick are cultured in Maximov assemblies, the spinal cord and skeletal components continue to differentiate and neuromuscular junctions will be formed after 3-4 weeks in vitro (Peterson et al., 1965; Crain, 1966; Bornstein et al., 1968; Veneroni and Murray, 1969; Crain, 1970; and for reviews: Shimada and Kano, 1971 and Crain and Peterson, 1974). Neurites originating from the spinal cord were found to reach and to penetrate into the mass of muscle tissue. Dorsal en ventral root nerve fibers could be discerned, and the large caliber axons were myelinated. By the use of this system it appeared possible to obtain a preparation in which the neuromuscular junctions closely resembled the mature neuromuscular junction in vivo. In such cultures, however, there is a possibility that early neuromuscular connections had already been established prior to explantation. The thick tissue mass makes it impossible to observe both the innervating neurite and the innervated muscle fiber.

2.2.2. Organ culture of explants coupled in vitro

Neuromuscular junctions can also be formed de novo between spatially separated explants of embryonic nerve and muscle tissue. With the aid of electron microscopy James and Tresman (1968) and Pappas et al. (1971) studied neuromuscular junctions formed between spinal cord and skeletal muscle explants of chick and rat or mouse, respectively. Nakai (1969) was able to identify neuromuscular junctions by light microscopy and histological techniques in chick nerve-muscle cultures. The presence of functional neuromuscular junctions was demonstrated with the aid of extracellular electrophysiological measurements (Crain, 1970; Peterson and Crain, 1970; Crain et al., 1970 and Peterson and Crain, 1972). In addition, they showed that in heterospecific (between different species) and heterotypic (between 'wrong' tissues) combinations functional neuromuscular junctions are formed. In the following mixed cultures functional neuromuscular junctions have been demon-

strated in vitro: (1) fetal rodent spinal cord with adult rodent skeletal muscle, (2) fetal rodent cord with adult human muscle, and (3) fetal rat cord with fetal mouse muscle.

Robbins and Yonezawa (1971 a,b) showed with intracellular electrophysiological studies of rat neuromuscular junctions that in an early stage of synapse formation chemical transmission in tissue culture begins with discrete, localized release of transmitter about the time when nerve-muscle contacts are first visible with light microscopy. Cross striation appeared to be related to innervation: every cross striated fiber tested was found to be innervated, and denervation in vitro led to a decrease of distinct cross striations.

The culture method of separated explants has the special advantage that all neuromuscular junctions are really formed de novo and are not a further development of pre-existing nerve-muscle contacts. In addition this method permits the study of the specificity of the process of formation of nerve-muscle connections.

2.2.3. Cell cultures of muscle cells with neuronal explants

The formation of neuromuscular junctions has been demonstrated in mixed cultures of dissociated skeletal muscle cells together with explants of spinal cord. Veneroni and Murray (1969) reported that 'a high degree of differentiation was achieved by both nervous and reaggreated muscle tissue. Exploring nerve fibers from the cord explants made contact with the sarcoblasts, and areas of cholinesterase activity appeared in the sarcolemma of the newly integrated fibers. Simple neuromuscular junctions formed de novo'. Electron microscopy of junctions in this culture system has been done by James and Tresman (1969). 'Neuromuscular junctions comparable to those found in the developing chick embryo (Fischman, 1967) formed de novo between homologous explants'. Electrophysiological investigations have been carried out in cultures of spinal cord explants together with muscle cells by Kano and Shimada (1971 a,b) and by Robbins and Yonezawa (1971 b) in chick and rat material, respectively.

The innervation of chick striated muscle fibers by chick ciliary gan-

glia was demonstrated electrophysiologically by Hooisma et al. (1975) and by Betz (1976 a,b). Kidokoro and Heinemann (1974) and Kidokoro et al. (1976) demonstrated synapse formation between clonal muscle cells and rat spinal cord explants by morphological and electrophysiological techniques. Innervation of chick muscle cells by mouse spinal cord explants was demonstrated by Hooisma et al. (1977).
Advantages of these systems are the good visibility of muscle cells and nerve endings.

2.2.4. Cell culture of muscle cells and dissociated neurons

Neuromuscular junctions can develop in mixed cultures of dissociated skeletal muscle cells together with dissociated neuronal material. Synaptogenesis in this system was demonstrated by electron microscopy by Shimada and Fischman (1966). Shimada (1968) and Shimada et al. (1969 a,b) were able to demonstrate end plate-like structures by staining and light microscopy. The first signs of functional synaptogenesis were reported by Fischbach (1970, 1972). He carried out intracellular electrophysiological measurements on neuromuscular junctions formed between dissociated chick spinal cord neurons and chick muscle cells. Further studies on synaptogenesis and parameters that seemed to be involved in synaptogenesis have been made eversince with the aid of intracellular electrophysiology (Cohen, 1972; Fischbach and Cohen, 1973; Bird and James, 1973, 1974; Stevens et al., 1974 and Engelhardt, 1976).
Fischbach et al. (1976) presented a way to separate neuronal cells by velocity sedimentation. In mixed cultures of muscle together with a fraction of neurons enriched in larger cells more nerve-muscle synapses were present.

Besides the obvious combination of spinal cord neurons with skeletal muscle cells some other combinations have been investigated in culture. Harris et al. (1971) have attempted the formation of synaptic contacts between mouse neuroblastoma cells and muscle fibers from a cell line of rat skeletal myoblasts. Although it was not pos-

sible to demonstrate neurotransmission, these authors were able to show regions of increased acetylcholine (ACh) sensitivity at areas of contact between neuroblastoma cells and muscle fibers. These regions of enhanced ACh-sensitivity were in sharp contrast with the diffuse ACh-sensitivity along myotubes in cultures devoid of neuroblastoma cells. Steinbach (1974) demonstrated that muscle activity is not required for the appearance of regions of high ACh-sensitivity on the clonal muscle fibers. Nurse and O'Lague (1975) presented electrophysiological evidence that neurons obtained by dissociation of rat superior cervical ganglia formed synapses with rat myotubes. Vogel and Nirenberg (1976) demonstrated that cells dissociated from chick embryo retina and cultured in vitro formed aggregates with a cellular arrangement similar to that of intact retina. Most of the nicotinic acetylcholine receptors of these aggregates were found associated with those regions where neurites were found. The receptor distribution on these cultured retina cells thus resembled that in the intact retina. Chamley et al. (1973) made use of phase contrast microscopy, time lapse cinematography, catecholamine fluorescence histology, and scanning and transmission electron microscopy, to demonstrate interactions between sympathetic nerve fibers and smooth guinea pig muscle cells in tissue culture.

As mentioned before, cell cultures of dissociated neuronal and muscle material offer the distinct advantage that morphological details of both types of cells are visible in the living culture. The dimensions of various cells can be estimated under the microscope, their surface areas can be approximated and subsequently their specific membrane properties can be calculated. The responses of individual cells on added biochemical, pharmacological and toxicological agents can be studied at the level of the individual cell. Under appropriate conditions there will be no or only a few fibroblasts or glial cells present in the culture. A disadvantage of such cultures is, however, that the organized structure of the original nerve tissue is lost, essential interactions at the cellular and organ level

may have been disturbed and identification of cells on the basis of the cytoarchitecture is no longer possible.

2.3. Parameters for neuromuscular maturation

At present a variety of features of the neuromuscular junction formed in tissue culture are well established. The results obtained in cultures of avian and mammalian cells will be summarized below. The data will be presented according to the different techniques used, which of course will make that many studies will be mentioned more than once.

2.3.1. Light microscopy

The morphology of junctions between nerve and muscle cells has extensively been studied. In all four culture systems the endings have been demonstrated by silver impregnation. In organ cultures of whole cross sections of embryos, dorsal and ventral root nerve fibers could be discerned. In combinations of spinal cord explants and muscle explants buttonshaped and branched endings and bulbous swellings have been shown (Bornstein et al., 1968; Veneroni and Murray, 1969; Nakai, 1969; Crain et al., 1970; Peterson and Crain, 1970; Robbins and Yonezawa, 1971 a,b and Peterson and Crain, 1972). Evidence for comparable structures on myotubes formed of dissociated muscle cells after innervation by spinal cord explants is presented by Kano and Shimada (1971 a). In cell culture of dissociated neuronal and muscle material Shimada et al. (1969 a,b) and Bird and James (1973) showed multiple bulbous nerve endings in contact with myotubes. At later stages the endings looked like complexly branched, finger-like projections in direct contact with the muscle. It is difficult to demonstrate end plate-like structures by light microscopy in the living cell culture; no authors give details on such observations.

2.3.2. Acetylcholinesterase

Histochemical demonstration of acetylcholinesterase (AChE) at the neuromuscular junctions have been successful in highly organized organotypic explants (Bornstein et al., 1968; Nakai, 1969; Peterson

and Crain, 1972; Robbins and Yonezawa, 1971 b and Crain and Peterson, 1974). The methods employed have been those of Koelle and Friedenwald (1949) and Karnovsky and Roots (1964). In chick muscle cell cultures confronted with spinal cord fragments, Veneroni and Murray (1969) demonstrated strong AChE activity at the site of the myoneural contacts by 26 days in vitro. However, in short-term cultures Shimada and Kano (1971), Fischbach (1972) and Fischbach et al. (1974 a) failed to demonstrate AChE positive loci on functionally innervated muscle fibers. Peterson and Crain (1972) showed that at neuromuscular junctions formed de novo between explants of spinal cord and muscle, AChE was functionally active. The end plate potentials (epp's) generated upon local cord stimulation showed the normal pharmacological sensitivity to eserine, a substance which interferes with cholinesterase activity and thus prolongs the action of the transmitter substance, resulting in increase of epp amplitude and duration. In cultures of dissociated chick spinal cord neurons and chick myotubes Fischbach (1972), Fischbach et al. (1974 a) and Fischbach et al. (1976) were not able to demonstrate physiologically active cholinesterease. Application of an anticholinesterase had no marked effect on the size and the time course of the end plate potentials in nerve-muscle cultures less than 2 weeks old. In combined cultures of chick skeletal muscle and chick ciliary ganglia Hooisma et al. (1975) also were not able to demonstrate physiologically active AChE.

2.3.3. Electron microscopy

Ultrastructural studies of neuromuscular synaptogenesis in vitro (Shimada and Fischman, 1966; Bornstein et al., 1968; James and Tresman, 1968, 1969; Shimada et al., 1969 a; Shimada and Kano, 1971; Pappas et al., 1971; Shimada and Fischman, 1973; James and Bird, 1973, 1974 and Fischbach et al., 1974 b) suggest a pattern of development which is similar to what is supposed to occur in vivo (Hirano, 1967; Teräväinen, 1968; Kelly and Zacks, 1969 and Lentz, 1969, 1970). The course of the development is a reconstruction based on individual electron micrographs taken at selected times of cultivation. Electron

microscopy on marked individual synapses which had previously been analyzed by electrophysiology has not yet been performed. Mature end plates have been observed only in the highly organized 'organotypic' explants (Pappas et al., 1971). These junctions possessed the characteristic postsynaptic infoldings of fibers with disc or plate-like endings. In the light microscope these end plates would be classified as 'en plaque' types.

2.3.4. Electrophysiology of muscle innervation in vitro

There is strong electrophysiological evidence of formation of functional neuromuscular junctions in vitro. Both spontaneously and artificially evoked end plate potentials have been recorded by extracellular (Crain, 1966, 1970; Crain et al., 1970 and Peterson and Crain, 1972) and intracellular techniques (Fischbach, 1970, 1972; Kano and Shimada 1971 a; Robbins and Yonezawa, 1971 a,b; Bird and James, 1974; Fambrough et al., 1974; Nurse and O'Lague, 1975; Hooisma et al., 1975, 1977; Fischbach et al., 1976 and Betz, 1976 a).

2.3.5. End plate potentials

Intracellular stimulation of the neurons gave rise in the muscle cells to epp's that had reversal potentials between -15 mV and -5 mV (Fischbach 1970). Robbins and Yonezawa (1971 a,b) showed that epp's occurred as a result of nerve stimulation and also arose spontaneously in explants of muscle together with spinal cord. They reported that transmitter output at the junctions increased with development. Kano and Shimada (1971 a) demonstrated that in cultures of dissociated chick myoblasts and fragments of chick spinal cord miniature end plate potentials (mepp's) with average frequencies of around one per second or more could be recorded. D-tubocurarine reversibly suppressed the mepp's and the stimulus evoked epp's. Fischbach (1972) demonstrated that the small potentials (1-10/min) share the following characteristics with miniature end plate potentials recorded from adult neuromuscular junctions in vivo: (1) the lengths of the intervals between subsequent depolarizations are exponentially

distributed; (2) the amplitudes are not greatly altered by reduction of extracellular Ca^{2+} or by an increase of extracellular Mg^{2+} (del Castillo and Katz, 1956 and Hubbard et al., 1968); and (3) the depolarizations are still observed in the presence of tetrodotoxin (TTX), a drug that blocks nerve action potentials but does not affect the random release of transmitter packets (Katz and Miledi, 1967).

It is known (Katz and Miledi, 1967) that, even if action potentials are blocked by TTX, transmitter release can be evoked from motor nerve terminals by applying graded depolarizing pulses to the terminals. With the aid of this technique Fischbach et al. (1976) were able to localize the sites of transmitter release (synapses) in tissue culture. They reported the incidence of varicosities and synapses, but in most cases varicosities turned out to be no synapses. Thus the identification of synapses by light microscopy alone is not feasible. Nurse and O'Lague (1975) presented electrophysiological evidence that neurons dissociated from rat superior cervical ganglia developed cholinergic synapses with rat myotubes. Hooisma et al. (1975) described synapse formation between chick ciliary ganglion explants and chick muscle cells. The end plates appeared to be cholinergic nicotinic. Betz (1976 a) has confirmed these findings.

2.3.6. Acetylcholine sensitivity and acetylcholine receptor distribution

Acetylcholine sensitivity and ACh-receptor density is evenly spread over the whole myotube in noninnervated and in innervated myotubes (Kano et al., 1971; Hartzell and Fambrough, 1973; Cohen and Fischbach, 1973). However, spots with enhanced receptor densities and thus increased ACh-sensitivities (Hartzell and Fambrough, 1973) have been demonstrated on noninnervated fibers (Fischbach and Cohen, 1973; Sytkowski et al., 1973 and Prives et al., 1976). These so-called 'hot spots' make it very difficult to demonstrate the development of end plate regions in innervated myotubes by their enhanced ACh-sensitivity.

Fischbach and Cohen (1973) showed that innervation of myotubes did not reduce their mean ACh-sensitivity. Fischbach et al. (1976) demonstrated areas of high ACh-receptor density at neuromuscular junctions, that were identified as true synapses.

2.3.7. Quantal transmitter release

At the neuromuscular junctions formed in tissue culture the ACh release is of a quantal nature (Robbins and Yonezawa, 1971 a,b and Fischbach, 1972). The amplitude histograms of evoked end plate potentials are multimodal, i.e.: the relative peaks in the histograms coincide with multiples of the mean quantum size.

2.3.8. Multiple innervation

It has been noted that, in some cases, stimulation of a single neurite elicits a series of end plate potentials with different rates of rise, demonstrating multiple innervation (Robbins and Yonezawa, 1971 b; Kano and Shimada, 1971 a; Fischbach, 1972; Hooisma et al., 1975; Fischbach et al. 1976 and Betz 1976 a). This type of innervation also occurs in regenerating and developing muscle in vivo (Letinsky, 1974; Benoit and Changeux, 1975; for a review see Dennis and Ort, 1976).

2.4. Innervation of chick muscle by ciliary ganglia

The present report describes the innervation in tissue culture of chick myotubes by explants of the chick ciliary ganglion and by fragments of the chick ciliary ganglion in which only a small number of neurons and sometimes even only one neuron is present. In vivo the chick ciliary ganglion contains two distinct types of neurons: the so-called 'ciliary' and the 'choroid' neurons, innervating the striated musculature of iris and ciliary body, and the choroidal smooth muscle fibers, respectively (Hess, 1965; Marwitt et al., 1971). The use of the ciliary ganglion neurons offers the great advantage above that of the spinal cord neurons, of being source of purely cholinergic neurons of only two types whereas the spinal cord possesses a great variety of cells which make use of an unknown number of differ-

ent transmitters. Moreover, about half of the neurons of the chick ciliary ganglion innervate striated musculature in vivo.

The innervation of chick muscle cells in tissue culture by the rabbit ciliary ganglion is also described and compared with innervation by the chick ciliary ganglia.

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Chapter 3

3. Innervation of chick skeletal muscle fibers by the chick ciliary ganglion in tissue culture

3.1. Introduction

3.1.1. Synaptogenesis in tissue culture

The knowledge of the formation of the cholinergic neuromuscular junction is largely based on experiments in which denervated striated muscle fibers are reinnervated by their proper nerve or by heterotypic nerves (Gaze, 1970; Landmesser, 1972; Bennett et al., 1973; Vyskočil and Vyklický, 1974 and Bennett and Pettigrew, 1976). In tissue culture the formation of neuromuscular junctions between striated muscle cells and neuronal cells has been studied in different laboratories (Fischbach, 1970, 1972; Shimada and Fischman, 1973; Nelson, 1975 and Fischbach et al., 1976). The tissue culture technique offers the opportunity of continuous observation and control of the experimental conditions. In tissue culture functional end plates are formed between embyronic spinal cord neurons and embryonic striated muscle cells. This most natural combination has the disadvantage that the spinal cord contains a great variety of neurons which make use of different transmitter substances. So far it has not been possible to identify motoneurons in cultures of dissociated spinal cord. An alternative source of cholinergic neurons is offered by the chick ciliary ganglion. The formation of neuromuscular junctions between the chick ciliary ganglion and chick skeletal muscle fibers in tissue culture has been demonstrated in this laboratory (Hooisma et al., 1975) and by Betz (1976).

3.1.2. Chick ciliary ganglion in vivo

The ciliary ganglion is an autonomic ganglion, in the chick situated just distal to the point where the oculomotor nerve gives off medial and lateral branches to the extra ocular muscles (Landmesser and Pilar, 1974a). The ciliary ganglion contains only two classes of neurons,

which both produce acetylcholine (ACh) (Marwitt et al., 1971); the larger neurons are called 'ciliary neurons', the smaller ones are called 'choroid neurons'. The ciliary nerves, which contain the myelinated axons that originate from the ciliary neurons (Hamburger, 1962), emerge from the ganglion and immediately penetrate through the sclera to run along the optic cup to the sphincter iridis and ciliary muscles. Medial to the ciliary nerves a second group of two or three strands of thinner non-myelinated axons coming from the choroid neurons emerge from the ganglion, pierce the sclera, and branch profusely in the vascular choroid tissue where they innervate smooth muscle fibers (Landmesser and Pilar, 1974a).

Each ciliary neuron is innervated by a single preganglionic fiber which originates from a neuron in the Edinger-Westphal nucleus and forms a calyciform ending on it. Each preganglionic fiber, on the other hand, innervates more than one ciliary neuron. The choroid neurons are multiply innervated; each receiving two or three preganglionic fibers. The adult ciliary ganglion contains approximately 3000 neurons of each type (Landmesser and Pilar, 1974b). The ciliary neurons are large spheroidal cells with pale staining cytoplasm and a distinctive perinuclear ring of Nissl substance. The choroid neurons possess angular and irregular somata with uniformly stained cytoplasm while the Nissl substance is scattered in individual clumps. At an embryonic age of 4-5 days the ciliary and choroid neurons cannot be distinguished but at 8 days in ovo the ciliary neurons are already larger than the choroid ones (Landmesser and Pilar, 1974a). At that stage of development both types of cells are innervated by bouton type synapses. At day 10 the synapses of the ciliary neurons begin to attain their ultimate calyciform shape. At that time the ciliary neurons are about 25 μm in diameter and are surrounded by glial cells. In the adult state the ciliary and choroid neurons have an average diameter of 45 μm and 25 μm, respectively. The ciliary neurons are cholinergic nicotinic (Pilar and Vaughan, 1969) whereas the choroid neurons are cholinergic muscarinic (unpublished observations cited by

Landmesser and Pilar, 1970). In addition Cantino and Mugnani (1974) have presented evidence which suggests that adrenergic fibers form a kind of 'distance à synapse' with the choroid neurons, with the preganglionic fibers or with both.

3.1.3. Neuromucular junctions of the ciliary ganglion in vivo

In the chick the ciliary body and the sphincter iridis are striated and differ in this respect from the corresponding smooth muscles in mammals (Ovio, 1927). Electron microscopic studies (Hess, 1966) have demonstrated a difference between the ultrastructure of avian iris muscle fibers and those of all other striated muscle fibers examined so far. Normal twitch fibers have large amounts of sarcoplasmic reticulum, well developed triads, and many postjunctional folds of the sarcolemma under the individual nerve endings, whereas slow fibers have scant amounts of sarcoplasmic reticulum, a poorly developed (if any) T-system, and hardly any folds of the sarcolemma under the multiple nerve terminals. The striated muscle fibers of the chick iris have an extensive sarcoplasmic reticulum and the T-system is well developed. Hence, the internal structure of these muscle fibers is entirely 'twitch' in character. However, the presence of elongated multiple terminals on the muscle fibers and the absence of postjunctional sarcolemmal infoldings under the nerve terminals are definitely characteristics of the 'slow' muscle fibers.

An interesting feature of the neuromuscular junction between the ciliary ganglion and the iris in the adult pigeon, as studied by Pilar and Vaughan (1969) and Pilar (1969) is the response on the addition of tetrodotoxin (TTX), a drug which selectively blocks the Na-channels in the nerve membrane and thus the generation of action potentials (Katz and Miledi, 1967). In the pigeon iris spontaneous junctional potentials (sjp's) of up to 18 mV can be recorded which persist in the presence of TTX at a concentration at which nerve conduction is completely blocked (Pilar, 1969). The frequency of occurrence of the sjp's is reversibly increased (1.9 - 4.7 times)

during TTX treatment. The amplitude distribution of the sjp's does not alter after TTX administration. In 30% of the muscle cells the administration of TTX caused a reduction of the resting membrane potential of 10-12 mV.

3.1.4. Present investigations

In this chapter a more detailed description will be given of the electrical properties and the pharmacological characteristics of the neuromuscular junctions which develop between ciliary ganglion neurons and muscle cells in tissue culture.

