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CHOLINERGIC MOTOR CONTROL OF SEA URCHIN TUBE FEET: EVIDENCE FOR CHEMICAL TRANSMISSION WITHOUT SYNAPSES*

BY E. FLOREY AND M. A. CAHILL

Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, Federal Republic of Germany and Friday Harbor Laboratories of the University of Washington, Friday Harbor, Washington 98250, U.S.A.

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SUMMARY

Isolated tube feet of *Strongylocentrotus franciscanus* contract briefly when the outer epithelium is touched. Similar twitch-like contractions can be induced by electrical stimulation of the outer surface of the tube foot. These responses appear to be chemically mediated. The following evidence indicates that the transmitter substance may be acetylcholine (ACh): ACh causes muscle contraction. This effect and that of electrical stimuli is potentiated by anticholinesterase agents and is antagonized by cholinergic blocking agents. Anaesthesia with chloralhydrate or chloretone abolishes responsiveness to mechanical or electrical stimulation but not to ACh. Desensitization with carbachol prevents responses to ACh and to mechanical or electrical stimulation.

There are no neuromuscular synapses and no axons can be detected which cross the connective tissue layer which separates the muscle fibres from the subepithelial nerve plexus. The latter is known to contain conspicuous amounts of ACh; nerve terminals containing clear vesicles invest the outer surface of the connective tissue layer. All evidence indicates that chemical transmission involves diffusion of ACh (released from activated nerve terminals) across this connective tissue layer which is around 5 μm thick in fully extended tube feet but may have a thickness of 20 or even 25 μm in less extended ones. Calculations based on equations describing transmitter diffusion prove the feasibility of such a mechanism.

INTRODUCTION

Isolated sea urchin tube feet have been shown to shorten when treated with acetylcholine (ACh) (Florey, Cahill & Rathmayer, 1975). The effect is potentiated by anticholinesterases and is reduced or blocked by cholinergic blocking agents. It was further shown that the outer layer (consisting of epithelium and subepithelial nerve plexus) contains up to 75 μg of ACh per g wet weight and the remaining

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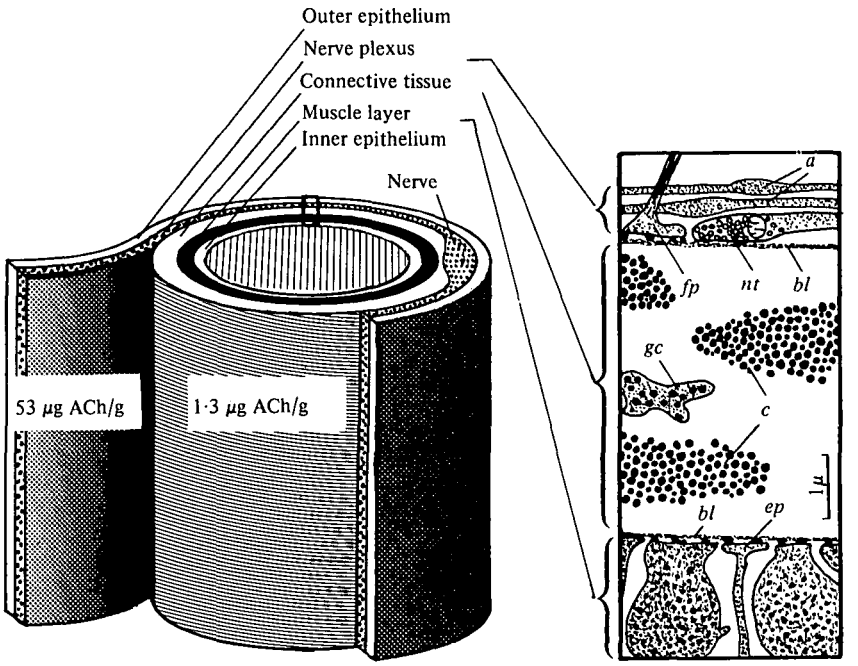