3.2. Materials and methods

3.2.1. Tissue culture

Leg muscles were removed from six 11-day old chick embryos under sterile conditions and cut into small pieces. The fragments were incubated at 37° for 30 min. in 5 ml of a 0.05% solution of trypsin (Grand Island Biological Company; Gibco) in Tyrode's solution prepared without Ca- and Mg-chloride. Subsequently, 5 ml tissue culture medium (see below) was added. Muscle cells were obtained by trituration of muscle pieces with the aid of a 10 ml pipette. The cell suspension was filtered through several layers of gauze and pelleted (5 min., 1000 rpm). The upper layer of the cell pellet was resuspended in 10 ml tissue culture medium. The mononucleated cells were counted in a hemocytometer. The cells were plated on collagen coated (Bornstein, 1958) 'cellfinder' object glasses (i.e. glass plates provided with a grid marked with figures and letters to facilitate the localization of cells; made by Micropure, Driebergen, The Netherlands) in 60 mm Falcon dishes at a cell-density of 5×10^4 cells/cm^2. The mononucleated cells were allowed to settle in 0.5 ml medium during 1 hour and subsequently cultured in 3 ml medium. Cultures were maintained for two days at 37° and pH 7.2 and thereafter at 35° and pH 7.2 in a watersaturated 95% air - 5% CO_2 atmosphere.

The tissue culture medium was composed of 70 parts Eagle's minimum es-

sential medium in Earle's balanced salt solution (10.96 g. Gibco powder, containing glutamine and Earle's salts but without bicarbonate, in 1 l distilled water), 10 parts of NaCl solution (8.07 g/l), 15 parts horse serum (Flow, Scotland), 5 parts freshly prepared embryo extract. The medium was supplemented with biotin (0.007 mg/l), ferric nitrate (0.7 mg/l), $NaHCO_3$ (1.71 g/l), glucose (0.7 g/l), penicilin (7000 U/l) and streptomycin (7 mg/l). Osmolarity was adjusted to a final value of 320 mOsm.

The proliferation of many of the mononucleated stellate cells, presumably fibroblasts, was inhibited by addition of 10^{-5} M D-arabinofuranosylcytosine (ara-C) to the medium. This drug inhibits DNA-synthesis and reduces the viability of dividing cells (Graham and Whitmore, 1970). The drug was added in fresh medium after 2 days when myotubes were beginning to form, but background stellate cells were still isolated and few in number (Fischbach, 1970). The drug was removed two days later by washing with tissue culture medium. Subsequently the medium was changed every 2-3 days.

Ciliary ganglia were dissected from chick embryos of various ages under sterile conditions and allowed to settle in 0.5 ml medium on collagen coated cellfinder object glasses. After attachment of the ganglia, medium was added to a final volume of 3 ml. For mixed cultures, ciliary ganglia were dissected from 7-day old chick embryos (unless otherwise stated) under sterile conditions. The ganglia were allowed to settle in 0.5 ml medium on top of muscle preparations that had been in culture for 2-6 days. After attachment of the ganglia, medium was added to a final volume of 3 ml. Sometimes the ganglia were mechanically divided into small pieces or dissociated by gentle trituration with the aid of a Pasteur pipette.

In order to select a myotube suitable for electrophysiological measurements, morphological criteria for acceptance were applied. The length of the myotube should be more than 200 µm, the cell should not be too flat and should have distinct and abrupt contours in phase-contrast microscopy. The cell should not contain vacuoles.

3.2.2. Electrophysiology

3.2.2.1. Recording set up

A cellfinder object glass carrying cultured cells was removed from the Petri dish and placed in a perspex chamber which permitted continuous perfusion with prewarmed (37°) Tyrode's solution (osmolarity 320 mOsm; pH 7.2) (fig. 1).

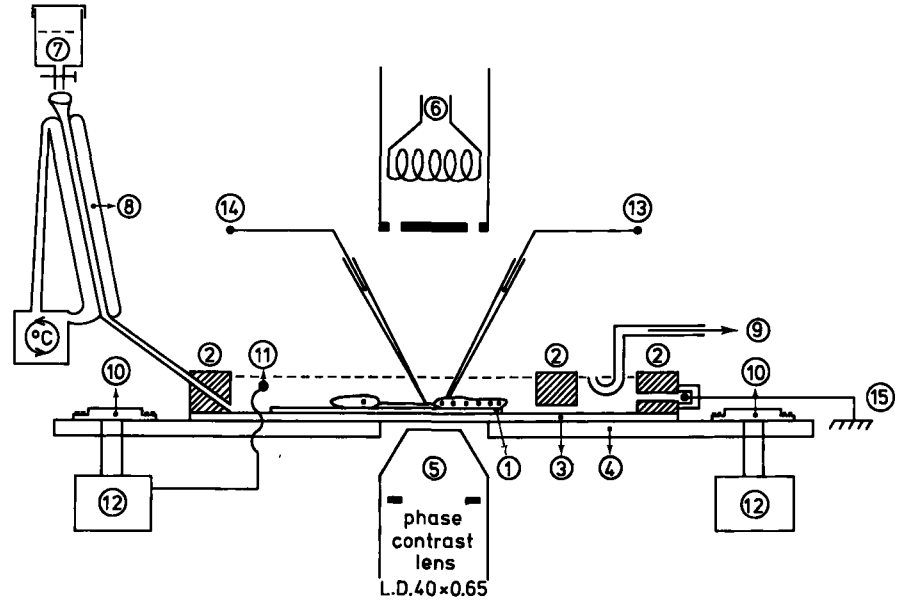


Fig. 1: Detail of the set-up for intracellular recording

A cellfinder object glass with cultured cells (1) is placed in a perspex chamber (2) attached to a glass plate (3) which is mounted on the heated stage (4) of a modified Zeiss phase contrast microscope (5, 6). Tyrode's solution (7) is led, via a warmed watermantle (8), through the chamber and sucked away at the other end (9). Power transistors (10) heat the stage under thermistor (11) control (12). Cells are impaled by a microelectrode (13) and signals led to the electronics. The other electrode (14) is an electrode for ACh-iontophoresis or an extracellular stimulation electrode. The reference electrode (15) is grounded. In some cases an extra reference electrode in combination with the ACh or extracellular stimulation electrode is present. The perspex chamber can be moved across the stage.

The chamber was mounted on the stage of a modified Zeiss phase-contrast inverted microscope. The stage was heated under thermistor control by 2 power transistors at $37.0^{\circ} \pm 0.5^{\circ}$. The cells were visualized at a magnification of 400x with the aid of a 40x long distance phase-contrast lens. Recording and stimulating electrodes, mounted on combined Brinkmann and Microcontrole micromanipulators, were introduced from above. A condensor with a long working distance allowed easy manipulation of the electrodes. Mechanical vibrations were minimized by mounting the microscope and the manipulators on a heavy steel plate which was hanging from a steel frame by 8 springs. The fine control of the electrode movements was effected by a hydraulic system consisting of 2 syringes interconnected by a flexible tube and filled with oil. This system had good mechanical stability. Intracellular potentials were recorded with 3 M KCL filled electrodes which had DC-resistances of 10-20 MΩ. The cells were impaled by a sudden advancement of the microelectrode over a distance of 1-3 µm by means of a piezo-electric driving device (Rikmenspoel and Lindemann, 1971). Pipettes were drawn from glass capillaries with a glass fiber melted to its inner wall (Clark, Electromedical Instruments, England) and filled with the aid of a hypodermic needle (Tasaki et al., 1968). Extracellular stimulation electrodes were filled with Tyrode's solution and had DC-resistances of 5-15 MΩ. Signals from the recording electrode were led to a 10x gain high impedance input, negative capacitance amplifier via a chlorided silver wire and were displayed on a storage oscilloscope and stored on magnetic tape. The DC-potentials were also recorded by a penrecorder (see fig. 2). Direct electrical stimuli were supplied via a stimulus isolation unit and fed through amplifier and recording electrode. Recording of the resulting potential transients was effected with the amplifier in a bridge circuit.

If current stimulation was performed through the cell, the resulting potential change was the sum of the potential changes over electrode and cell respectively. Since the RC-time of the electrode was much shorter than that of the cell, both signals could be separated

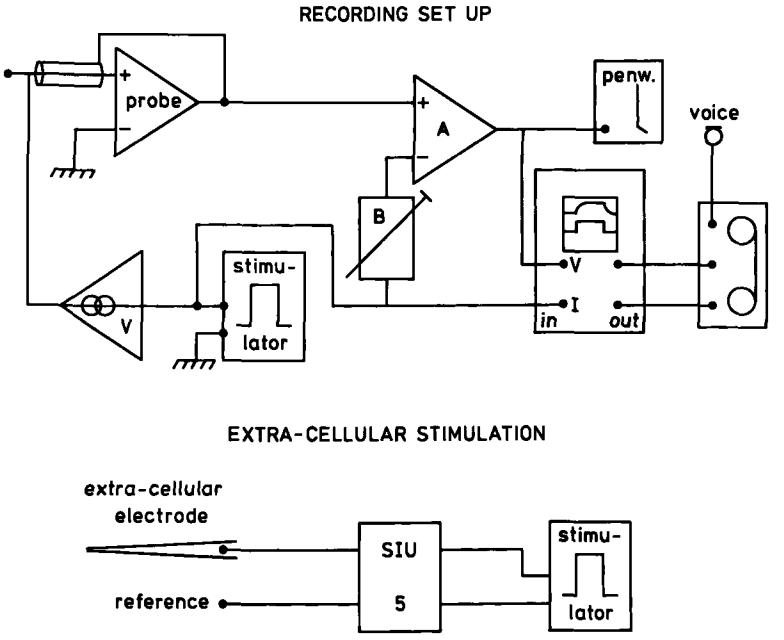


Fig. 2: Schematical representation of recording and stimulating devices

The recorded signals are led to a guard-shielded probe, also providing negative capacitance. Further amplification occurs at the amplifier A. DC-signals are recorded by the penwriter (penw.). AC and DC-signals are displayed on a storage oscilloscope and after further amplification by the oscilloscope stored on tape. A stimulator provides a signal which after voltage to current conversion is fed into the cell via the microelectrode. The same signal is fed to amplifier A, which enables balancing of the signal with the aid of device B (DC and transient balance). Extracellular stimulation is performed via a stimulus isolation unit (SIU 5) and the extracellular electrode with a separate reference ground electrode.

by using a step current. A copy of the signal over the electrode was electronically made with the aid of a RC-circuit and substracted from the potential change measured, thus leaving only the potential change over the cell (see fig. 3). This procedure, called 'intracellular balancing', is carried out with the electrode in the cell (Eisenberg and Johnson, 1968). In case of extracellular stimulation the return path for the current was formed by a separate reference electrode in order to minimize the stimulus artefact. A small artefact remained that made it possible to determine the relative stimulus strength.

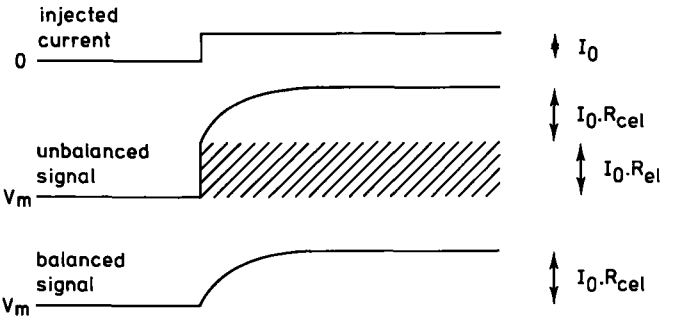


Fig. 3: Intracellular balancing

The upper line shows the current step supplied by the stimulator. The second line shows the resulting potential recorded with the electrode. The fast (shaded) part is the potential caused by the current going through the electrode. The exponentially increasing part is the real potential transient in the cell. If the fast (shaded) part is electronically subtracted, the real potential over the cell membrane remains (line 3).

The pharmacological substances in the bath reached a concentration within a few percents of the final value within 5 minutes after changing the perfusion fluid. The solutions of d-tubocurarine, atropine, TTX or physostigmine were always freshly prepared. When the Mg^{2+} concentration of the Tyrode's solution was raised by a factor of 10 and the Ca^{2+} concentration lowered by a factor of 4, the osmolarity changed only 5 mOsm.

3.2.2.2. Analysis of drug effect on end plate potential distribution

The amplitude distributions of the spontaneously occurring end plate potentials have been analysed with the aid of a PDP-8 computer. The discrimination level of the computer program had to be set a little above the noise level (0.2 - 0.4 mV). Amplitude distributions were printed out and converted into reversed cumulative representations. A normal cumulative distribution is obtained by adding to the number of potentials in an amplitude class, the number of all smaller potentials. In this way an undue significance is assigned to the small potentials. Since it is more appropriate to assign greater

significance to the large potentials, the calculation was reversed (Huybregts and Schreurs, 1975). The number of all larger potentials was added to the number of amplitudes in a certain class of amplitudes. When the reversed cumulative distribution is plotted on a double logarithmic scale, a change of the amplitude or the frequency by a certain factor does not affect the shape of the distribution, but only shifts the plot along the abscissa and the ordinate, respectively. It must be born in mind that a shift in frequency also changes the chance to observe epp's with the highest amplitudes. A shift in amplitude causes the total number of epp's above the discrimination level of the computer program to increase or decrease. Comparing two distributions on double logarithmic scale by superimposing the plots reveals whether a change in distribution had been due to a shift in frequency or in amplitude. The method favours the detection of changes in the occurrence of higher amplitude epp's. Changes in frequency and amplitude of the lower amplitude epp's can not easily be detected. Effects of pharmacological substances were interpreted by comparing the changes in the amplitude distribution and the reversed cumulative representation.

3.3. Results

3.3.1. Morphology

3.3.1.1. Differentiation of muscle cells

The fusion of myoblasts started during day 1. On day 3 many myotubes had formed with nuclei lying in line in the centre. Cross striations, faintly visible on day 4, became pronounced and abundantly present on day 5. On day 8 most myotubes had many branches and were several milimeters long. Hypolemmal nuclei were seen in most thick myotubes. The presence of ara-C in the tissue culture medium during days 3 and 4 greatly reduced the proliferation of the fibroblasts. Not whithstanding this treatment dividing cells grew to confluency in 8 to 10 days. After 10 to 12 days the amount

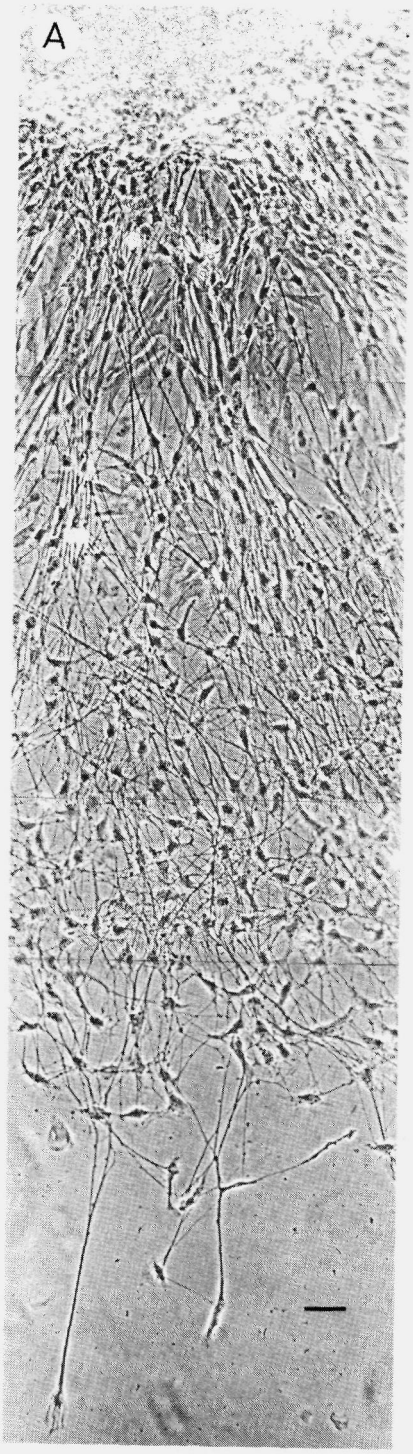
of vacuoles in the myotubes became conspicuous and myotubes began to disappear.

3.3.1.2. Influence of embryonic age on growth of neurites from the ganglia

The most suitable developmental stage of the ciliary ganglia was established by comparing the results of the radial growth of neurites from ganglia dissected from embryos in different developmental stages in culture. Ganglia attached themselves to the collagen coated coverslips within a few hours after explantation. Within 24 hours, branching neurites were growing radially from 6- and 7-day old ganglia. After two days in culture a corona of neurites with a diameter of 500 - 1000 µm surrounded the ganglia. At that time fibroblasts started to grow from the ganglia, obscuring the neurites. The growth of neurites from ganglia dissected from 8 day old embryos was much slower. The growth of non-neuronal cells then obscured the neurites to a greater extent. The older the gestational stage of the ganglia the less neurites grew from them. Ganglia taken from 14 day old embryos produced hardly any neurites.

3.3.1.3. Mixed cultures

The number and length of the neurites in mixed cultures with muscle cells was greatly influenced by the overall density of fibroblasts around the ganglion and by the number of myotubes. In crowded cultures fewer neurites grew out from the ganglion and especially at places where thick myotubes were lying opposite to the ganglion a somewhat disturbed growth pattern was observed. Often a neurite, just emerging from the ganglion seemed unable to cross a myotube. On the whole the neurites preferred to run parallel with the myotubes. However, the number of axons emerging from the ganglion was usually so large that after some days neurites were coursing across or on myotubes and often seemed to end on them. Sometimes, morphologically well differentiated myotubes in the immediate vicinity of the ciliary explant were not contacted by outgrowing nerve fibers, whereas many nerve fibers seemed to end upon mononucleated cells. Fig. 4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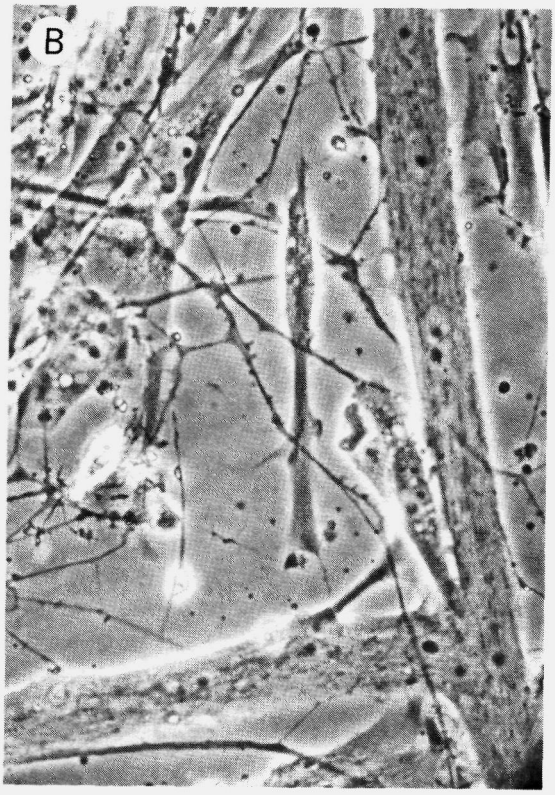
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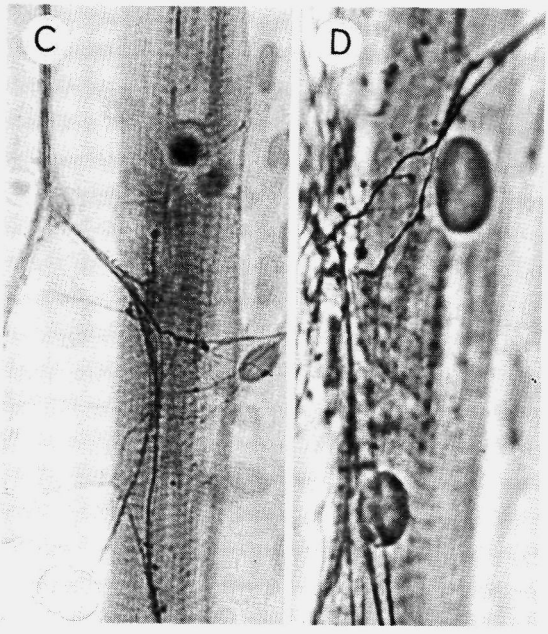
C
D

shows a sector of a ganglion explant, a few myotubes and the outgrowth of numerous neurites. Neither bright field, phase contrast nor differential interference contrast microscopy revealed distinct end plate-like structures on the myotubes. Often, a number of neurites or bundles of neurites seemed to end on one myotube suggesting multiple innervation. No gross changes in overall morphology of the myotubes were observed as a consequence of innervation. After silver impregnation according to a modification of the Bielschowsky method (Sevier and Munger, 1965) varicosities of the nerve fibers could sometimes be seen (fig. 4).

3.3.2. Electrophysiology

3.3.2.1. Electrical properties of cultured muscle cells

In noninnervated myotubes the membrane resting potential V_m was -81.4 $\pm$ 5.3 mV ($\pm$ S.D.) in 51 cells in 19 different culture. Active potential transients could be elicited in the myotubes. In young myotubes slow plateau potentials with a duration of about 5 seconds were observed (Kidokoro, 1973). After an initial fast transient (100 msec) the potential remained at a plateau (-30 mV to -40 mV) and decayed after 3 seconds in about 2 seconds to the membrane resting potential. In some cultures these plateau potentials occurred spontaneously at such short intervals that other electrical phenomena were obscured. As the maturation of the cells proceeded

Fig. 4: Ciliary ganglion, neurites and muscle cells

A. At the top of the figure a segment of a ganglion. Many branching neurites grow radially from the explant. Ciliary ganglion 4 days in vitro.
B. A branching multinucleated myotube in which the nuclei are scattered over the middle of the myotube. Neurites are coursing over and on the myotube. Ciliary ganglion and muscle cells 1 and 3 days in vitro, respectively.
C. Innervated cross striated myotube. Neurites coming from the left seem to make small beads where they contact a myotube. Ciliary ganglion and muscle cells 1 and 6 days in vitro, respectively.
D. Innervated myotube with cross striations and nuclei. In the central part neurites coming from upper right show varicosities where they contact the myotube. Same preparation as in C.

A and B: unstained living preparations, phase contrast 10x lens.
C and D: Bielschowsky silver impregnation, phase contrast lens, 40x and 100x oil, respectively.

Bar applies to all figures: A: 50 µm; B: 20 µm; C: 8 µm and D: 4 µm.

the duration of the plateau potentials became reduced and action potentials appeared on top of them (fig. 5).

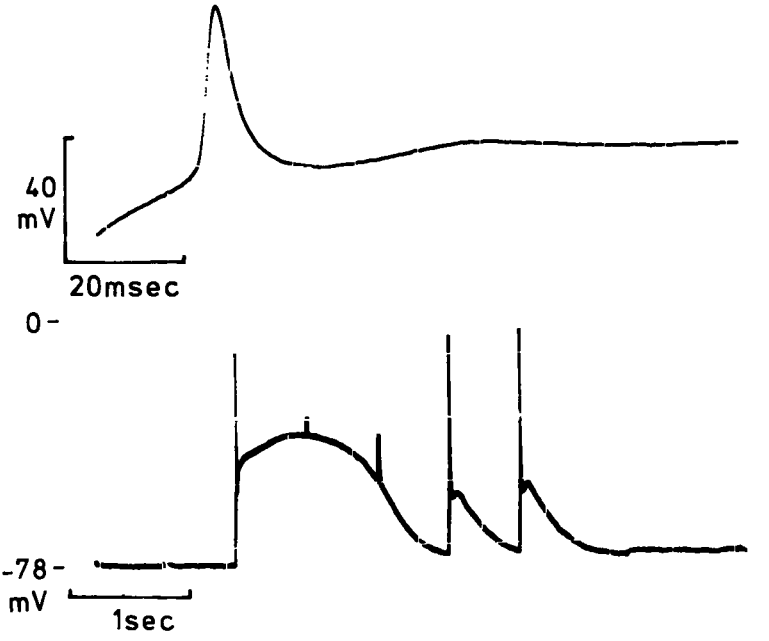

Fig. 5: Plateau potential with action potential

The lower line represents the responses of the muscle cell to 5 subsequent stimuli. The first stimulus evoked a plateau potential, the fourth and fifth did not succeed in evoking a full grown plateau potential but elicited only a rudimentary one, demonstrating the presence of a refractory period. Stimuli 1, 4 and 5 elicited action potentials; the amplitude of the passive potential transients at these stimuli cannot be estimated from this sweep. The reduced passive potentials transients evoked by stimuli 2 and 3 indicate the lowered cell resistance during the plateau potential at an extended time scale. After the first stimulus a rudimentary action potential is seen, on top of the plateau potential, with a maximal rate of rise of only 23 V/sec. Muscle cell 7 days in vitro; V_m = -78 mV.

In older cultures the slow plateau potential had disappeared and action potentials were sometimes recorded (fig. 10^b), a phenomenon also observed by Betz (1976).

3.3.2.2. Influence of innervation on membrane resting potential

Neither innervation nor age in tissue culture changed the membrane resting potential significantly. The mean membrane resting potential of 139 innervated myotubes was -80.7 mV $\pm$ 5.7 mV ($\pm$ S.D.).

Table 1 Influence of gestational age and days in tissue culture of ciliary ganglia and days in vitro of muscle cells on V_m. Figures are mean $V_m \pm$ S.D.; number of measurements between brackets.

Gestational age of ciliary ganglia	Muscle cells days in vitro	ciliary ganglia, days in vitro				
		1	2	3	4	5
6 days	6	-79.8 ± 6.2 (16)				
	7		-80.5 ± 7.0 (22)			
	8			-77.4 ± 6.0 (7)		
7 days	6	-83.9 ± 5.1 (18)				
	7		-81.2 ± 4.8 (31)	-77.6 ± 3.9 (7)		-82.7 ± 3.1 (3)
	8			-78.3 ± 7.5 (6)	-79.6 ± 5.4 (7)	
8 days	6	-82.1 ± 3.0 (9)				
	7		-79.9 ± 6.3 (13)			
irrespective of gestational age	6	-82.0 ± 5.4 (43)				
	7		-80.7 ± 5.8 (66)	-77.6 ± 3.9 (7)		-82.7 ± 3.1 (3)
	8			-77.9 ± 6.4 (13)	-79.6 ± 5.4 (7)	

The means for various groups of myotubes that had been cultured together with ciliary ganglia of different gestational age for different periods varied from maximally 83.9 mV ± 5.1 mV (± S.D.) to minimally -77.4 mV ± 6.0 mV (± S.D.) (see table 1). A systematic shift could not be demonstrated.

3.3.2.3. Influence of gestational age on innervation

In the vicinity of explanted ciliary ganglia many visually contacted myotubes appeared to be functionally innervated. Spontaneous end plate potentials (epp's) could be recorded from myotubes within 24 hours after explantation of ciliary ganglia on top of the cultured muscle cells (Hooisma et al., 1975). Spontaneous epp's were never recorded in the absence of a ciliary ganglion or in myotubes situated far away from the ganglion. The influence of the gestational age on the ability of the ganglia to form functional neuromuscular connections was investigated. Ciliary ganglia with a gestational age of 6-, 7- or 8-days were explanted on top of comparable cultures of muscle cells. In 23 cultures a total of 133 myotubes was investigated. The frequency of spontaneous epp's and their amplitude distribution were used to determine the degree of innervation. The number of epp's and the amplitude varied significantly between the individual myotubes in one culture. The percentage of myotubes with more than 100 epp's per 2 min. varied from 0 to 100% for the same gestational age and innervation time in different cultures. There was a slight decrease from 6 to 8 days gestational age in efficiency of innervation. All further experiments were done with 7-day old ciliary ganglia.

3.3.2.4. End plate potentials in newly formed junctions

The formation of neuromuscular junctions around an explanted ganglion was thought to be dependant on the time during which the ciliary ganglion was in culture. A series of muscle cell cultures was prepared on which ciliary ganglia were explanted on day 4 or 5 of cultivation. At various days of co-cultivation, innervation was studied

by intracellular recording. Spontaneous epp's were recorded for periods of 2 minutes. It appeared that the variation in amount of neuromuscular transmission between different series of similar experiments was great compared to the variation due to the variation of the time of co-cultivation. No significant change in amplitude distribution or maximal amplitude of the epp's was found. The percentage of myotubes with more than 100 epp's per 2 min. increased with the length of the period that the ciliary ganglia had been in the culture. When the neuronal cells had been in culture for one day the percentage was 38% (n = 37), whereas the percentages at days 2 and 3 were 60% (n = 66) and 70% (n = 20), respectively. Apparently, the influence of the duration of co-cultivation on the amplituce is negligible, but a small effect on the frequency was observed. For practical reasons all further data have been treated irrespective of the length of the period that the ciliary ganglia had been in culture. However, a check on possible influence of the timing of the explantation was usually made afterwards.

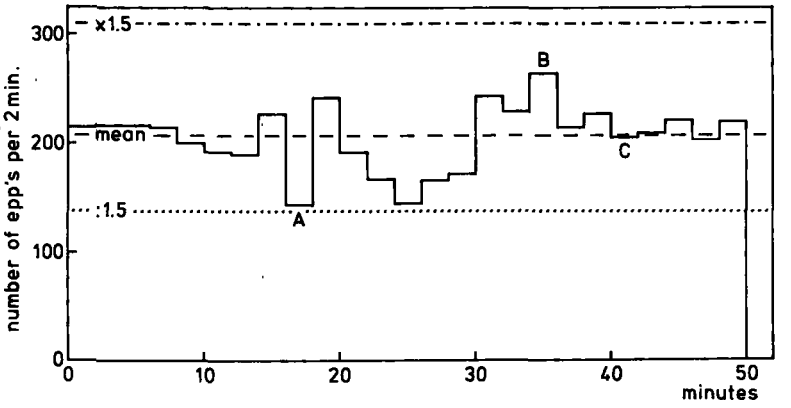

Fig. 6: Stability of frequency of occurrence of spontaneous epp's

The number of epp's in a period of 2 min is plotted for the duration of the experiment. In the whole 50 min period the average number was 209 per 2 min. All values remained within the limits of a factor of 1.5. Ciliary ganglion and muscle cells 2 and 3 days in vitro, respectively. V_m = -90 mV, discrimination level 0.4 mV. Periods A, B and C are presented in fig. 7 and 8 in different representations.

3.3.2.5. Stability of end plate potential generation during electrophysiological experiments

The amplitudes of the spontaneous epp's varied from the noise level (about 0.2 mV) up to 20 mV. Occasionally epp's were observed with amplitudes that exceeded the threshold for action potential generation. Fig. 6 shows results obtained in a typical experiment. In the tachogram each bar represents the number of spontaneous epp's recorded in a period of two minutes. Clearly, in this experiment the average frequency of occurrence was stable within ⁑ 1.5. Fig. 7 shows a different representation of the same experiments.

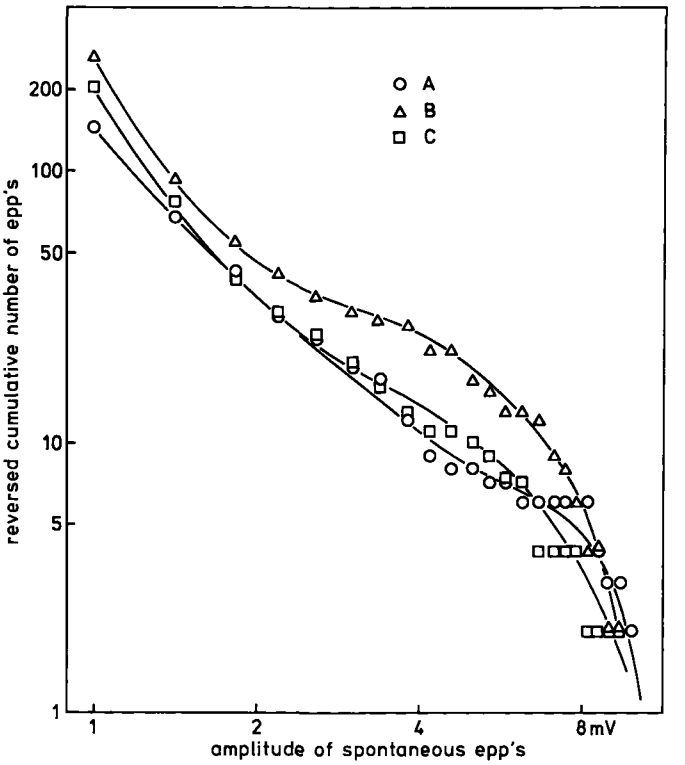

Fig. 7: Reversed cumulative representation of the epp amplitude distribution

The number of epp's in the 2 min period is cumulated from the higher to the lower ones. The three distributions are well within a factor of 1.5 which on the logarithmic scale corresponds with a displacement of 0.18. Curves fitted by eye.

The distribution of the amplitudes in a period of 2 minutes is plotted on a double logarithmic scale. On the abscissa the midclass amplitudes are plotted. On the ordinate the reversed cumulative number is represented (see paragraph 3.2.2.2.). The figure shows 3 distributions that are representative for the variance over the period of 50 minutes. The variation stays well within the limits of ⁑ 1.5, that is within a displacement of 0.18 on the double logarithmic scale. Fig. 8 shows the amplitude distribution of the 2 min. periods represented in fig. 6.

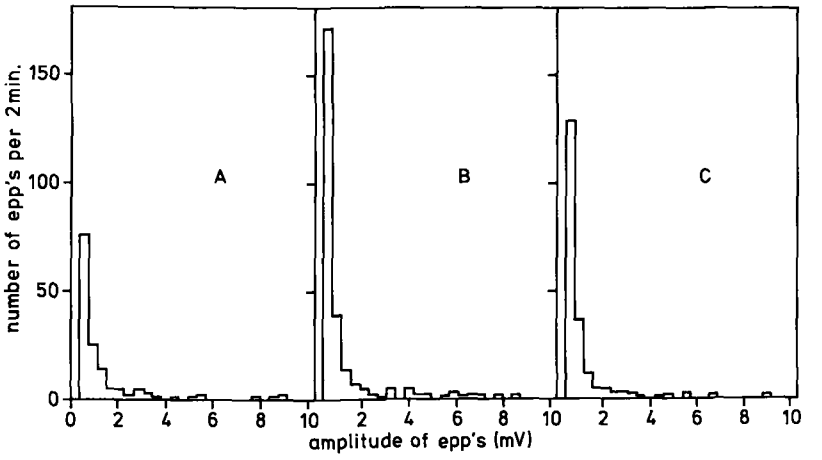

Fig. 8: Histograms showing amplitude distributions
Histograms are shown of periods A, B and C of fig. 6.

In another 4 comparable experiments the variation during the experiments never exceeded a factor of ⁑ 1.5. Moreover, in most experiments carried out, the frequency of occurrence of end plate potentials during control periods before and after an experimental condition were within these limits. When the effects of drugs on the occurrence of spontaneous epp's was studied, such effects were taken to be significant if the number of epp's per period of 2 min. was shifted over more than a factor of 1.5.

3.3.2.6. Multiple innervation

From the morphological data it is known that myotubes in the vicin-

ity of the ganglion generally receive innervation from more than one neurite. It can be demonstrated electrophysiologically that this innervation is functional. In fig. 9 parts of the registration from a single myotube are shown.

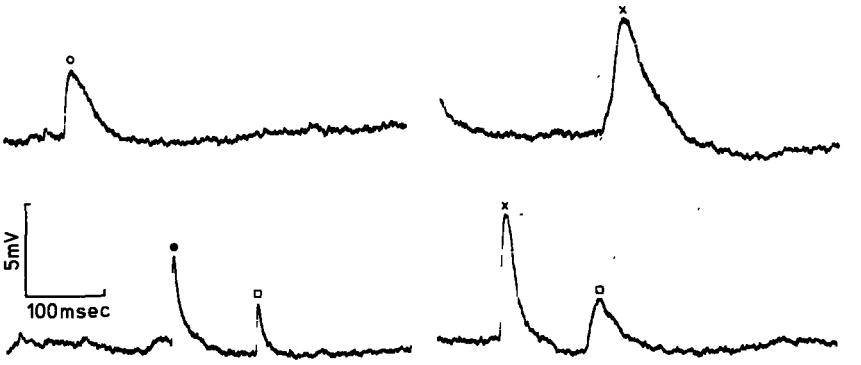

Fig. 9: Multiple innervation

The traces represent the membrane resting potential and show various spontaneous epp's occurring in the same cell. Epp's of approximately the same amplitude (0, □, X) differ markedly in time course.

It appears that epp's of approximately equal amplitude clearly exhibit different rates of rise and decay times. The electrical properties of the myotubes cause the attenuation of the amplitude and shape of the epp's if they are recorded at some distance from their site of origin. The further away the location of the microelectrode, the lower the amplitude, the slower the rate of rise and the longer the decay time will be. Epp's of approximately equal amplitude and clearly different shapes are thus indicative of multiple innervation. The fastest epp's had times to peak ranging from 2-5 msec whereas the slowest epp's could be a factor of 2-4 slower. The half decay time ranged from 5-40 msec.

3.3.2.7. Quantal nature of end plate potentials

The release of ACh at the neuromuscular junctions in discrete amounts could be demonstrated in a few experiments in which a singly innervated myotube happened to be impaled. In fig. 10 spontaneous epp's are shown that have been recorded from a myotube. There

were small epp's up to 4 mV (fig. 10[a]) and large ones that sometimes triggered action potentials (fig. 10[b]).

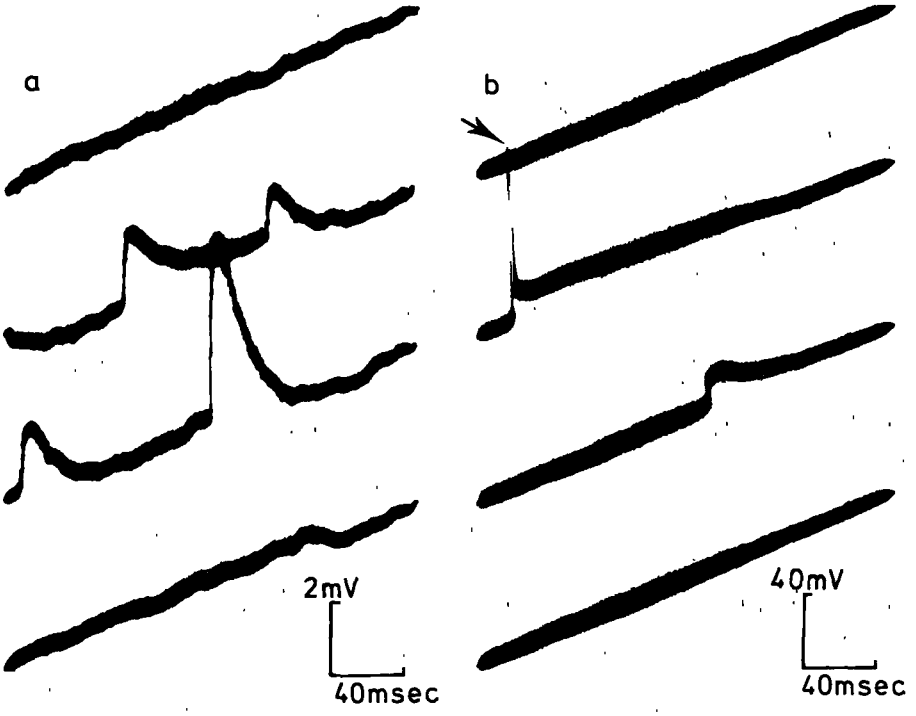

Fig. 10: Small and large spontaneous epp's

In a) recordings with small epp's of up to 4 mV are shown. In b), recorded from the same cell, a large epp (15 mV) is shown and one epp that triggered an action potential (arrow) with an overshoot of 5 mV. V_m = -88 mV. Dissociated ciliary ganglion cells together with muscle cells, 8 and 12 days in vitro, respectively.

A histogram of the smaller epp's is given in fig. 11 which shows distinct peaks at multiples of the smallest epp's with a unit size of about 0.7 mV. This multimodal amplitude histogram demonstrates quantal release of the ACh.

3.3.2.8. Pharmacological nature of receptors of end plates

The nature of the receptors at the neuromuscular junctions was investigated by the determination of the sensitivity of the epp-generating synapses to d-tubocurarine and atropine, substances which

preferentially block nicotinic and muscarinic ACh-receptors, respectively (Beránek and Vyskočil, 1967). Fig. 12 shows the results of a typical experiment.

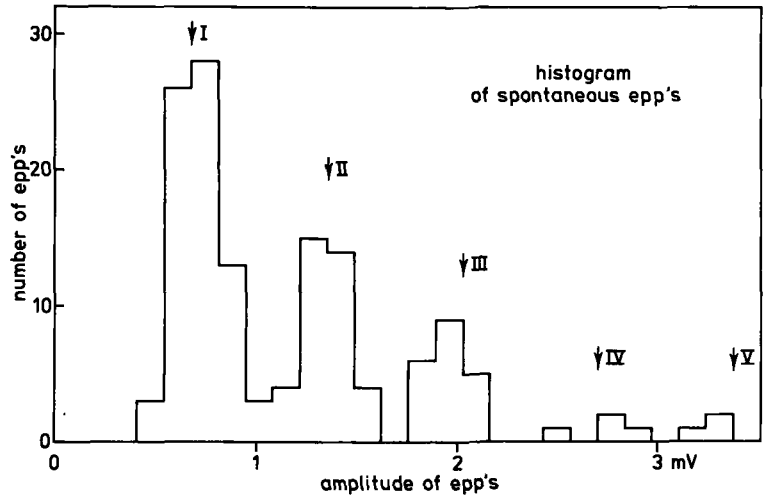

Fig. 11: Multimodal amplitude histogram of spontaneous epp's

Histogram of the epp's in the experiment described in fig. 10. There are distinct peaks at multiples of the unit epp's, demonstrating the quantal nature of the epp's. Noise level 0.4 mV.

After a control period of 4 min. in which the number of spontaneous epp's was about 400 per 2 min., d-tubocurarine at a concentration of 0.05 µg/ml reduced the amplitude of nearly all spontaneous epp's to a value below the discrimination level. After 20 min. of washing, the amplitudes of the epp's were back to about the initial value. Atropine, at a concentration of 10 µg/ml, had no marked effect. When more atropine was added, to a final concentration of 100 µg/ml, about 80% of the epp's became reduced below the discrimination level. In 20 different cultures d-tubocurarine was tested in concentrations ranging from 0.05 µg/ml to 5 µg/ml. At the level of 0.1 µg/ml the number of epp's per 2 min. remained mostly within the stability limits of $\frac{x}{x}$ 1.5, but 1 µg/ml almost completely blocked the epp's. Atropine was tested in concentrations ranging from 1 to 100 µg/ml. Atropine at a concentration of 1 µg/ml did not affect the amplitude of the epp's. The effect of 10 µg/ml was only slight, whereas 100 µg/ml

resulted nearly always in a reduction of the largest epp's nearly to the noise level. In 7 experiments the sensitivity of the receptors at nerve muscle junctions to d-tubocurarine and atropine was tested on one and the same myotube. In all these cases the concentration of atropine needed to block the epp's was at least 100 times that of d-tubocurarine.

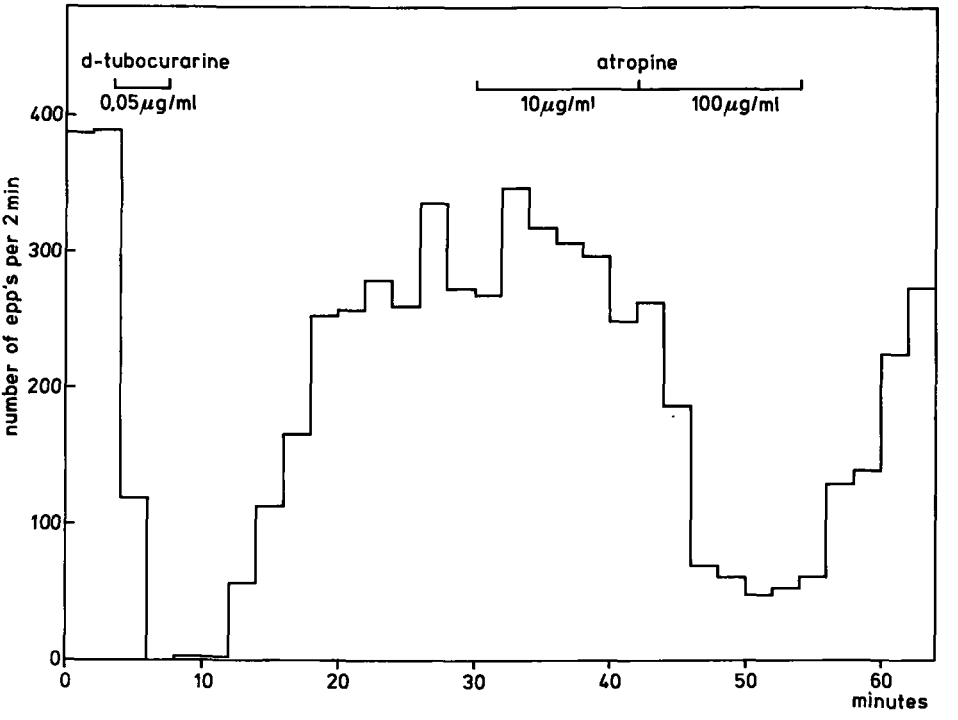

Fig. 12: Nature of end plate receptors

The effect of d-tubocurarine and atropine on the occurrence of spontaneous epp's. After 4 min of control activity, administration of d-tubocurarine (0.05 µg/ml) reduced the amplitude of nearly all epp's to a value below the discrimination level of the computer program; in this case set at 0.8 mV. After washing, the amplitude of the epp's returned to normal. Atropine had no marked effect at 10 µg/ml but about 80% of the epp's became smaller than 0.8 mV when the atropine concentration was raised to 100 µg/ml. Ciliary ganglion and muscle cells 1 and 3 days in vitro, respectively; V_m = -88 mV.

The sensitivity of the epp's for physostigmine at concentrations of 1-20 µg/ml was studied in order to obtain information about the presence of functional acetylcholinesterase (AChE) at the newly formed junctions. The rate at which ACh is hydrolysed determines the time

during which ACh is interacting with the ACh-receptors and thus the duration of the epp's. Physostigmine blocks AChE by interaction with its catalytic site. If AChE is active at the newly formed neuromuscular junction, inhibition of this activity should result in a prolonged interaction of ACh with the receptors and a prolonged duration of the epp's. Tested in the present cultures physostigmine did not influence the duration of the epp's at day 1, 2 or 3 of co-cultivation of the ciliary ganglion with the muscle cells. It is known that in vivo higher concentrations of physostigmine exert a curare-like depression of the amplitude of the epp's while the longer duration of the epp's remains. In the present experiments, physostigmine caused a reversible depression of the epp amplitude at a concentration of 20 µg/ml, but even under these conditions the epp's were not lengthened.

3.3.2.9. Stimulus evoked end plate potentials

An attempt has been made to impale ciliary neurons and to use them to stimulate the neuromuscular junctions.

Fig. 13: Action potential of a ciliary ganglion neuron

The action potential was triggered by a step of current of 100 msec duration, with the electrode in a balanced circuit. The action potential had a maximal rate of rise of 100 V/sec and an overshoot of +20 mV. In the repolarizing phase a hyperpolarizing after potential was seen. Ciliary ganglion neuron 12 days in vitro; V_m = -60 mV.

Only in two neurons it was possible to obtain a membrane potential that remained stable for a period sufficiently long to perform a good intracellular measurement. In both cells action potentials could be elicited by depolarization of the cell membrane. Fig. 13 shows a tracing from one of the two neurons. The action potential had a maximal rate of rise of about 100 V/sec and an overshoot of +20 mV. Since intracellular stimulation of ciliary neurons and subsequent recording from a connected myotube appeared to be impractical, two other techniques were employed to demonstrate conduction of action potentials from the neuron through the neurites to the neuromuscular junctions:

a. extracellular stimulation of neurites or bundles of neurites, and
b. iontophoretic application of ACh on the ciliary explant.

It proved possible to apply an extracellular stimulus to neurites that visibly contacted a myotube. End plate potentials could be recorded in that myotube shortly after extracellular stimulation. It was often found that what was thought to be one neurite in fact often consisted of a bundle of neurites. Fig. 14 shows the results of such an experiment. A schematical drawing clarifies the experimental situation in culture; the myotube seemed to be innervated by 3 processes. Each of the processes was stimulated extracellularly with different stimulus intesities. The epp's recorded from the myotube had different amplitudes, rates of rise and latencies. Epp's were abolished if the extracellular stimulation electrode was shifted only a few microns away from the processes. On a graded increase of the strength of the stimulus applied to one process (or bundle of processes), abrupt changes in epp-amplitude were seen. When bundle no. 1 was stimulated, at least three different types of epp's could be distinguished, indicating that the bundle consisted of at least 3 neurites. Process 3 consisted probably of one neurite, since a comparatively high stimulus strength produced either a larger epp, or a failure.

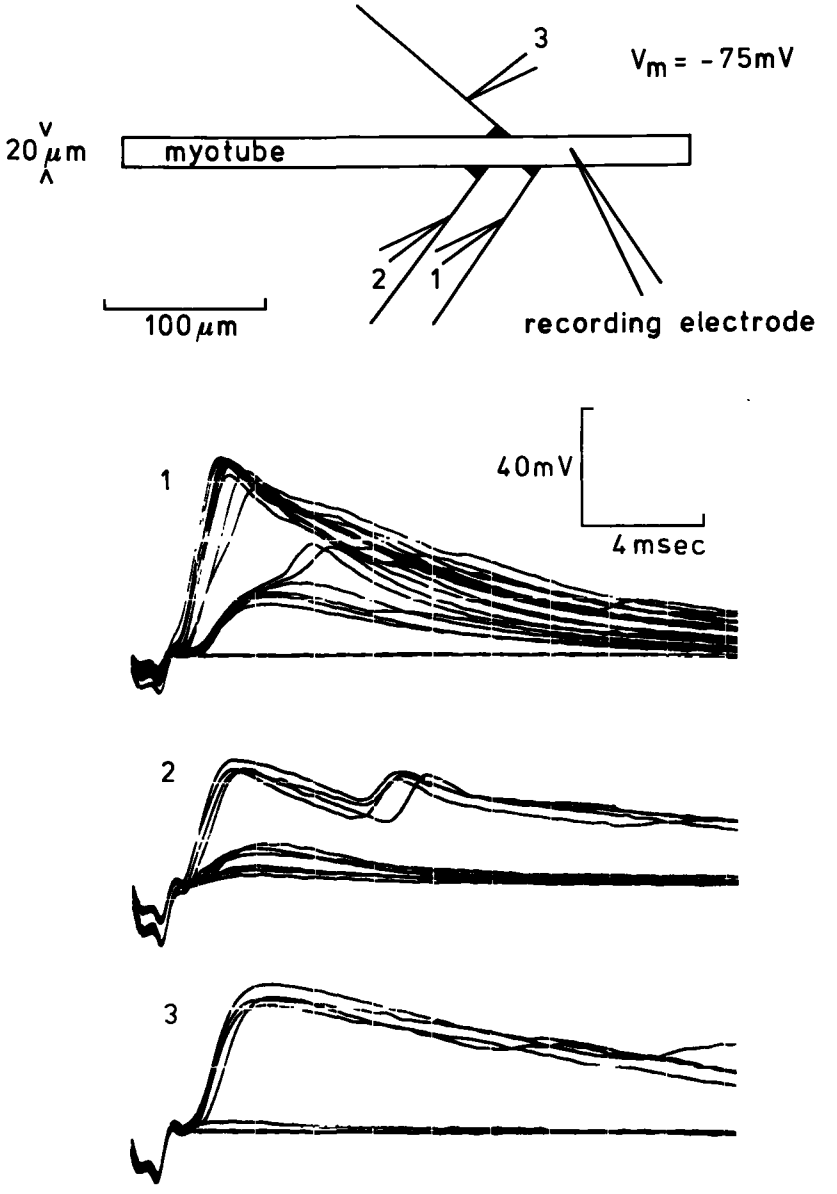

Fig. 14: Extracellular stimulation

Upper part: a schematical representation of the situation in the culture. Three neurites or bundles of neurites (1, 2, 3) are shown and the position of the recording electrode. Lower part: traces recorded with different positions of stimulating electrode. Graded stimulus intensity change resulted in abrupt potential transient changes. Note different latency times. Ciliary ganglion and muscle cells 2 and 5 days in vitro, respectively; V_m = -75 mV.

Since in the chick the ciliary ganglia are cholinoceptive, it was tried to induce action potentials in the cultured ciliary ganglion cells by ACh-iontophoresis on the explant and in this way to study the ability of the neurites to conduct action potentials to the neuromuscular junction. At low magnifications (100x) it was observed that by iontophoretic application of pulses of ACh on the ganglion, contractions could be induced in myotubes synchronous with the ACh pulses. It was observed that the application of ACh only resulted in contractions in the myotubes if the ACh was deliverd to

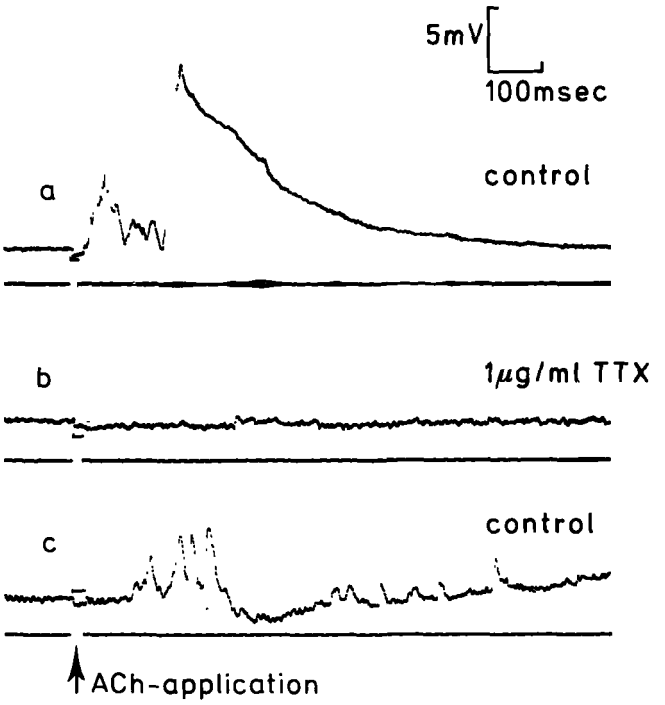

Fig. 15: Influence of TTX on conduction of action potentials

a. ACh applied iontophoretically on the ciliary ganglion (pulse on lower trace). Upon ACh-application, complex epp-responses could be recorded in a myotube (upper trace). The complex response varied from stimulus to stimulus.
b. No respons to ACh could be elicited in the myotube 5 min after addition of TTX to the perfusion fluid.
c. After washing, the stimulus evoked epp's returned. Washing time was to short to regain the total response.

Ciliary ganglion and muscle cell 8 and 12 days in vitro, respectively; V_m = -82 mV.

certain parts of the explants. If a contracting myotube was impaled with a microelectrode, depolarizations were seen synchronous with the ACh pulses. These depolarizations were not caused by leakage of ACh to the myotubes, since it was found that bringing the ACh pipette closer to the myotube, but away from the explant abolished these epp's. The presence of epp's following ACh-application to the explant indicated that the neurons in the cultures ciliary ganglion are sensitive to ACh and that the nerve processes formed in vitro are capable to conduct action potentials. The epp's occurred at different latency periods after the stimulus, indicating the existence of more synapses on one and the same myotube. Fig. 15 shows the response of a myotube to ACh application. If TTX was added to the bath in a concentration of 1 µg/ml the epp's which occurred synchronous with the iontophoretic pulses of ACh to the explant, were abolished. This effect could be reversed by washing out the TTX. In four other experiments the blocking effect of TTX was confirmed. In the presence of TTX, application of ACh to any part of the explant did not result in eliciting contractions in the myotubes, whereas after washing out of the TTX several parts of the explants responded to ACh again. Concentrations of 1 µg/ml TTX were sufficient to prevent the occurrence of epp's generated by nerve impulses conducted from the neurons to the neuromuscular junction.

3.3.2.10. Influence of tetrodotoxin on spontaneously occurring end plate potentials

The spontaneously occurring end plate potentials have been used as parameter to study the sensitivity of the neuromuscular junction to pharmacological substances. It was assumed that the total population of spontaneous depolarizations consisted of epp's evoked by spontaneous neuronal activity as well as mepp's due to spontaneous release of one package of ACh at the neuromuscular junction. TTX was routinely applied to the bath at concentrations of 1 µg/ml or more. TTX had neither an effect on the frequency of occurrence nor on the amplitude distribution of the spontaneous epp's in 8 out of 13 experiments (see

table 2, which is situated on the back cover of this thesis), even when the concentration of TTX was raised to 100 µg/ml (exp. 15). The absence of an effect of TTX indicates that only mepp's, due to the spontaneous release of ACh vesicles, occurred. In 3 experiments TTX blocked the larger spontaneous epp's, whereas in 3 experiments the smaller epp's decreased in frequency (see table 2). Because of the multiplicity of innervation even the smaller spontaneous potentials could be neurally evoked. A selective block of the larger spontaneous epp's as well as a decrease in frequency are indicative for the presence of neurally evoked epp's.

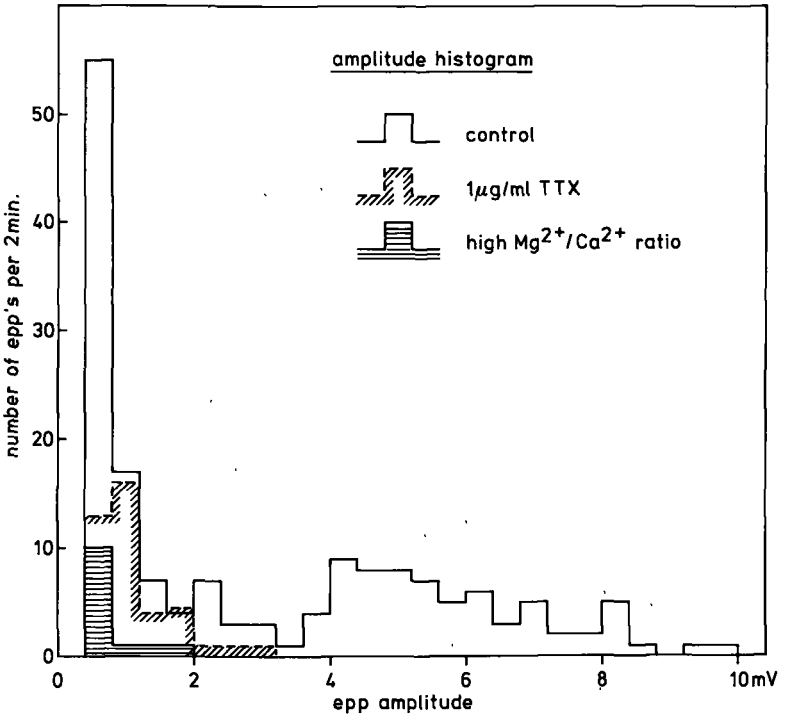

Fig. 16: Amplitude histogram of experiment 14 table 2

The figure shows a histogram of the control situation, in which distinct peaks can be seen for neurally evoked epp's (> 3 mV) and mepp's (< 3 mV). In the presence of TTX, all epp's have disappeared. A high Mg^{2+}/Ca^{2+} ratio in the perfusion fluid affects also the amplitude distribution of the mepp's (see also fig. 17). Ciliary ganglion and muscle cells 9 and 12 days in vitro, respectively; V_m = -70 mV.

In most experiments the multiplicity of innervation gave rise to a skewed amplitude distribution (compare fig. 8), but fortunately the spontaneous epp's in experiment 14 were remarkably uniform. The amplitude distribution showed distinct peaks for mepp's evoked by neuronal activity. In the presence of TTX all larger (neurally evoked) epp's had disappeared (fig. 16, 17).

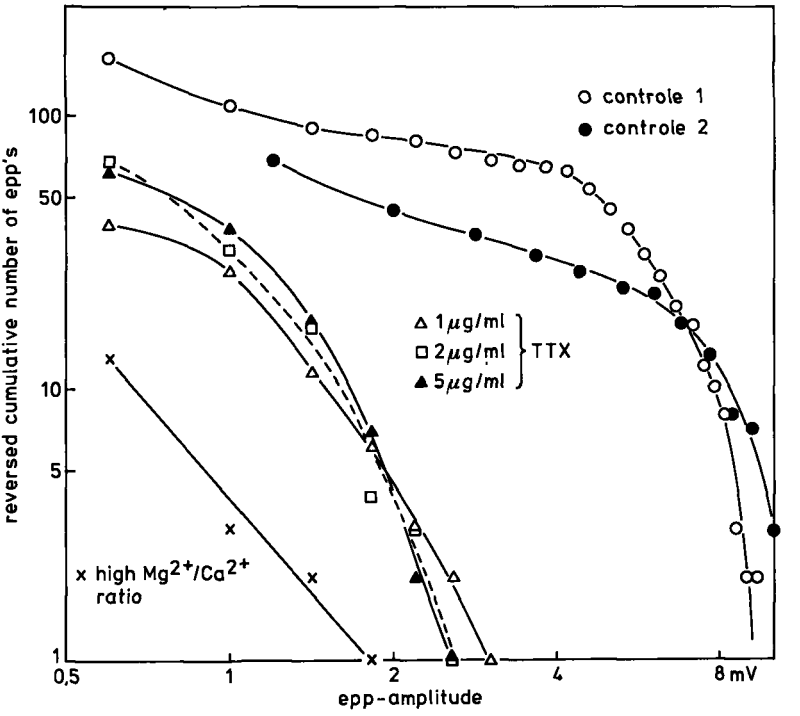

Fig. 17: Reversed cumulative distribution of the same experiment

The reversed cumulative distribution is explained in paragraph 3.2.2.2. The experiment started in perfusion fluid with a high Mg^{2+}/Ca^{2+} ratio. After returning to perfusion with normal Tyrode's solution (control period 1), TTX was added for 1 µg/ml and there after the concentration of TTX was raised to 2 and 5 µg/ml. At the end of the experiment the perfusion was carried out with normal Tyrode's solution (control period 2).

The amplitude of the spontaneous epp's did hardly change with the period of co-cultivation. In younger cultures TTX had no effect but in the older ones the spontaneous epp's that persisted in the presence of TTX had lower amplitudes. The figures suggest that the mepp-amplitude had decreased with co-cultivation time and that the quan-

tum content had increased.
If the effects of TTX are compared with the age of the myotubes at the time of explantation of the ganglia it seems that the younger the myotubes, the better the innervation.

3.3.2.11. Influence of a high Mg^{2+}/Ca^{2+} ratio on spontaneous end plate potentials

In 7 experiments the Mg^{2+} concentration was raised by a factor of 10, whereas the Ca^{2+} concentration was lowered by a factor of 4 (see table 2). Only in exp. 6 the change in Mg^{2+}/Ca^{2+} ratio was carried out in the presence of TTX. In all other experiments a washing period separated perfusion with TTX and exposure to a high Mg^{2+}/Ca^{2+} ratio. It was expected that as a result of the change in the Mg^{2+}/Ca^{2+} ratio the quantum content of the epp's would decrease and that only mepp's would remain with the same frequency of occurrence and amplitude distribution as the mepp's observed in the presence of TTX.
In 6 experiments this Mg^{2+}/Ca^{2+} ratio change resulted in a decrease of the amplitudes of the spontaneous depolarizations by a factor of 2-8. In one experiment (exp. 7) a shift in frequency occurred. In exp. 6 the change of the Mg^{2+}/Ca^{2+} ratio was studied in the presence of TTX. Tetrodotoxin had no influence on the amplitudes of the spontaneous depolarizations, suggesting that only spontaneous mepp's were present. The change in the Mg^{2+}/Ca^{2+} ratio, however, decreased the amplitude by a factor of 2. In this case the high Mg^{2+}/Ca^{2+} ratio influenced the mepp-amplitude. If for the 6 experiments the amplitudes of the depolarizations that remained in the presence of a high Mg^{2+}/Ca^{2+} ratio were compared with those that remained in the presence of TTX, the amplitude appeared to decrease by a factor of about 2. In fig. 16 and 17 the effect of a high Mg^{2+}/Ca^{2+} ratio is shown in a normal plot of the amplitude distribution and in a reversed cumulative representation, respectively.
The experiments show that a high Mg^{2+}/Ca^{2+} ratio affected the amplitude of those potentials that remained in the presence of TTX and thus had to be interpreted as mepp's. Unexpectedly, the amplitudes of these mepp's were reduced two-fold by a high Mg^{2+}/Ca^{2+} ratio.

3.4. Discussion

3.4.1. Influence of gestational age on synaptogenesis

The innervation of chick myotubes by ciliary ganglion explants in tissue culture was demonstrated by Hooisma et al. (1975) and Betz (1976). The present investigations showed that the growth of neurites from ciliary ganglion explants decreased when the ganglia were taken from embryos older than 6-8 gestational days. This could be due to the progressive differentiation of the ganglionic neurons in vivo or to the fact that from gestational day 8 the number of living neurons decreases in 4 days by a factor of 2, as reported by Landmesser and Pilar (1974b). These authors found that if in vivo ciliary ganglia were deprived of their postganglionic structures by removal of the optic vesicle between gestational day 3 and day 6, the neurons were influenced in an unfavourable way. Under those circumstances most cells die, a process which begins at gestational day 8 and leads to degeneration of 90% of the cells within a week. Thus without a target most cells survived in vivo only a limited number of days. If deprived of their target, as occurs in tissue culture they might therefore be expected to show less power to regenerate.

3.4.2. Membrane resting potential

Contrary to expectation, innervation by ciliary ganglion neurons had no influence on the membrane resting potential, V_m, of the myotubes. Albuquerque and Thesleff (1968) reported that in denervated muscles the V_m was about 15 mV lower than in innervated ones. In the present study V_m ranged from -60 mV to -95 mV with a mean of -81 mV. Fischbach (1970, 1972) and Betz (1976) reported membrane resting potentials ranging up to -75 mV, while most of the values they found were between -50 mV and -60 mV. However, these investigators used pectoral muscle as the source for the myoblasts whereas in the present study leg muscle was used. It is known (Karzel, 1968) that in the chick at birth the membrane resting potential of breast muscle is lower than that of thigh muscle; -25 mV and -58 mV respectively. In 60 days old chicks no difference is left,

the values being -93 mV and -96 mV, respectively. This could account for the high membrane resting potentials found in the present experiments.

3.4.3. Influence of co-cultivation time on innervation

The outgrowth of neurites started immediately after attachment of the ganglion. Within one day many outgrowing neurites were within the vicinity of myotubes. At this early stage the synapses formed were already able to generate end plate potentials, but membrane specializations were not present. The number of epp's and the amplitude distribution could have been influenced by: (1) the gestational age of the ciliary ganglion and (2) the period of co-cultivation of myotubes and ciliary ganglia. The percentage of impaled myotubes that showed epp's was not influenced by variation in the gestational age (6-8 days) of the ciliary ganglion which was used as source of innervating neurons. This could be due to an unintentional selection for impalement of those myotubes that visually seemed to have contact with neurites. The frequency of occurrence of epp's was related to the period of co-cultivation. Possible further age related differences may have been obscured by the great differences between various series of experiments. Moreover, a neuromuscular contact observed three days after explantation might have been formed any time following explantation. From the experiments in which TTX was applied, it can be concluded that the mean mepp amplitude decreased with innervation time. Since the amplitude of all spontaneous epp's did not change with innervation time, it must be concluded that the quantum content of the epp's did increase. The decrease of the mepp amplitudes must have been caused by a decrease of the input resistances of the myotubes, which could readily be caused by the fact that the width of the myotubes increased with age.

3.4.4. The nature of the receptors

The nature of the receptors was established by determination of their differential sensitivity to d-tubocurarine and atropine. In all experiments the receptors were at least 100-fold more sensitive to d-tubo-

curarine than to atropine. This means that all neuromuscular junctions investigated were of the nicotinic type (Beranék and Vyskočil, 1967). The question can be raised whether only the ciliary neurons in the ciliary ganglion innervated the skeletal muscle cells, since in vivo the ciliary neurons form nicotinic synapses. In chapter 5 data will be presented on the innervation of cultured skeletal muscle cells by chick or rabbit ciliary ganglia, which in vivo innervate muscle fibers with either nicotinic and muscarinic, or only muscarinic receptors, respectively. Since both types are able to form nicotinic synapses, the fact that the chick neurons only induced nicotinic synapses is no proof for innervation exclusively by the ciliary neurons. The junctions formed between the chick ciliary ganglia and the myotubes were immature. With the aid of physostigmine no functional acetylcholinesterase could be demonstrated, which confirmed findings of other investigators (paragraph 2.3.2.).

3.4.5. Action potential generation and conduction by the neurons

Ciliary neurons were very fragile and difficult to impale. In the two cases in which a rather stable registration could be obtained, the neurons were be able to generate action potentials upon intracellular stimulation. ACh application to the explants gave rise to activity in the myotubes showing that the neurites could conduct impulses but also that the neurons were cholinoceptive. TTX reversibly blocked the evoked epp's and thus conduction of action potentials in the neurites. The application of ACh was only effective at distinct areas of the ganglia. This suggested that only few neurons took part in the innervation. Others might have died because they did not find a suitable target at the right time. Conduction of action potentials through the neurites to the end plates could also be demonstrated by extracellular stimulation of the nerve processes which resulted in epp's and contractions of the myotubes.

3.4.6. Effect of tetrodotoxin on spontaneous end plate potentials

In frog and mammalian nerve-skeletal muscle preparations, TTX blocks

nerve conduction and thus abolishes all neuronally evoked end plate depolarizations. Only mepp's remain and their amplitude and frequency is hardly affected by TTX. In the present experiments the majority of the myotubes (8 out of 13) examined did not respond with a change in amplitude or frequency of its spontaneous end plate potentials at concentrations of TTX that had proven to block nerve conduction. In five experiments out of 13 either a selective block of high amplitude spontaneous depolarizations was found or an overall decrease in frequency of occurrence of all spontaneous depolarizations. In the multiply innervated myotubes, junctions situated far away from the tip of the microelectrode may produce multiquantal potentials (epp's) which are not recognized as such due to the attenuation by the distance. If such a myotube is treated with TTX, loss of these epp's may look like a mere reduction of the frequency of all spontaneous potentials over the whole amplitude range.

The results led to the conclusion that more than half of the myotubes examined produced only potentials of a mepp-like character, i.e. not due to impulses coming from the neurons. The other myotubes showed, besides mepp's, also epp-like potentials which could be abolished by TTX. The results are in substantial agreement with those obtained by Betz (1976) in similar cultures. Definite proof for the occurrence of large spontaneous junctional potentials (sjp's) as reported by Pilar and Vaughan (1969) and Pilar (1969) in the isolated preparation of the ciliary ganglion-iris muscle of the pigeon was not obtained in the present experiments.

3.4.7. Influence of a high Mg^{2+}/Ca^{2+} ratio

The results of the experiments with TTX have to be compared with those obtained during perfusion with a solution with a high Mg^{2+}/Ca^{2+} ratio. Del Castillo and Katz (1954) showed that in the frog the quantum content of the epp's is drastically lowered in the presence of a high Mg^{2+}/Ca^{2+} ratio. The effect on mepp's, however, is quite different. Hubbard et al. (1968) showed that in mammalian motor nerve terminals under comparable circumstances the mepp frequency decreased less than

20% and the mepp amplitude less than 30%. The effects of a high Mg^{2+}/Ca^{2+} ratio on the frequency and the amplitude were found to be of presynaptic and postsynaptic origin, respectively. The presynaptic effects of Mg^{2+} and Ca^{2+} were counteracting on another, but there existed no simple relation between the effects. If at a certain Ca^{2+} concentration the Mg^{2+} concentration was raised, the mepp frequency decreased but with further increase of the Mg^{2+} concentration, the mepp frequency increased again. The effects of increasing the Mg^{2+}/Ca^{2+} ratio on the postsynaptic membrane, however, are uniform, i.e. an increase in Mg^{2+} concentration decreases the mepp amplitude irrespective of the concentration of Ca^{2+}. In tissue culture Fischbach (1972) studied the influence of a high Mg^{2+}/Ca^{2+} ratio on epp's in mixed cultures of dissociated chick spinal cord neurons with chick skeletal muscle cells. In that preparation the amplitudes of the epp's decreased from about 10 mV to the level of the mean amplitude of the mepp's (2 mV), whereas the mepp amplitude decreased less than 20% and the mepp frequency remained unaltered.

In the present experiments with a high Mg^{2+}/Ca^{2+} ratio a reduction in amplitude of the epp's was expected in those myotubes in which spontaneous epp's could be blocked by TTX. In the two myotubes in which TTX caused a block of epp's (exp. 12 and 14) this prediction came true. It was expected that a high Mg^{2+}/Ca^{2+} ratio would have little or no effect in myotubes that generated only mepp-like potentials. Similarly, little or no influence was expected on the mepp-like potentials in those myotubes that had their epp's blocked by TTX. In all 7 myotubes tested in the presence of a high Mg^{2+}/Ca^{2+} ratio, however, the amplitude of the mepp-like potentials was reduced. In 6 myotubes the high Mg^{2+}/Ca^{2+} ratio reduced the amplitudes by a factor of about 2 compared with the effect of TTX. This change in amplitude of the mepp-like potentials is much greater than that obtained in vivo by Hubbard et al. (1968) and in vitro by Fischbach (1972). Such a large change could be explained if the postsynaptic membrane, where the mepp's are formed, was more sensitive to changes in the Mg^{2+} and/or the Ca^{2+} concentration than the mammalian

nerve muscle junction, as described by Hubbard et al. (1968). Another possibility is that the mepp's in these nerve-muscle junctions are not uniquantal but multiquantal. The high Mg^{2+}/Ca^{2+} ratio change could than act on the mechanism by which vesicles are dragged along with the vesicle that starts a mepp (Martin and Pilar, 1964). The quantum content of the multiquantal mepp-like potentials would then decrease like the quantum content of the epp's. If such an explanation were correct the change in Mg^{2+} and Ca^{2+} would only reduce the amplitude of the mepp-like potentials and not their frequency. In fact, a high Mg^{2+}/Ca^{2+} ratio had little effect on the frequency in the present experiments, which is compatible with the hypothesis. Up to now clustering of mepp's as the result from dragging of vesicles has been observed only incidentally in vivo, but recently, Van der Kloot et al. (1976) have demonstrated that clustering (= dragging) of mepp's occurs more often than believed up till now.

3.5. Summary

1. Electrophysiological and pharmacological properties of neuromuscular junctions formed in tissue culture between chick ciliary ganglia and chick skeletal muscle cells have been determined.
2. Functional neuromuscular junctions are formed within 24 hours.
3. The neurons in the ciliary ganglion are cholinoceptive and capable of generating action potentials. The neurites conduct the action potentials to the neuromuscular junction, where epp's are generated.
4. The newly formed end plates are cholinergic nicotinic in nature. No functional AChE is present at the end plates.
5. TTX blocks nerve conduction in tissue culture. In the presence of TTX mepp-like potentials remain whose amplitudes are strikingly affected by a high Mg^{2+}/Ca^{2+} ratio in the medium.
6. It is suggested that these mepp-like potentials are multiquantal and that this multiplicity is affected by a high Mg^{2+}/Ca^{2+} ratio.
7. The figures suggest that the number of spontaneously generated epp's and the quantum content of the epp's increases with co-cultivation time.

3.6. References

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Chapter 4

4. Acetylcholine sensitivity of chick muscle cells in the absence and presence of innervation by the chick ciliary ganglion in tissue culture

4.1. Introduction

4.1.1. Measurement of acetylcholine sensitivity

Transmission of nerve impulses from motor axons to muscle cells occurs at a specialized synapse, the motor end plate. This structure consists of a nerve and a muscle part, separated by the synaptic cleft. Upon the arrival of depolarizations from the nerve cells at the presynaptic part, acetylcholine (ACh) is released into the cleft. The ACh-molecules diffuse through the synaptic cleft and reach the postsynaptic membrane, which contains a great number of ACh-sensitive receptor sites (for a review see Hubbard, 1973). Interaction of ACh with the ACh-receptor leads to an increase of the permeability of the postsynaptic membrane for several small ions and thereby to a drop in the local membrane resistance. Synchronous operation of a large number of ACh-molecules at many receptor sites produces a considerable increase in permeability leading to a flow of current which causes a substantial transient drop of the potential across the postsynaptic membrane, the end plate potential (epp). The density of ACh-receptor sites in the muscle membrane is not constant, but changes during development in early life as well as after denervation and subsequent reinnervation.

The ACh-receptor density of the muscle membrane can be determined in different ways:

a) Intracellular recording of the change in the membrane potential caused by iontophoretic application of ACh (Nastuk, 1953; del Castillo and Katz, 1955). The ACh-sensitivity is usually expressed as the number of mV depolarization per nCoulomb of charge released from the iontophoresis pipette filled with ACh-chloride.

b) Autoradiography after labelling of the receptors with radioactive ^{125}I-α-bungarotoxin, a neurotoxin which is known to bind specifically and irreversibly to the ACh-receptor (Chang and Lee, 1963). If it is assumed that one molecule α-bungarotoxin binds to one receptor, this method allows the receptor density to be expressed in receptors per μm^2.

Hartzell and Fambrough (1972) demonstrated a correlation between the ACh-sensitivity, iontophoretically measured, and the ACh-receptor density as measured by autoradiography using ^{125}I-α-bungarotoxin.

c) Staining of the receptors with fluorescent α-bungarotoxin (Anderson and Cohen, 1974; Axelrod et al., 1976), horse radish peroxidase labelled α-bungarotoxin or indirectly, by the detection of ACh-receptor-α-bungarotoxin complexes, with the aid of fluorescent anti-α-bungarotoxin antibodies (Ringel et al., 1975; Daniels and Vogel, 1975).

4.1.2. Iontophoretic methods for acetylcholine application

Del Castillo and Katz (1955) made the first steps towards a quantitative analysis of ACh-sensitivity of frog skeletal muscle. In their study, however, the rise time of the ACh-potentials, elicited by iontophoretical application of ACh on the muscle, was much slower than that of intracellularly recorded epp's. Krnjević and Miledi (1958) demonstrated ACh-sensitivity at the end plates of the rat diaphragm and were able to elicit ACh-potentials with a time course only two times slower than that of the epp's. The use of Nomarski optics made it possible to perform the precise positioning of the iontophoresis pipette near the postsynaptic membrane required for very accurate measurements. With this improved technique Kuffler and Yoshikami (1975) succeeded in mapping the ACh-sensitivity distribution on μm scale. They were able to elicit ACh-potentials that were only 20% slower in time course than the fastest miniature end plate potentials (mepp's) recorded in the same fiber.

4.1.3. Acetylcholine sensitivity of skeletal muscle in vivo

Miledi (1960) showed that the maximal sensitivity to ACh in the innervated fiber coincided with the end plate as defined by the site at which focal mepp's were recorded. The ACh-sensitivity fell off at either side of this region and could be several thousand times lower a few hundred microns away. Axelsson and Thesleff (1959) demonstrated the influence of denervation on the ACh-sensitivity of the non-end plate region of cat leg muscle (see table 1). No detectable ACh-potential was found in the innervated muscle outside the end plate region, but two weeks after denervation the whole muscle was sensitive to ACh to the level of the end plate region, which maintained its original responsiveness to the drug. In their study of foetal rat diaphragm Diamond and Miledi (1962) demonstrated an ACh-sensitivity over the entire length of the muscle fibers. At birth the chemo-sensitive properties of the muscle started to recede from the tendon ends towards the middle of the fiber, where the end plate is located. The localized sensitivity as seen in the adult animal was attained a few weeks after birth.
Miledi and Potter (1971) showed in rat and frog muscle that α-bungarotoxin irreversibly blocked the ACh-receptors in the end plate as well as the newly developed ones upon denervation in the non-end plate region. The number of receptor sites present in the non-end plate area after denervation was 200 times the number in innervated muscles. Reinnervation led to the disappearance of the enhanced ACh-sensitivity of the non-end plate area. After some time the ACh-sensitivity was localized again in the newly formed motor end plate regions. Fambrough and Hartzell (1972) and Hartzell and Fambrough (1972) found in the adult rat diaphragm in the end plate region and in the non-end plate area receptor densities of $10^4/\mu m^2$ and less than $5/\mu m^2$, respectively. The density appeared to fall off by a factor 10^3 within 150 μm of the neuromuscular junction. Denervation of the muscle resulted in an increase of receptor sites in the non-end plate area from 5 to 1700 receptors per μm^2 in two weeks. After 45 days of denervation the number of receptor sites had fallen to $530/\mu m^2$. Albuquerque et al. (1974) determined ACh-sensitivity and ACh-receptor densities at vertebrate end plates and correlated their

Table 1. Distribution of acetylcholine receptors in vivo

Authors	Preparation	Experimental condition	Location of ACh-receptors on muscle membrane	ACh-sensitivity (iontophoresis)	ACh-receptor density α-bungarotoxin autoradiography
Axelsson and Thesleff 1959	cat leg muscle	control	junctional area		
		denervation	entire surface		
Diamond and Miledi 1962	rat diaphragm	before birth	entire surface		
		1 week after birth	junctional area		
Miledi and Potter 1971	rat diaphragm	control	end plate		5.10^{11} /hemidiaphragm
		denervation	entire surface		10^{13} /hemidiaphragm
		reinnervation	end plate		5.10^{13} /hemidiaphragm
Berg et al. 1972	rat diaphragm	neonatal	entire surface		
		adult	junctional area		
		denervation	entire surface		
Fambrough and Hartzell 1972	rat diaphragm	control	junctional area		10^4 /μm²
			extrajunctional area		< 500/μm²
Hartzell and Fambrough 1972	rat leg muscle	control	end plate	< 0.01 mV/nC	< 5/μm²
		14 days after denervation	spreads over entire surface	310 mV/nC	1700/μm² extrajunctional
		45 days after denervation	decrease of sensitivity		530/μm²
Albuquerque et al. 1974	mouse diaphragm	control	junctional area	2860 mV/nC	8500/μm²

results with ultrastructural specializations. Berg et al. (1975) confirmed the results of Diamond and Miledi (1962) with the ^{125}I-α-bungarotoxin method. They found that chronically denervated adult muscle and muscle from neonatal rats bind substantial amounts of toxin over the whole membrane.

4.1.4. Possible factors influencing the acetylcholine sensitivity

Recently, investigations of the distribution of ACh-sensitivity on muscle fibers were carried out to study the influence of activity and that of trophic neuronal factors. Some authors state that muscle activity is the only factor controlling extrajunctional ACh-sensitivity (Drachman and Witzke, 1972; Lømo and Rosenthal, 1972; Berg and Hall, 1975; Lømo and Slater, 1976). It was shown by Jones and Vrbová (1974) that direct muscle stimulation prevented the onset of the development of extrajunctional hypersensitivity during the first 2-3 days after nerve section. Thereafter, electrical stimulation only reduced the actual ACh-sensitivity of the denervated muscles. Lavoie et al. (1976) demonstrated that the increase in amount of α-bungarotoxin bound to skeletal muscles after muscle inactivity was less than that induced by denervation. In their experiments the muscle inactivity was caused by local application of tetrodotoxin, which does not cause nerve degeneration or a block of axonal transport. By quantitative studies on ACh-receptor densities in disused muscles Pestronk et al. (1976) showed that muscle activity has a prominent but not exclusive role in the regulation of extrajunctional ACh-receptor distribution. Frank et al. (1976) reported that the number of junctional receptors at denervated end plates was quite stable, suggesting that the persistence of these receptors is not immediately dependent on the presence of a functional motor nerve terminal. The extrajunctional receptors, however, show a high degree of fluidity; they are able to appear and disappear rapidly (Axelrod et al., 1976).

4.1.5. Acetylcholine sensitivity of muscle cells in tissue culture

The ACh-sensitivity of embryonic muscle cells has been studied in tissue culture. In chick noninnervated myotubes Kano et al. (1971) found

an ACh-sensitivity of 0.1 to 1 mV/nC, evenly distributed over the myotubes.

Fambrough and Rash (1971) reported that the ACh-sensitivity of noninnervated rat myotubes increased with their age in culture. Mononucleated cells (myoblasts) exhibited a low ACh-sensitivity of 0.01 mV/nC, whereas in myotubes with 10 or more nuclei, after 5-6 days in culture, the maximal overall sensitivity of 1500 mV/nC was attained. Harris et al. (1973) found an ACh-sensitivity of 4-50 mV/nC in noninnervated chick myotubes. Later Hartzell and Fambrough (1973) found an ACh-sensitivity of 300-400 mV/nC and an ACh-receptor density of 1500-2000/μm^2 in chick and rat myotubes after 1 week in tissue culture. Cohen and Fischbach (1973) demonstrated that chick muscle fibers, that had been stimulated intermittently during prolonged periods, were less sensitive to iontophoretically applied ACh and bound less ^{125}I-α-bungarotoxin than inactive fibers.

Different nomenclature is used in the litterature for the areas of high sensitivity to ACh demonstrated by iontophoresis and clusters of ACh-receptors visualized by α-bungarotoxin autoradiography. In this chapter the cluster of ACh-receptors and the areas of high ACh-sensitivity in noninnervated myotubes will be called 'hot spots'. Clusters of ACh-receptors and areas of high ACh-sensitivity related to innervation will be called 'hypersensitive loci'.

In noninnervated chick myotubes Fischbach and Cohen (1973) demonstrated areas of high sensitivity to ACh, so called hot spots. The sensitivity of these hot spots could be as high as 10^3 mV/nC. Peaks in sensitivity were usually located in the immediate vicinity of muscle nuclei and the membrane near most nuclei was more sensitive than that of other regions along the same cell. In noninnervated chick myotubes Sytkowski et al. (1973) demonstrated hot spots with densities of 9000 ACh-receptors per μm^2; values much higher than the average number of 900 receptors per μm^2. Mononucleated cells did not bind α-bungarotoxin, but after 1 week 1% of the myotubes showed hot spots, with surface areas of about 125 μm^2. After 11 days hot spots were found on 80% of the cells. Prives et al. (1976) described a different timing of the occurrence of hot spots.

After 4 days in tissue culture, the myotubes derived from chick muscle showed a homogeneous ACh-receptor distribution and no cross striations. After 7 days faint cross striations were observed as well as receptor clusters of 5-15 µm in diameter. After 10 days the hot spots had disappeared and it was observed that the denser the cross striations on the myotubes, the lower the ACh-receptor density.

4.1.6. Innervated muscle cells in tissue culture

In chick myotubes innervated by spinal cord explants, Kano and Shimada (1971) found areas of increased ACh-sensitivity with areas of low sensitivity in between. In the studies of Fischbach and Cohen (1973) innervation of the myotubes by spinal cord cells did not reduce the mean ACh-sensitivity of the muscle cells. On the membranes of individual functionally innervated myotubes these authors found peaks of ACh-sensitivity near the sites where nerve terminals made contact. Fischbach et al. (1976) have demonstrated areas of high ACh-receptor densities at neuromuscular junctions in tissue culture, the latter being identified as true synapses by their ability to generate end plate potentials after extracellular depolarization of nerve terminals in the presence of tetrodotoxin. The peaks in sensitivity were extremely localized: 5-10 µm in diameter. However, in many instances no synaptic potentials could be evoked, even though the ACh-sensitivity at the site of stimulation was comparable to that found at proven synapses. Fischbach thus demonstrated the presence of hot spots, not related to innervation, and hypersensitive loci, related to innervation, on one and the same myotube.

Harris et al. (1971) and Steinbach et al. (1973) demonstrated restriction of the ACh-sensitive area in clonal muscle cells in the presence of neuroblatoma cells. Although the neuroblastoma neurites were firmly attached to the muscle cells no signs of functional innervation were found.

4.1.7. Experimental approach

In the experiments described in this chapter the ACh-sensitivity was

studied of chick muscle cells in tissue culture grown in the presence and absence of fragments of the chick ciliary ganglion. The ciliary ganglion consists of cholinoceptive parasympathetic neurons which in situ form cholinergic end plates on the muscle fibers of the ciliary body and the sphincter iridis (Marwitt et al., 1971). In tissue culture functional cholinergic neuromuscular junctions are formed between the neurons in the ciliary ganglion and skeletal muscle fibers (Hooisma et al., 1975; Betz, 1976; this thesis, chapter 3).

4.2. Materials and methods

4.2.1. Tissue culture

Culture methods are the same as described in chapter 3, paragraph 3.2.1.

4.2.2. Electrophysiology

4.2.2.1. Intracellular measurements

The intracellular recording set up and methods were the same as described in chapter 3, paragraph 3.2.2.1.

4.2.2.2. Iontophoretic application of acetylcholine

ACh-sensitivity was measured according to the method described by Nastuk (1953) and del Castillo and Katz (1955), and further developed by Kuffler and Yoshikami (1975) with extracellular application of ACh and intracellular recording of the resulting potential transients. Iontophoretic current was supplied by a push-pull current source. This arrangement resulted in a separation of the circuits for recording and iontophoresis and this gave a minimal stimulus artefact. Current pulses of 0.5-1 msec duration were used to release ACh from the pipette. The duration of the current pulses was kept constant throughout the experiment. The current as well as the voltage drop over the ACh-pipette were monitored in order to observe changes in resistance of the pipettes due to damage or contamination of the tip. The current

was so adjusted that the pulses of ACh maximally caused a depolarization of the muscle fiber of 5 mV. Standard inward directed braking currents through the pipettes of 5nA were applied routinely in order to prevent leakage of ACh by diffusion from the tip. Fresh Tyrode's solution was constantly running through the bath to remove traces of ACh that might inadvertently escape from the pipette. A negative testpulse preceeded the iontophoresis pulse to enable discrimination between electrotonic effects of current pulses and iontophoretical stimulation. In all cases the recording electrode was positioned within 300 μm from the iontophoresis electrode. Electrotonic conduction of the ACh-induced membrane depolarization, from the site of generation to the site of recording, resulted in a decrease of the amplitude of the ACh-potential transient. Since the electrotonic length constant λ of the myotube was greater than 800 μm (unpublished observations), the attenuation of the amplitude of the signal was less than 30%.

4.2.2.3. Criteria for acceptance of a measurement

When a cell was selected for impalement on morphological criteria (see paragraph 3.2.1.), further criteria came into play. After penetration by the recording electrode no long-lasting contraction, loss of cytoplasm or marked change of clearness around the tip of the electrode were accepted. Cells were also discarded if vacuoles appeared. In an acceptable myotube the membrane potential should rise as a fast jump from the base line. Very often the membrane sealed slowly around the tip of the microelectrode, accompanied by a more gradual increase in the membrane potential after initial jump. This increase should be accomplished within 5 minutes. After sealing, the membrane resting potential, V_m, should be stable and exhibit no decrease greater than 5 mV.

The ACh-pipette was placed slightly above the membrane of the myotube by lowering the pipette along the vertical axis. If the tip of the ACh-pipette touched the muscle membrane a local change in brightness of the myotube was observed with phase optics. The electrode was

subsequently lifted until the brightness change had disappeared. If the membrane depolarization that followed a pulse of ACh had a delay of more than 2 msec, the height of the pipette above the myotube was readjusted in order to obtain an optimal response. A long delay is expected if the distance from the pipette to the receptors is too large, which either means that the electrode is too high above the membrane, or that no receptors are in the immediate vicinity of the ACh-pipette.

The braking current was adjusted to the lowest value that left the membrane resting potential unaltered (usually 3-5 nA). Subsequently, testpulses (0.5-1 msec duration) were delivered to check the adjustment of the braking current. Care was taken to assure that no long-lasting effects were caused by these testpulses; i.e., at the end of the ACh-potential the membrane potential should regain its original value. Each time before a myotube was screened for its ACh-sensitivity the braking current was adjusted. If a braking current exceeded 5 nA the ACh-pipette was discarded.

4.2.2.4. The 'precise' and 'relative' method

For the investigation of the acetylcholine sensitivity 2 different

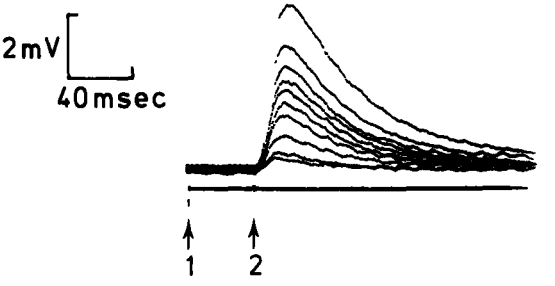

Fig. 1: Precise method for ACh-sensitivity

The upper bundle of traces shows ACh-potentials elicited by different charges of ACh applicated at the moment indicated by arrow 2 to the myotube. At arrow 1 a negative going test pulse was applied. Clearly no potential transient was seen in the myotube, indicating the absence of direct electrical stimulation. Duration of the ACh-pulse was 0.6 msec; delay between the onset of the ACh-potential and the end of current pulse was less than 1 msec; time to peak 20 msec. Calculation of the sensitivity in fig. 2.

procedures have been employed. The precise method described by Kuffler and Yoshikami (1975) and a relative method.

a. The precise method. A series of current pulses was given that elicited ACh-potentials of amplitudes from the noise level up to 5 mV. The slopes of dose-response relationships thus obtained were expressed in mV/nC and used as an index of ACh-sensitivity. Figure 1 shows a series of superimposed ACh-potentials elicited by different amounts of current at a constant pulse duration. Figure 2 presents the amplitudes of the ACh-potentials plotted against the amounts of charge required to elicite them. The slope of the graph obtained is a measure for the ACh-sensitivity.

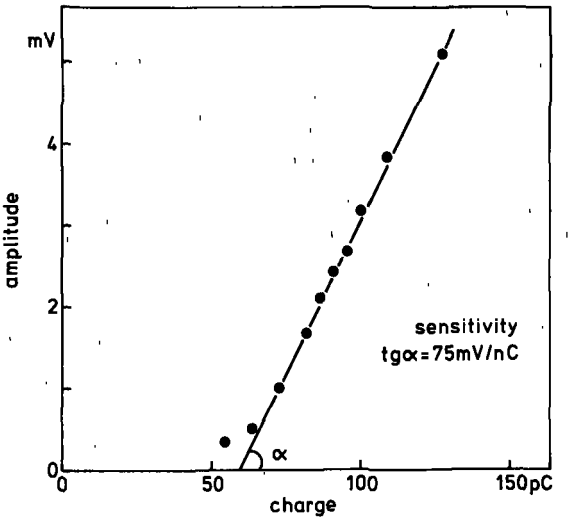

Fig. 2: Graphical determination of ACh-sensitivity

The amplitudes of the ACh-potentials of fig. 1 have been plotted against the charges used to elicit them. The linear relationship is fitted by eye. The tangent of the linear relationship (tgα) corresponds with the ACh-sensitivity, *in this case 75 mV/nC.*

b. The relative method. After applying the precise method the current pulse was so adjusted that an ACh-potential of about 2 mV resulted. The ACh-pipette was then moved in steps over distances of 3-5 µm. With current pulses of the same magnitude ACh-poten-

tials were elicited. At those sites where differences of more than a factor of 3 were found, the precise method was again applied to check the ACh-sensitivity of that particular area more accurately. The factor by which the ACh-sensitivity at a certain site differs from the mean ACh-sensitivity of an individual myotube is called the 'deviation factor'.

4.3. Results

4.3.1. Morphology

The development of the muscle cells is described in detail in chapter 3. On the fifth day nuclei are seen situated in line alongside the membrane immediate beneath the sarcolemma. Most myotubes exhibit cross striations. At day 6 hypolemmal nuclei can be seen in most myotubes. In mixed cultures with ciliary ganglia thin branching neurites grow radially from the ganglion within a few hours after explantation. The number and length of the neurites are largely influenced by the overall density of the fibroblasts and myotubes around the ganglion. In crowded cultures fewer neurites grew out of the ganglion. Many nerve fibers seemed to end on myotubes or on mononucleated muscle cells, although sometimes morphologically well differentiated myotubes in the immediate vicinity of the ciliary explant were not contacted. Neither bright field nor phase-contrast microscopy did reveal distinct end plate-like structures on the myotubes.

4.3.2. Electrophysiology

4.3.2.1. Noninnervated myotubes

ACh-sensitivity was measured in noninnervated control cultures. In 19 different cultures, 51 cells were measured that had been in tissue culture for 6-13 days. The membrane resting potentials of these cells, ranging from -68 mV to -92 mV, had an average of -81.4 $\pm$ 5.3 mV ($\pm$ S.D.). No correlation between age and V_m was found.

In 45 cells the ACh-sensitivity has been measured according to the precise method on 326 different positions at intervals of about 20 µm. The mean sensitivity at these positions ranged from 30 mV/nC to 670 mV/nC and had an average of 230 mV/nC (see fig. 3).

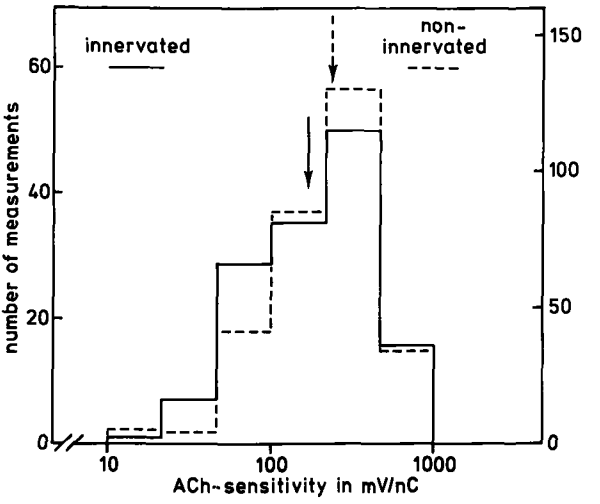

Fig. 3: Acetylcholine sensitivity of innervated and noninnervated myotubes

The histogram shows the distribution of the sensitivity of all spots measured, determined with the precise method. The solid line represents the innervated myotubes, the broken line the noninnervated ones. Scales are so adjusted that the total surface areas of both histograms are equal. Arrows indicate the mean ACh-sensitivity for innervated and noninnervated myotubes.

If the sensitivities measured on one myotube were compared, the maximal deviation factor ranged from 1.1 to 3.2 with a mean of 1.5. Figure 4 shows the distribution of ACh-sensitivity along a myotube. The average sensitivity is reflected by the broken line. In this case the maximal deviation factor was 1.3. Special attention was paid to membrane specializations such as nuclei. The ACh-sensitivity of the membrane overlying nuclei was determined with the precise method in 8 myotubes in 6 different cultures. The ratio of the ACh-sensitivity of the nucleus membrane and the average value ranged from 0.68 to 2.24 with a mean ratio of 1.07 $\pm$ 0.43 ($\pm$ S.D.; n = 13). The mean deviation factor on the 8 myotubes was 1.96 $\pm$ 0.63 ($\pm$ S.D.). Apparently no correlation existed between the presence of nuclei

under the membrane and ACh-sensitivity.

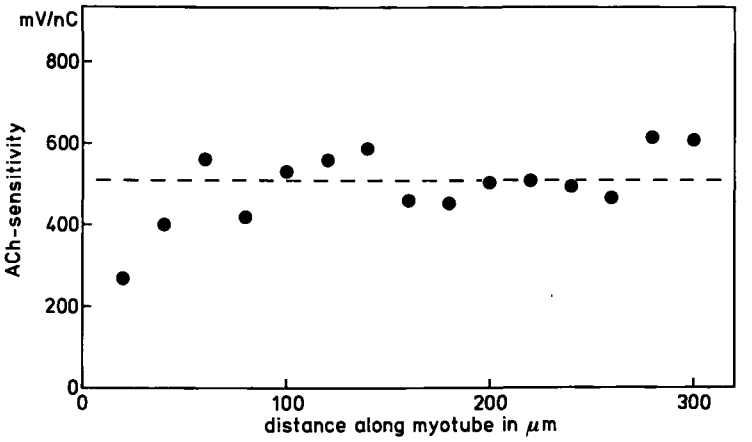

Fig. 4: Acetylcholine sensitivity along a myotube

Each dot represents the sensitivity of one point along the axis of the myotube, determined with the precise method. The broken line represents the mean sensitivity of 509 mV/nC. Muscle cell 6 days in vitro; V_m = -77 mV; width of myotube 26 μm.

Because variations in measured ACh-sensitivity could be due to variations in width of the myotubes, the ACh sensitivities of myotubes with various width were compared. The width of 46 myotubes varied from 16 μm to 43 μm with an average of 28.2 μm ± 6.9 μm (± S.D.). Even very wide fibers did not differ notably in sensitivity from the average muscle cells. In all cases, the negative going pulse that preceeded the testpulses did not result in any electrotonic potential (see fig. 1). This demonstrates that no current flow through the muscle membrane occurred. The delay between the onset of the ACh-potential and the testpulses in figure 1 was less than 1 msec. In this particular case the time from start to peak of the ACh-potential was 20 msec but in general this parameter ranged from 20 to 50 msec. After applying the precise method, a total area of 81.000 μm^2 in 17 cells was examined according to the relative method. After adjusting the current pulse to obtain an ACh-potential of 2 mV, the membrane of the myotube was tested over its entire width and over a certain length. Special attention was again paid to membrane specializations.

Changes in the ACh-sensitivity were always gradual and never exceeded a factor of 3. The ACh-induced potentials were fairly constant for individual noninnervated fibers.

4.3.2.2. Innervated myotubes

By iontophoretic application of ACh to muscle fibers the general sensitivity of these fibers to ACh has been measured and evidence has been obtained for the existence of hypersensitive loci.

a. General ACh-sensitivity of innervated myotubes

ACh-sensitivity was measured in myotubes that were cultured together with ciliary ganglion neurons and exhibited spontaneous epp's of at least 3 mV at a frequency of about 1 Hz or more. Superthreshold epp's were seldom seen. In 11 different cultures, 32 cells were investigated that had been in tissue culture for 7-9 days and co-cultured with ciliary neurons for 2-5 days. In 4 different cultures, 7 cells were tested that had been in tissue culture for 12-14 days and co-cultured with ciliary neurons for 8-9 days. The overall mean membrane resting potential was: V_m = -78.4 $\pm$ 6.5 mV ($\pm$ S.D.; n = 39). Essentially no differences were found between sensitivities of the younger and older cultures. In 31 cells the ACh-sensitivity was measured according to the precise method on 146 spots at distances of about 20 µm along the myotube. The average sensitivity to ACh of the individual myotubes ranged from 25 to 450 mV/nC with a mean of 175 mV/nC (see fig. 3). The maximal deviation factor per myotube ranged from 1.1 to 2.4 with a mean of 1.5. Therefore, gradual variations were the same as on noninnervated myotubes, as was confirmed with the relative method on a total surface area of over 35.000 μm^2.

b. Evidence for hypersensitive areas

In cells on which the precise method was applied a search for peaks in ACh-sensitivity was performed subsequently with the relative method. While searching for spots with high ACh-sensitivity, special attention was paid to regions where neurites were coursing over myotubes or ending on them. On a total surface area of over

35.000 μm^2 comprising 15 myotubes in 6 cultures, 14 hypersensitive areas were found on 6 different cells in 6 different cultures. In all 14 cases the hypersensitivity was restricted to small areas of about 10 µm in diameter. The sensitivity of all hypersensitive loci was again determined with the precise method. The deviation factors of the 14 spots varied from 4.1 to 36.4 and had an average of 10.2. On one particular cell that was tested with the relative method, two spots were detected with a deviation factor of 5.5. Background ACh-potentials rose in 28 msec while at the hypersensitive loci the ACh-potentials rose in 5 and 4 msec respectively (see fig. 5).

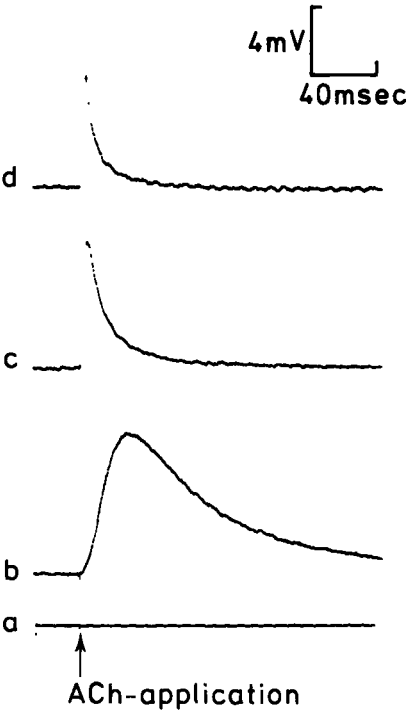

Fig. 5: ACh-potentials of hypersensitive loci and background

Line a represents the iontophoretic current. The arrow indicates the time of application; duration of current 0.6 msec. Different charges have been used at the three spots, 184 nC, 46 nC and 61 nC in b, c and d respectively. Sensitivities were calculated to be 52 mV/nC, 283 mV/nC and 285 mV/nC respectively. In line b a background ACh-potential is shown, which rose in 28 msec to its maximum. In c and d ACh-potentials are shown of hypersensitive loci on the same myotube, which rose in 5 msec and 4 msec, respectively.
Ciliary ganglion and muscle cell 8 and 6 days in vitro, respectively; V_m = -75 mV.

The time to peak of the ACh-potential at the hypersensitive loci was 4-15 msec. In the same myotubes spontaneous epp's with approximately the same amplitudes had a time to peak ranging from 2 to 5 msec. It is difficult to compare the time to peak of an ACh-potential with that of an epp of equal amplitude. Due to multiple innervation epp's were generated at various distances from the tip of the recording electrode. The amplitude and the time to peak of these epp's was attenuated depending on the distance to the electrode. It was not clear whether the sites of epp-generation were closer to or more distant from the electrode than the ACh-pipette. This made it uncertain which time to peak had to be taken into account for an epp originating from a particular hypersensitive locus. ACh-potentials at hypersensitive loci were about 2-4 times slower than the epp's but 3-6 times faster than ACh-potentials in membrane areas of average sensitivity.

4.4. Discussion

4.4.1. The absence of hot spots in noninnervated myotubes

The acetylcholine receptor is one of the few functional components of the surface membrane of differentiated or differentiating muscle cells which can be identified unambiguously. In cultures of noninnervated muscle cells the overall distribution of the ACh-receptors is diffuse, but several investigators reported the presence of so-called hot spots, i.e. restricted areas of enhanced ACh-sensitivity of areas which show enhanced binding of α-bungarotoxin. In the present study the ACh-sensitivity of noninnervated myotubes was studied at various stages of development using ACh micro-iontophoresis.

The level of overall sensitivity and the occurrence of hot spots have been investigated. The following criteria were used to define a hot spot: (1) the ratio of the local ACh-sensitivity to the overall sensitivity should be more than 3; (2) the high sensitivity should be localized in a restricted spot with sharp boundaries; and (3) the delay between the iontophoretic pulse and the top of the ACh poten-

tial should be shorter than that in the surrounding parts of the cell membrane. The average value of the ACh-sensitivity of the cell membrane, 230 mV/nC, did not differ markedly from that found by Fischbach and Cohen (1973). The deviation factor of the ACh-sensitivity ranged from 1-3. This variation is in part due to the variations that occur during measurements, and will in part be caused by changes of the input resistance along the muscle fiber or by undulation of the surface membrane by which more or less membrane area is exposed to the ACh from the pipette. However, in 17 cells and on 81.000 μm^2 of membrane hot spots have not been detected. Because the membrane overlying the nuclei have been reported to have a high ACh-sensitivity, such membrane areas were carefully tested for their ACh-sensitivity. Higher sensitivity was never found in the vicinity of nuclei.

4.4.2. Possible reasons for the absence of hot spots

The reason for the absence of hot spots in noninnervated myotubes studied in the present investigation is unknown. It could be due to minor differences which always exist in tissue culture methods or in medium components used in different laboratories. The absence of hot spots could be related to special morphological and electrophysiological properties of the muscle cells cultured under the conditions described here. In our hands the development of the cross striations and the electrical properties of cultured muscle cells was comparatively rapid. Cross striations were visible in many tubes at day 4 of cultivation and very pronounced at day 5. Fischbach and Cohen (1973) and Prives et al. (1976) described the appearance of cross striations at day 7. Moreover under our conditions also the membrane resting potential reached a high value at an early state. On the third day of cultivation the membrane resting potential was already -80 mV and remained at that level for at least two weeks. Fischbach and Cohen reported values of membrane resting potentials of breast muscle cells of the chick that reached a maximum of -60 mV only at day 5-6. It must be taken into account, that in vivo membrane resting potentials of chick breast muscles are lower than those of chick thigh muscle; at birth -25 mV and -58 mV, respectively (Karzel,

1968). In 60 day old muscle, the difference is very low, -93 mV and -96 mV, respectively.

The absence of hot spots could be related to this high rate of differentiation or might be caused by the factors which also led to the rapid appearance of cross striations or the early high membrane resting potential.

4.4.3. ACh-sensitivity in innervated myotubes

The mean overall ACh-sensitivity was only slightly lower in the innervated myotubes (175 mV/nC) than in the noninnervated ones (230 mV/nC). This is in good agreement with Fischbach and Cohen (1973) and Fischbach et al. (1976). In innervated myotubes Kano and Shimada (1971) reported sensitive loci around nerve terminals and absence of ACh-sensitivity distant from these loci. Fischbach et al. (1976) made a very elegant study of the presence of hypersensitive loci near nerve terminals in contact with myotubes. They demonstrated a coincidence of local hypersensitive with the place where mepp's were generated. However, these authors also found hot spots at places where no active nerve terminals could be demonstrated.

In the present study 14 hypersensitive loci were found on 6 myotubes in 6 different cultures. The differences in ACh-sensitivity and in time to peak of the induced ACh-potentials on these loci with those of the surrounding membrane were striking. Hypersensitivity was always restricted to very small areas with a diameter of about 10 µm. Fibers on which hypersensitive loci were located seemed to have lower values for the general ACh-sensitivity, but the number of cells is too small to substantiate this conclusion. Since it was not possible to determine the exact time of innervation, innervation might have been in existence for too short a period to induce a significant decrease in background ACh-sensitivity. The amplitude of the spontaneous epp's in the myotubes was subthreshold, and only occasionally muscle action potentials were recorded. Cohen and Fischbach (1973) reported that activity induced by extracellular stimulation decreased the ACh-sensitivity by a factor of 10. In the present experiments the absence of an effect on background sensitivity might be due to the lack of superthreshold epp's.

4.4.4. The reliability of the method

The fact, that hot spots have not been found in noninnervated cultured muscle cells could be due to a technical failure with the technique of micro-iontophoresis. The iontophoretic pipettes used were rather low in resistance and thus had a relative large opening at the tip. This resulted in approximately 2 times slower ACh-potentials than those found by Fischbach. These pipettes, however, proved good enough to demonstrate hypersensitve loci on innervated myotubes. It has been reported by several authors that at some stages of muscle development most of their cultured myotubes contained hot spots or clusters of ACh-receptors. Therefore, considering the number of fibers investigated, if hot spots had existed on the noninnervated myotubes they should have been detected. Moreover, the total surface area of membrane examined on noninnervated fibers was more than twice that covered in the study of innervated fibers. Hot spots were not found in 17 noninnervated myotubes, against 14 hypersensitive loci on 6 out of 15 innervated fibers.

4.4.5. Hypersensitive loci and innervation

The fact that the hypersensitive loci were found exclusively in mixed cultures of ciliary ganglia and muscle cells and not in cultures of muscle cells proves that the hypersensitive loci were induced by the nerve cells present in the culture. It is tempting to speculate that the clusters of ACh-receptors were induced in newly formed neuromuscular junctions. Although the hypersensitive loci have been found in the vicinity of the ciliary explants on muscle fibers that received innervation, the proof that they coincide with neuromuscular junctions awaits further experimentation in which iontophoretic and other electrophysiological techniques will be applied to the same innervated myotubes. Also autoradiography might be helpfull to answer this point.

For reasons yet unknown hot spots were not found in muscle fibers cultured under the conditions described here. Their absence is a great advantage for further studies of neurally induced clustering of ACh-receptors in cultured myotubes.

4.5. Summary

1) ACh-sensitivity was determined by iontophoretical application of ACh to chick myotubes cultured in the presence or absence of innervation by the chick ciliary ganglion.
2) Myotubes grown in tissue culture from dissociated chick leg muscle cells exhibited an evenly distributed ACh-sensitivity. With microiontophoretical application of ACh, spots of high ACh-sensitivity, so-called hot spots (Fischbach and Cohen, 1973), could not be demonstrated. Over large membrane areas the differences in ACh-sensitivity never exceeded a factor of 3 and were always gradual.
3) The innervation of the myotubes by chick ciliary ganglion neurons did not alter the overall ACh-sensitivity.
4) Hypersensitive loci were found on functionally innervated myotubes. Hypersensitivity was located within sharply defined areas of about 10 μm in diameter.
5) Apparently, innervation in tissue culture was accompanied by the formation of regions of high ACh-sensitivity.

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Chapter 5

5. Innervation of cultured skeletal muscle fibers by neurons, that innervate smooth muscle in vivo

5.1. Introduction

The formation of neuromuscular junctions between striated muscle cells and neurons has been studied using different kinds of neuronal cells. Recently, the formation of neuromuscular junctions between the chick ciliary ganglion and chick skeletal muscle cells in tissue culture has been demonstrated by Hooisma et al. (1975), Betz (1976) and Slaaf (this thesis chapter 3). The avian ciliary ganglion offered the distinct advantage that all neurons present in the ganglion were cholinergic (Marwitt et al., 1971). The mammalian ciliary ganglion offers another advantage. The muscles innervated by this ganglion are exclusively smooth in character. In the present experiments it has been investigated whether neurons in the ciliary ganglion of the rabbit are able to form functional connections with cultured muscle fibers. In birds, the ciliary ganglion contains only two classes of neurons, the so-called ciliary neurons which are cholinergic nicotinic (Pilar and Vaughan, 1969) and the so-called choroid neurons which are cholinergic muscarinic in nature (unpublished observations cited by Landmesser and Pilar, 1970). In the chick the ciliary body and the sphincter iridis, muscles that are innervated by the ciliary neurons, are striated and differ in that respect from the corresponding muscles in mammals (Ovio, 1927) which are smooth. The choroid neurons send profusely branching axons into the vascular choroid tissue where they innervate smooth muscle fibers (Landmesser and Pilar, 1974).

The mammalian ciliary ganglion is a parasympathetic relay station in the nervous pathway from the Edinger-Westphal nucleus in the midbrain to the smooth muscle fibers of the sphincter iridis muscle and the ciliary muscle (Kerr, 1973). Only nerve branches leaving the oculomotor nerve have endings on the neurons in the ciliary ganglion. Other axons, sensory and sympathetic, merely use the ganglion and its efferent short

ciliary branches as a guide to the eyeball. The innervation of both ciliary muscle and sphincter iridis is cholinergic muscarinic in nature (Westheimer, 1974). Little is known about the ciliary ganglion of the rabbit in particular, but the anatomy and physiology of this ganglion are comparable with those in other mammals.

In these studies the ciliary ganglion of the rabbit has been co-cultivated with chick skeletal muscle fibers for which type of muscle cells conditions are known for successful development in tissue culture. The formation of functional junctions in a heterospecific system has been observed in several cases (Crain, 1970; Stevens et al., 1974; Nurse and O'Lague, 1975 and Hooisma et al., 1977).

It was demonstrated that neuronal cells that in vivo innervate exclusively smooth muscle cells are able to form functional junctions with skeletal muscle cells in culture. Pharmacological properties of these junctions have been investigated.

5.2. Materials and Methods

5.2.1. Dissection of the rabbit ciliary ganglion

Newborn rabbits were killed by a blow in the neck and washed in 70% ethanol for 3 x 5 minutes in three different jars. The dissection was carried out in a laminar flow cabinet. The head was rinsed with Tyrode's solution and freed from skin. The skull, the brains and the cartilage of the eye chamber was removed. The optic nerve was dissected free after cutting the extra ocular muscles. The ciliary ganglion became visible as a tiny transparant body, rather firmly attached to the oculomotor nerve, close to the bifurcation of the oculomotor nerve just frontal from the optic nerve and at the origin of a tiny nerve connection between the oculomotor nerve and the optic nerve. The ganglion was dissected with the aid of a pair of microscissors.

5.2.2. Tissue culture

Muscle cells were prepared from 11-day old chick embryos as described

before (this thesis, paragraph 3.2.1.). In mixed cultures ciliary ganglia of rabbit embryos (24 and 27 gestational days) and newborn (0-6 days) rabbits were explanted on top of the muscle cell preparation.

5.2.3. Electrophysiology

The recording set up was the same as that described before (Hooisma et al., 1975; this thesis paragraph 3.2.2.1.). Measurements were carried out with a microelectrode (10-20 MΩ DC-resistance) placed intracellularly in a myotube. Administration of pharmacological substances and washing was done as previously described (this thesis, paragraph 3.2.2.1.).

5.3. Results

5.3.1. Influence of gestational age on growth of neurites

In preliminary studies ciliary ganglia were taken from rabbit foetuses (gestational age 24 and 27 days) as well as from newborn rabbits (1-6 days old). Around the foetal ciliary ganglia only few neurites were observed 3-5 days after explantation. In cultures of the ganglia obtained from the newborn rabbits a significant but not abundant amount of neurites was observed after the same period of cultivation. There appeared to be no essential difference between the outgrowth from the ganglia of newborn rabbits of various age. Routinely, newborn rabbits of 1 or 2 days have been used.

The growth pattern differed considerably from the pattern observed with ciliary ganglia of the chick. Whereas the chick ganglia formed an extensive network of neurites within 48 hours, it took the rabbit ganglia a few days to produce 10-50 tiny neurites. In addition to these thin neurites, thick processes were formed that in phase contrast optics had the appearance of thin myotubes without any detectable substructure. It is not yet known whether these were neurites.

As a consequence of the slow time course of process formation, cells of the connective tissue that had been inadvertently brought into culture together with the ganglion, had the opportunity to proliferate and to

overgrow the neurites and myotubes. For this reason it was only occasionally possible to follow a neurite over some distance and to observe the point where it contacted a muscle fiber.

5.3.2. Electrophysiology

5.3.2.1. Electrical properties of chick cells cultured with or without rabbit ciliary ganglia

a. Muscle cells grown in the presence of rabbit ciliary ganglia

In 15 different cultures the membrane resting potentials (V_m) of 40 myotubes have been measured. The mean V_m of all 40 myotubes was -81.8 $\pm$ 9.5 mV ($\pm$ S.D.). The myotubes that showed signs of innervation had an average V_m = -84.0 $\pm$ 5.2 ($\pm$ S.D.; n = 16). Those myotubes that did not show spontaneous epp-like depolarizations had an average V_m = -80.3 $\pm$ 11.4 mV ($\pm$ S.D.; n = 24). Clearly, co-cultivation with the ciliary ganglion had no influence on the membrane resting potential.

b. In absence of neuronal material

The mean membrane resting potential of 51 cells in 19 different cultures was -81.4 $\pm$ 5.3 mV ($\pm$ S.D.). No correlation was found between V_m and age in vitro.

5.3.2.2. End plate potentials

In myotubes situated in the vicinity of outgrowing neurites spontaneous depolarizations could be recorded that very much resembled end plate potentials. Not all myotubes exhibited such epp's. There was a great variation in the frequency of occurrence of epp's in myotubes. The number of epp's in an innervated myotube was usually very low (a few per minute). In a few cases the number of epp's was about one per second. Signs of multiple innervation have been found in several myotubes. Fig. 1 shows several epp's some of which have equal amplitudes but show different rates of rise. The electrical properties of the myotube cause the attenuation of the amplitude and shape of the epp's. The more distant from the place of

generation the epp is recorded, the lower the amplitude, the slower the rate of rise and the longer the decay time. Epp's of approximately equal amplitude and clearly different shapes are thus indicative for multiple innervation.

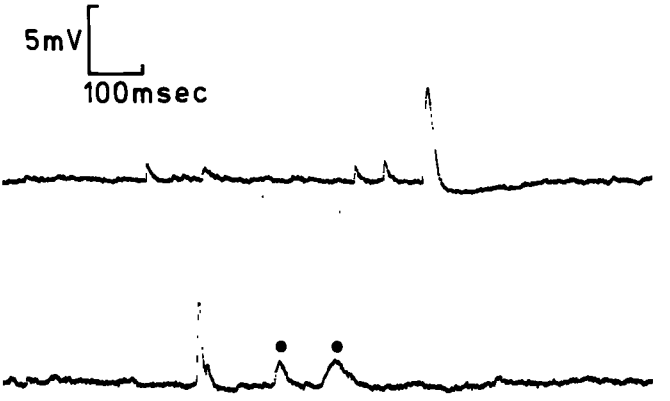

Fig. 1: Spontaneous end plate potentials

The two lines show epp's as they spontaneously occurred in an innervated myotube. The dots mark epp's of equal amplitude but with striking differences in rate of rise, indicating the presence of multiple innervation. Ciliary ganglion and muscle cell 5 and 12 days in vitro, respectively; V_m = -72 mV.

5.3.2.3. Characterization of the end plate

The nature of the receptors in the newly formed neuromuscular junctions has been determined by a differential test of the sensitivity of the epp-generating system for d-tubocurarine and atropine, repectively.

In 6 experiments a concentration of 1 µg/ml d-tubocurarine appeared to block neuromuscular transmission. In 2 experiments 10 µg/ml atropine only produced a slight decrease in epp amplitude. A concentration of 100 µg/ml atropine almost completely blocked the epp's in these experiments. Fig. 2 shows a typical experiment; after a control period of 10 minutes the administration of 1 µg/ml d-tubocurarine caused almost all epp's to submerge in the noise. After a washing period most of the epp's regained amplitudes above the noise lev-

el. The subsequent administration of 10 µg/ml atropine caused only a slight decrease whereas 100 µg/ml caused nearly all epp's to disappear below the noise level.

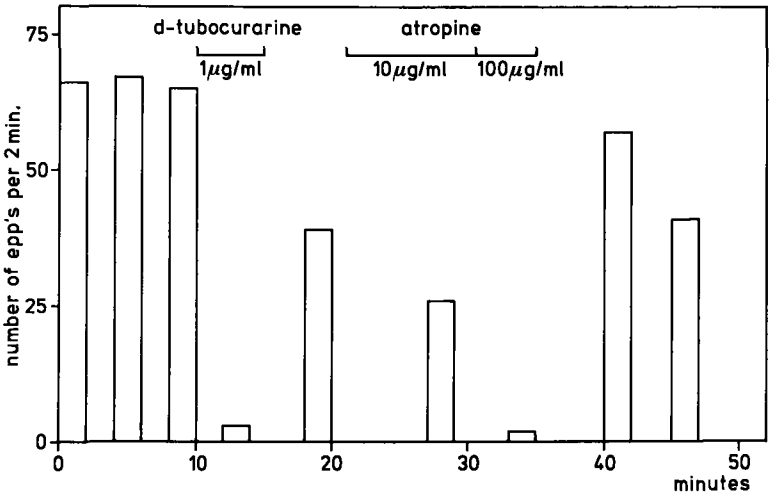

Fig. 2: Nature of end plate receptors

The effect of d-tubocurarine and atropine on the occurrence of the spontaneous epp's. After 10 min of control activity, the administration of 1 µg/ml d-tubocurarine reduced the amplitude of nearly all epp's below the discrimination level of 0.4 mV. Washing restored most of the epp's. Atropine had no marked effect at 10 µg/ml, but 100 µg/ml almost completely abolished epp's with an amplitude above the noise level. The occurrence of plateau potentials made it impossible to perform epp analysis continuously during the experiment.
Ciliary ganglion and muscle cell 5 and 7 days in vitro, respectively; V_m = -86 mV.

5.4. Discussion

5.4.1. Heterotypic and heterospecific innervation

The innervation in tissue culture of chick skeletal muscle cells by chick ciliary ganglion explants was demonstrated by Hooisma et al. (1975), Betz (1976), and is described in detail in this thesis (chapter 3). The present investigation showed that rabbit ciliary ganglia also contain neurons which are able to innervate chick skeletal muscle under comparable tissue culture conditions. Heterotypic and heterospecific innervation have been demonstrated in a variety of systems:

fetal rodent cord with adult human muscle (Crain et al., 1970), fetal rat cord with fetal mouse muscle (Peterson and Crain, 1970), mouse spinal cord with chick skeletal muscle (Stevens et al., 1974), rat sympathetic neurons with skeletal myotubes (Nurse and O'Lague, 1975), chick ciliary ganglia with chick skeletal muscle (Hooisma et al., 1975) and chick ciliary ganglia with mouse muscle cells (Slaaf, unpublished observations).

5.4.2. Influence of innervation

Chick ciliary ganglia in vivo innervate both smooth and striated muscle fibers, whereas rabbit ciliary ganglia in vivo only innervate smooth muscle cells. It was observed that the outgrowth of neurites from rabbit ciliary ganglia is sparse and few connections are formed with skeletal muscle fibers. On the contrary, the chick ciliary ganglion provides neurons that form many neurites and abundantly innervate muscle cells. Chamley et al. (1973) showed, with time lapse cinematographical techniques, the favourable influence of the presence of smooth muscle target cells on the outgrowth of guinea pig sympathetic ganglia. A comparable effect could be responsible for the slow development of the rabbit ciliary ganglia which had to cope with heterotypic muscle cells. It has been reported that outgrowth of neurites to heterospecific, homotypic cells occurs about equally well as in a homospecific, homotypic combination (Crain and Peterson, 1970, 1974; Peterson and Crain, 1970). Therefore it is possible that the sparse outgrowth of the rabbit ciliary ganglion is due to the absence of the homotypic target.

Innervation of the muscle cells by rabbit or chick ciliary ganglion neurons had no influence on the membrane resting potentials. Membrane resting potentials in innervated myotubes were equal to those found in myotubes without neuronal material in the culture. It could be that innervation existed too short a period to exert an influence. In cultures both with rabbit or chick ciliary ganglia multiple innervation has been demonstrated. Multiplicity of innervation

seems to be common in tissue culture, since it has been demonstrated by many other authors. Multiple innervation also occurs in regenerating and developing muscle in vivo (for review, Dennis and Ort, 1976).

5.4.3. Nature of receptors

The nature of the receptors involved in the neuromuscular transmission is cholinergic nicotinic. Whether the neurons were rabbit or chick in origin appeared to be of no importance. In vivo the receptors of the rabbit smooth muscle cells that are innervated by the ciliary ganglia are muscarinic cholinergic in nature. In the chick the receptors of the striated muscles of the ciliary body and the sphincter iridis are nicotinic whereas those of the smooth muscles of the vascular choroid tissue are muscarinic in nature. From the data presented it must be concluded that there was no appreciable non-nicotinic component in the epp's. The present data demonstrate that innervation of skeletal muscle can occur by neurons that in vivo innervate striated muscle or smooth muscle cells. It appears that the nature of the receptors in the muscle membrane is determined by the muscle fiber and not altered by innervation by neurons that in vivo form muscarinic neuromuscular junctions.

5.5. Summary

1. Electrophysiological measurements were carried out in cultures of chick skeletal muscle cells and rabbit ciliary ganglia.
2. Chick skeletal muscle cells can be innervated by chick and by rabbit ciliary ganglia in tissue culture.
3. The receptors at the places of neuromuscular transmission are cholinergic nicotinic.
4. It is suggested that the nature of the muscle receptors is determined by the muscle properties and not by the ciliary ganglion neurons.

5.6. References

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Chapter 6

6. General summary

The investigations with neurons and muscle cells in tissue culture described in this thesis have been focussed on the formation of neuromuscular junctions, the distribution of the acetylcholine receptors on the muscle membrane and the effects that innervation exerts on the nature of receptors. Ciliary ganglia were used as the source of neurons whereas skeletal muscle cells were offered as target cells. In vivo chick ciliary ganglion neurons are cholinoceptive as well as cholinergic and innervate smooth and striated muscle cells. The junctions formed are muscarinic and nicotinic in nature, respectively. When dissociated chick muscle cells were cultured, multinucleated myotubes were formed (3.3.1.). The muscle cells had high membrane resting potentials and in an early stage of development were able to generate slow plateau potentials whereas with increasing age they acquired the ability to generate real action potentials (3.3.2.1.). In mixed cultures of chick ciliary ganglia together with chick myotubes neurites grew from the explants and contacted the myotubes within 24 hours. These contacts appeared to be capable of inducing end plate potentials (epp's) in the myotubes as was demonstrated by intracellular electrophysiological measurements (3.3.2.4.). The neurons from the ciliary ganglion were found to be cholinoceptive and were able to conduct action potentials through the neurites which ran to the neuromuscular junctions (3.3.2.9.). The nature of the receptors of the end plates proved to be cholinergic nicotinic (3.3.2.8.). Most myotubes were multiply innervated, a phenomenon also encountered in regenerating and developing muscles in vivo (3.3.2.6.; 5.3.2.2.). The number of spontaneous epp's generated in the myotubes increased with co-cultivation time while the quantal content of the epp's also increased. The amplitude of the spontaneous epp's did not increase markedly, but the amplitude of the miniature epp's (mepp's) - that is the spontaneous depolarizations that persisted in the presence of tetrodotoxin - decreased with innervation time (3.3.2.10.). This de-

crease in mepp amplitude was probably due to a decrase of the input resistance which can be attributed to the increase of the width of the myotubes with age.

The mepp's that remained in the presence of tetrodotoxin decreased in amplitude by a factor of 2-3 when the Mg^{2+}/Ca^{2+} ratio in the superfusion fluid was increased 40-fold (3.3.2.11.). This decrease is large compared to the results that other investigators obtained in vivo or in vitro with comparable Mg^{2+}/Ca^{2+} ratio changes. The large decrease may be caused by a difference in the sensitivity of the mepp generating system in neuromuscular junctions in tissue culture for changes in the Mg^{2+}/Ca^{2+} concentration ratio. Another explanation could be that the mepp's were not uniquantal as usually but were instead of a multiquantal nature. This explanation would necessitate the assumption that in these preparations a high Mg^{2+}/Ca^{2+} concentration ratio leads to an inhibition of the mechanism which makes that one vesicle has a definite tendency to drag subsequent vesicles through the cell membrane of the nerve terminal.

Rabbit ciliary ganglia were also able to innervate cultured chick skeletal muscle cells (5.3.1.). While in vivo the receptors in the smooth muscles that are innervated by the rabbit ciliary ganglion neurons are cholinergic muscarinic in nature (5.3.2.3.), the present experiments showed that the junctions formed in mixed cultures with skeletal muscle cells were cholinergic nicotinic. Taken together with the findings obtained with chick ciliary ganglion cells, the results demonstrate that the nature of the receptors is a property of the muscle cells which is not altered by innervation by a foreign ciliary ganglion neuron.

Electrophysiological measurements showed that all noninnervated myotubes were sensitive to acetylcholine (ACh) over their entire surface membrane, like denervated muscle cells in vivo (4.3.2.1.). In our cultures no areas with localized increased ACh-sensitivity have been

found in the absence of innervation. This in contrast to the findings of other investigators who reported the presence of restricted areas of high acetylcholine sensitivity ('hot spots') in the absence of neuronal cells. So far hot spots have not been demonstrated in vivo. In the presence of innervation, loci were found that were definitely hypersensitive to ACh (4.3.2.2.). The probability that these end plate like hypersensitive spots had already been present before innervation was extremely unlikely. Apparently innervation in vitro - as in vivo - leads to the formation of loci hypersensitive to ACh. Between the hypersensitive loci the sensitivity of the myotubes to ACh was not changed by innervation. The fact that in our cultured noninnervated myotubes hot spots of ACh-receptors have not been found is a great advantage for further studies of induced clustering of ACh-receptors as the result of innervation.

Chapter 7

7. Samenvatting

In de verschillende laboratoria waar de eigenschappen van zenuwcellen en spiercellen in weefselkweek worden onderzocht, is in de afgelopen jaren overtuigend aangetoond dat in weefselkweek functionele zenuw-spier verbindingen kunnen worden aangelegd. De vorming van de zenuw-spier verbinding tussen gekweekte zenuwcellen en spiercellen uit het embryo van de kip die in dit proefschrift beschreven wordt, heeft hieraan mede bijgedragen.

Het onderzoek is uitgevoerd in gemengde culturen van zenuwcellen van het ganglion ciliare tesamen met dwarsgestreepte spiercellen uit de pootspier. Het ganglion ciliare is gelegen achter het oog en behoort tot het parasympatische zenuwstelsel. De zenuwcellen van het ganglion ciliare bedienen onder andere de lensspier en de iris-spier, beiden dwarsgestreepte spieren in de kip. Het ganglion ciliare werd gekozen omdat het slechts 2 soorten zenuwcellen bevat en hierover in vivo reeds veel onderzoek is verricht. In weefselkweek vormen de zenuwcellen van het ganglion ciliare van het 7 dagen oude embryo van de kip een netwerk van zenuwuitlopers rondom het ganglion. Skeletspiercellen, verkregen door dissociatie van skeletspieren van kippeëmbryos, fuseren in weefselkweek tot lange spiervezels. In gemengde culturen van het ganglion ciliare tesamen met spiervezels maken de uitlopers van de zenuwcellen binnen 24 uur zichtbaar contact met de spiercellen.

Met behulp van electrofysiologische meetmethoden is nagegaan of in dergelijke zichtbaar geïnnerveerde spiervezels ook zenuwprikkels kunnen worden overgedragen van de zenuwuitloper op de spiervezel. Hiertoe werden de electrische eigenschappen van de skeletspieren onderzocht met behulp van intracellulair geplaatste microëlectroden. Spiervezels in de omgeving van het ganglion hadden een hoge membraanpotentiaal (-80 mV) maar vertoonden bovendien kortdurende veranderingen in de membraanpotentiaal. Deze potentiaalveranderingen bleken eindplaatpotentialen (epp's) te zijn, die werden opgewekt in zenuw-

spier verbindingen. Blijkbaar is het ganglion ciliare in staat in weefselkweek functionerende zenuw-spier verbindingen te vormen met skeletspiervezels. De epp's werden reeds 24 uur na explantatie van het ganglion gevonden; dit betekent dat in weefselkweek functionele synapsen al binnen 24 uur worden aangelegd. Spiervezels ver verwijderd van het ganglion en waarmee op het oog door neurieten geen contact was gemaakt, hadden ongeveer dezelfde membraanpotentiaal maar vertoonden geen epp's.

Door middel van stimulatie van de zenuwcellen of van de zenuwuitlopers kon aangetoond worden dat zenuwprikkels vanuit de zenuwcellen naar de synaps werden voortgeleid. Vervolgens bleek dat de (chemische) signaaloverdracht van het zenuwuiteinde naar de spier plaatsvond door middel van de transmitterstof acetylcholine (ACh), die door interactie met de receptoren voor ACh in de spiermembraan eindplaatpotenialen opwekte. De meeste spiercellen bleken geïnnerveerd te worden via meerdere synapsen (multipele innervatie), hetgeen ook in vivo voorkomt tijdens de ontwikkeling van het zenuw-spier systeem, zowel tijdens de embryonale ontwikkeling als ook bij regeneratie van beschadigde zenuw-spier verbindingen. In de geïnnerveerde spiercellen konden vele spontaan optredende epp's worden afgeleid. Het aantal epp's nam toe met de tijd dat de zenuwcellen in de culturen aanwezig waren. De zenuwcellen in het ganglion ciliare in weefselkweek bleken zelf ook gevoelig voor ACh te zijn. Wanneer ACh op het ganglion gebracht werd, traden in de spiercellen contracties op. Hieruit blijkt dat in weefselkweek een miniatuur zenuw-spier systeem wordt aangelegd: de prikkeling van de zenuwcel wordt voortgeleid naar de zenuw-spier verbinding waar door middel van een eindplaatpotentiaal een contractie van de spiervezel wordt bewerkstelligd.

In vivo worden in spiervezels eindplaatpotentialen waargenomen met een amplitude van 20 mV tot 40 mV die worden opgewekt door prikkels die van een motorische zenuwcel naar het zenuwuiteinde worden

voortgeleid. In het zenuwuiteinde veroorzaakt de zenuwprikkel het tegelijkertijd vrijmaken van vele pakketjes ACh waardoor de eindplaatpotentiaal (epp) ontstaat. Ook kunnen er kleinere potentiaalveranderingen ontstaan (0.1 - 2 mV) die het gevolg zijn van het spontaan vrijkomen van een of enkele pakketjes ACh. Deze spontane potentiaalveranderingen worden miniatuur eindplaatpotentialen (mepp's) genoemd. Of de spontane potentiaalveranderingen die we in weefselkweek kunnen afleiden uit de spiercellen spontaan ontstaan (mepp's), dan wel door activiteit van de zenuwcel (epp's), kan bepaald worden wanneer men de voortgeleiding van de zenuwprikkel naar de synaps blokkeert met de stof tetrodotoxine (TTX). De dan overblijvende signalen zijn mepp's.

Wanneer TTX aan culturen van het ganglion ciliare en spiervezels werd toegevoegd, werd de voortgeleiding van de actiepotentialen geblokkeerd en verdwenen de contracties in de spiercellen. Door de potentiaalveranderingen in aanwezigheid van TTX te vergelijken met die in afwezigheid van TTX konden de spontane mepp's worden onderscheiden van de epp's die het gevolg zijn van activiteit van de zenuwcellen. Hierbij bleek dat als regel mepp's en epp's naast elkaar voorkwamen.

TTX had niet altijd een invloed op de spontaan optredende potentiaalveranderingen. In die gevallen hadden we dus ook vóór de TTX toediening al te maken met alleen mepp's. In andere experimenten verdwenen de grotere potentiaalveranderingen (dus de epp's) en bleven uitsluitend de wat kleinere over. Er werd geconstateerd dat de amplitudes van de mepp's kleiner werden naarmate de zenuwcellen langer in kweek gehouden werden. De daling van de mepp amplitude zou het gevolg kunnen zijn van de vermindering van de ingangsweerstand van de cellen die op zijn beurt het gevolg zou kunnen zijn van de toegenomen breedte van de spiercellen. Alle spontaan optredende potentiaalveranderingen tesamen veranderden echter nauwelijks in oudere culturen. Bij gelijkblijvende gemiddelde epp-amplitude en afnemende mepp (= eenheids)-amplitude betekent dit dat het gemiddeld aantal pakketjes ACh per epp (de quantuminhoud) toegenomen moet zijn. Deze toename van de quantum-

inhoud suggereert dat de synaps een rijpingsproces doormaakt en zich ontwikkelt tot een efficienter werkende synaps. De door ons meestal gemeten quantuminhoud van 1-3 is echter nog ver verwijderd van de 100-200 in het volwassen dier.

De mepp's in de gekweekte spiercellen waren relatief groot vergeleken bij mepp's gemeten in vivo. Dat zou het gevolg kunnen zijn van: (1) de relatief hoge ingangsweerstand van de spiercellen; (2) de aanwezigheid van een vergrote hoeveelheid ACh per pakketje en (3) het gezamenlijk vrijkomen van meerdere pakketjes ACh in plaats van één enkel pakketje.

Het is uit de litteratuur bekend dat een verhoging van de Mg^{2+}/Ca^{2+} verhouding in de badvloeistof met een factor 40 weinig of geen invloed heeft op de mepp's, maar de epp's nagenoeg doet verdwijnen. De spontane potentiaalveranderingen die in spiervezels overbleven bij een hoge Mg^{2+}/Ca^{2+} verhouding en die dus mepp's zouden moeten zijn, werden vergeleken met de potentiaalveranderingen in aanwezigheid van TTX. Naar analogie van de resultaten verkregen in vivo zouden deze potentiaalveranderingen beiden ongeveer even groot moeten zijn. De mepp's in aanwezigheid van de hoge Mg^{2+}/Ca^{2+} verhouding waren echter een factor 2 à 3 kleiner dan de mepp's in aanwezigheid van TTX. De gegevens zouden kunnen worden verklaard wanneer de aangebracht verandering in de Mg^{2+}/Ca^{2+} concentratie in vitro wèl een effect heeft op de amplitude van de mepp's. Een andere mogelijkheid is dat de mepp's in aanwezigheid van TTX niet veroorzaakt worden door één enkel pakketje ACh, zoals in het intakte dier, maar dat er een verhoogde kans is op meesleuren van meerdere pakketjes. Dit zou dan kunnen betekenen dat de mepp's na TTX uit 2 of 3 eenheidsmepp's bestaan. Deze verklaring maakt het dan wel noodzakelijk dat een hoge Mg^{2+}/Ca^{2+} verhouding een negatieve invloed uitoefent op het mechanisme dat ervoor zorgt dat pakketjes meegesleurd worden. Is dat zo, dan worden bij een hoge Mg^{2+}/Ca^{2+} verhouding slechts de enkelvoudige pakketjes ACh afgegeven en dus ook slechts enkelvoudige mepp's opgewekt.

Het is mogelijk gebleken om in weefselkweek innervatie tussen cellen uit verschillende species te bewerkstelligen. Zo is in ons laboratorium aangetoond dat ruggemergcellen van de muis spiercellen van de kip innerveren. Het nut van dergelijke vreemde zenuw-spier combinaties is dat hiermee kan worden nagegaan welke eigenschappen door zenuwcellen en welke door spiervezels bepaald worden.
In het konijn innerveert het ganglion ciliare alleen gladde spiercellen in het oog. In de kip innerveren sommige zenuwcellen van het ganglion ciliare gladde spiercellen terwijl andere zenuwcellen in het ganglion dwarsgestreepte spiercellen innerveren. In beide gevallen fungeert ACh als transmitterstof. De receptoren voor ACh op de plaatsen van de signaaloverdracht in glad spierweefsel worden geblokkeerd door atropine, terwijl de ACh receptoren in dwarsgestreepte spieren door curare worden geblokkeerd.
Zenuwcellen uit het ganglion ciliare van het pasgeboren konijn bleken eveneens in staat te zijn om dwarsgestreepte spiercellen van de kip te innerveren. De receptoren van de synapsen met de spiercellen bleken veel gevoeliger voor curare te zijn dan voor atropine, zowel in geval van innervatie door het ganglion ciliare van de kip als dat van het konijn. Het is hieruit duidelijk dat het type receptor in de spiermembraan een eigenschap is die door de spiercellen bepaald wordt en niet veranderd wordt wanneer een ander type zenuwcel de spiercel innerveert.

In het volwassen dier heeft de spiermembraan alleen veel ACh receptoren bij de synaps en is derhalve de spiermembraan bij de synaps veel gevoeliger voor op de membraan toegediende ACh dan buiten de synaps. Na denervatie blijken de spieren in vivo over hun gehele oppervlakte gevoelig voor ACh te worden. Een over de hele membraan verspreide ACh gevoeligheid wordt ook waargenomen in spiervezels gedurende de fase van de normale embryonale ontwikkeling die aan innervatie voorafgaat.
Door andere onderzoekers is beschreven dat in weefselkweek de niet geinnerveerde spiercellen over hun gehele oppervlak gevoelig zijn voor

ACh. Volgens een aantal onderzoekers zijn er echter ook plaatsen met een sterk verhoogde gevoeligheid voor ACh ('hot spots') op die niet geïnnerveerde spiercellen. Onder de door ons gebruikte kweekomstandigheden werden met behulp van electrofysiologische methoden hot spots niet gevonden.
In gemengde culturen van het ganglion ciliare en spiercellen, beiden van de kip, konden in cellen die epp's vertoonden en dus duidelijk geïnnerveerd waren, wèl plaatsen aangetoond worden met een hoge gevoeligheid voor ACh. Het moet erg onwaarschijnlijk geacht worden dat deze gevoelige plaatsen reeds voor de innervatie aanwezig waren. De gevoeligheid van de spier op de membraan tussen de gevoelige plaatsen werd niet veranderd door innervatie.

De afwezigheid van hot spots op niet geïnnerveerde spiervezels is een groot voordeel bij het onderzoek naar de vorming van acetylcholine gevoelige plaatsen ten gevolge van innervatie. Van gevoelige plaatsen die na innervatie worden gevonden behoeft dan niet eerst te worden bewezen dat ze vóór de innervatie niet reeds aanwezig waren.

Blijkbaar worden in gemengde culturen van zenuwcellen uit het ganglion ciliare en spiercellen, zenuw-spier contacten aangelegd die electrofysiologisch en farmacologisch een sterke gelijkenis vertonen met de zenuw-spier verbinding in vivo. Bovendien leidt innervatie in vitro evenals in vivo tot vorming van gebiedjes op de spiermembraan met grote gevoeligheid voor ACh. De vorming van de zenuw-spier verbinding in weefselkweek is daarom een geschikt modelsysteem voor verdere studie van het mechanisme van innervatie en voor onderzoek van de invloed van neuropathologische en neurotoxische stoffen op het zenuw-spier systeem.

Nawoord

Het zal iedereen duidelijk zijn dat een proefschrift dat de bestudering van electrofysiologische parameters van innervatie in weefselkweek beschrijft, niet het werk is van een enkele onderzoeker, maar tot stand gekomen is door de samenwerking van een groep onderzoekers van verschillende discipline tegen de achtergrond van een daartoe uitgerust laboratorium Het Medisch Biologisch Laboratorium TNO verschafte mij de gelegenheid om onder de vereiste omstandigheden te werken, waarvoor ik zeer erkentelijk ben. Een woord van dank is ook op zijn plaats voor de Stichting Promeso die mij de financiële middelen van het onderzoek verschafte.

Ik ben mij bewust van het feit dat velen hun bijdrage aan dit proefschrift in anonimiteit geleverd hebben. Ik wil hen daarvoor allereerst bedanken.
Gaarne wil ik persoonlijk bedanken mijn promotor Prof. Dr. D. de Wied.
Hooggeleerde Heer, hoewel U eerst in een later stadium dit onderzoek bent gaan begeleiden, bent U een drijvende en stimulerende kracht voor mij geweest.
De begeleiding van het hele onderzoek gedurende de gehele periode is in handen geweest van Dr. W.F. Stevens en Dr. E. Meeter. Vooral hun begeleiding heeft de realisatie van dit proefschrift mogelijk gemaakt.
Beste Wim, ik ben je erkentelijk voor de stimulerende invloed die je op het onderzoek in z'n totaliteit en mijn werk daarbinnen gehad hebt. De manier waarop je de projectgroep in voor- en tegenspoed hebt laten functioneren als een goed geöliede machine strekt mij tot voorbeeld. Bij de uiteindelijke tot stand koming van dit proefschrift ben je een voortreffelijke discussiepartner geweest.
Beste Dick, de discussie die ik met jou gehad heb, noopten mij tot zorgvuldiger overwegen en formuleren. Ook heb je mij geleid op het moeilijke pad van dat deel van de fysiologie dat betrekking had op

het onderzoek. Ik ben je erkentelijk voor de uren die je hebt willen steken in realisatie van dit proefschrift.

De opleiding in de electrofysiologie heb ik gekregen van Dr. J. Siegenbeek van Heukelom tijdens mijn studie in Utrecht.
Beste Jan, de tijd dat ik onder jouw leiding onderzoek heb verricht is voor mij het prettigste en meest waardevolle van mijn studie geweest. In die periode heb ik geleerd hoe belangrijk een goede wisselwerking in een team onderzoekers is.

De ondersteuning van het onderzoek kan grofweg in twee richtingen verdeeld worden.
Allereerst, en gezien mijn interesse waarschijnlijk niet onverwacht, natuurlijk de ondersteuning binnen het electrofysiologisch onderzoek. Vanaf het eerste uur heb ik samengewerkt met de Heer A.G. Beyersbergen van Henegouwen. De bouw van de electrofysiologische opstelling en de eerste metingen zijn ons gezamenlijk werk geweest. In een later stadium kwam Ing. R.A.G. van Ruler het team versterken en heeft hij van de opstelling een gestroomlijnd geheel gemaakt, zonder mystiek met een strakke planning van de beschikbare tijd.
De mechanische werkplaats onder leiding van de Heer J.B. Engelen en de electronische werkplaats in de personen van Ir. L. van den Steen en Ir. T. Magchielse, hebben een belangrijke bijdrage geleverd bij de realisatie van de electrofysiologie opstelling.

De tweede richting is de celbiologie geweest. Het is evident dat electrofysiologisch onderzoek in weefselkweek zonder een goede ondersteuning van die zijde onmogelijk was geweest.
De supervisie van de weefselkweek heeft gedurende het onderzoek gelegen bij Drs. J. Hooisma.
Beste Jakob, ik wil langs deze weg mijn dank betuigen voor de inbreng die je steeds gehad hebt om de culturen in zo goed mogelijke staat te houden. Onder leiding van Wim en jou heeft een heel team voor de voorziening van de weefselkweek gezorgd.

Mevrouw M. Remmelts heeft ervoor gezorgd dat het ganglion ciliare in kweek gebracht werd in die aantallen die nodig waren. Nadat Dr. A.J. van Eick de eerste preparaties verricht had, heeft zij het vrij prepareren van het ganglion ciliare tot kunst verheven.
Het verdere kweekwerk werd afwisselend verricht door een aantal mensen de Heren J.A.M. Glandorf, A.L. van der Laaken, E.P.B. Bierman en Mevrouw A.C. van Neerbos.
De histologische verwerking van de preparaten was in de goede handen van Mevrouw R.M.J. van Benthem.
Het knutselwerk binnen de electrofysiologie opstelling, de tekeningen in dit proefschrift en de verpozing dankzij en ten koste van Feyenoord waren afkomstig van de Heer A.W. Kluivers.
De fotografische ondersteuning was in goede handen bij de Heren H.E. Groot Bramel en M.J.M. Boermans. Hun adviezen voor de tekeningen en de lay-out van het proefschrift waren zeer waardevol.

Tijdens mijn langdurige verblijfperioden in Rijswijk terwijl ik reeds naar Eijsden verhuisd was, mocht ik voortdurend rekenen op de gastvrijheid van Theo Lammers, die ervoor zorgde dat die periodes niet te lang duurden.

Ook de Rijksuniversiteit Limburg heeft een belangrijke bijdrage geleverd. De capaciteitsgroepen Biofysica en Fysiologie hebben mij de gelegenheid gegeven het onderzoek in Rijswijk af te ronden door een deel van mijn verplichtingen in Maastricht over te nemen.
Het typewerk van dit proefschrift werd verzorgd door Mevrouw R.M. Borgman-Hanssen, secretaresse van de capaciteitsgroep Biofysica, daarbij indien nodig steeds ondersteund door het secretariaat van de capaciteitsgroep Fysiologie, Mevrouw G.J.C. van der Mars-Pastoors en Mejuffrouw E.G.M. Geurts.

Curriculum vitae

De schrijver van dit proefschrift werd in 1945 geboren te Rotterdam. Na het behalen van het eindexamen Gymnasium β aan het Grotius College te Heerlen in 1963 begon hij aan zijn studie in de wis- en natuurkunde aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen werd afgelegd in oktober 1967. In januari 1972 werd het doctoraal examen natuurkunde behaald; hoofdvak experimentele natuurkunde, waarvoor o.a. 12 maanden onderzoek werd verricht aan electrofysiologische eigenschappen van de epidermis van de mens onder leiding van Dr. J. Siegenbeek van Heukelom, en de bijvakken wiskunde en medische fysica. Voor het vervullen van zijn militaire dienstplicht werd hij na een verkorte officiersopleiding, gedetacheerd bij het Medisch Biologisch Laboratorium der Rijksverdedigingsorganisatie TNO te Rijswijk, alwaar hij begon aan het onderzoek waarop dit proefschrift betrekking heeft. In 1973 ontving hij een beurs van de Stichting Promeso, die het mogelijk maakte het onderzoek na afloop van de militaire dienstplicht voort te zetten. Sedert december 1975 is hij in dienst bij de Medische Faculteit van de Rijksuniversiteit Limburg te Maastricht, waar hij werkzaam is bij de capaciteitsgroep Biofysica.

Table 2 The effects of tetrodotoxin and of changes in the ratio of the Mg^{2+} and Ca^{2+} concentrations on the frequency and the amplitude of the spontaneous end plate potentials in muscle cells innervated by ciliary ganglia.

Exp. no.	Days in vitro		Control situation		Effects of tetrodotoxin (TTX)				Effects of raised $[Mg^{2+}]/[Ca^{2+}]$ in medium			
	Muscle cells	Ciliary ganglia	Frequency no./2 min	Max. epp[§] amplitude	Conc. (µg/ml)	Effect	$\frac{f_{TTX}}{f_{control}}$	Max. epp[§] in TTX (mV)	Ratio change	Effect: decrease in	Amplitude[Φ] shift by a factor of	Max. epp[§] in high Mg^{2+}/Ca^{2+}
1	7	1	460	12 mV	1	none	1	9 mV	-	-	-	-
2	7	1	423	7 mV	1 3	none none	1 1	7 mV 7 mV	-	-	-	-
3	7	2	165	12 mV	-	-	-	-	40	amplitude	1/2	5.5 mV
4	7	2	84	9 mV	1	none	1	8 mV	-	-	-	-
5	6	2	152	15 mV	1	block of high epp's	1	7 mV	-	-	-	-
6	8	2	310	8 mV	1	none	1	8 mV	40[Ψ]	amplitude	1/2	4.0 mV
7	7	2	373	10 mV	1	none	1	10 mV	40	frequency	-	4.5 mV
8	7	2	899	11 mV	1	none	1	11 mV	-	-	-	-
9	7	3	55	16 mV	1	decrease frequency	1/4	6 mV	-	-	-	-
10	8	4	237	6 mV	-	-	-	-	40[T]	amplitude	1/2	2.4 mV
11	8	4	184	6 mV	1	none	1	6 mV	40	amplitude	1/2	4.0 mV
12	9	5	330	7 mV	1	decrease frequency	1/2	4 mV	40	amplitude	1/4	1.6 mV
13	9	6	444	16 mV	1	block of high epp's	1	4 mV	-	-	-	-
14	12	9	107	10 mV	1 2 5	decrease frequency and a block of high epp's	1/3 1/3 1/3	3 mV	40	amplitude	1/8	1.8 mV
15	11	9	216	10 mV	1 10 100	none	1 1 1	10 mV	-	-	-	-

All experiments have been done with a washing period between TTX and high Mg^{2+}, low Ca^{2+} treatment or vice versa. In exp. 6 the administration of high Mg^{2+}, low Ca^{2+} was performed in the presence of 1 µg/ml TTX.

§ The maximal epp is determined from the intercept of the reversed cumulative distribution.

¶ Additional block of high epp's could not be excluded.

Ψ Experiment done in the presence of TTX.

T This experiment was done twice with the same result.

Φ Shift of the amplitude obtained by comparing the reversed cumulative distribution to that in the control situation.