Fig. 1. Schematic diagrams showing the structural components of echinoid tube feet. In the diagram on the left the outer layer, consisting of epithelium and nerve plexus, is shown partially peeled off. The small rectangle indicates the cross-section which is shown enlarged on the right. The latter is based on electronmicrographs. *a* = axon, *fp* = filamentous process of epithelial cell, *nt* = nerve terminal, *bl* = basal lamina, *gc* = granule containing cell, *c* = collagen fibrils, *ep* = endothelial cell process. The figures for acetylcholine (ACh) content represent the average values reported by Florey *et al.* (1975). For details of ultrastructure, consult Florey & Cahill (1977).

inner layer (consisting of connective tissue, smooth muscle and endothelium) has an ACh concentration nearly two orders of magnitude lower (1·9 μg/g). We suggested that the tube foot musculature is controlled by cholinergic motoneurones.

An analysis of the ultrastructure (Florey & Cahill, 1977) indicated that the tube foot musculature (which lies at the inner surface of the connective tissue layer) does not receive motor innervation but that the outer surface of the connective tissue cylinder is invested with nerve terminals of the subepithelial nerve plexus. The absence of neuromuscular synapses in echinoid tube feet had already been noted by Kawaguti (1964).

On the basis of our findings we suggested that motor control of sea urchin tube feet is by ACh released from nerve terminals of the subepithelial nerve plexus and that the transmitter has to diffuse through the layer of connective tissue before it reaches the cholinoceptive muscle. Fig. 1 summarizes the relevant structural features.

Our hypothesis differs from the interpretation of tube feet motor control offered by Kawaguti (1964) who considered the endothelial cells as motor cells. Cobb (1978) recently proposed that the granule containing branched cells which sporadically occur among the muscle cells serve as motoneurones. Such granule-containing cells pervade all tube foot structures (Coleman, 1969; Florey & Cahill, 1977); they have

been seen in a variety of echinoderm organs (haemal vessels: Doyle, 1967; gills: Cobb & Sneddon, 1977; gonads: Brusle, 1969; hyponeural tissue: Hehn, 1970). It is possible, of course, that some of these cells are indeed neurosecretory cells. They might even be neurones using an as yet unidentified transmitter. It is equally possible, however, that these cells play another role. As was noted earlier (Florey & Cahill, 1977), the granule cells in echinoid tube foot tissue are always singly placed and are never seen to receive synaptic connexions. They are certainly very different from typical cholinergic neurones whose terminals contain small, clear vesicles.

The hypothesis that motor control involves diffusion of the motor transmitter across a connective tissue sheath is not new. In 1970, Cobb suggested that the muscles of asteroid and echinoid tube foot ampullae are activated by nerve terminals located on the other side of the connective tissue sheath to which the muscle fibres are attached. This connective tissue layer is much thinner than what we previously reported for the tube feet. Further investigation has now shown that in more extended tube feet the connective tissue layer can be as thin as 4–5 μm. Cobb (1978) argues that chemical mediation of nerve impulses across a connective tissue layer several micrometers thick is very unlikely and suggests a non-cholinergic, possibly serotonergic, mechanism of motor control by neurosecretory cells which function as motoneurones. According to this hypothesis, the responses of tube foot muscle to ACh would be the result of a ubiquitous distribution of ACh-receptors and would not be proof of a normal involvement of this ACh-sensitivity in motor control.

Combined physiological and pharmacological experiments were conducted to resolve the question of whether or not motor control involves cholinergic motoneurones.

MATERIALS AND METHODS

As experimental animals we chose the very large species *Strongylocentrotus franciscanus* of the Northamerican west coast. Its tube feet reach a length of 10 cm and have a diameter of 2 mm at their base.

Fully extended lateral tube feet were snipped off about 2–3 cm from their base. The cut end was ligated and attached to a hook inside a small thermostated muscle bath. The other end was ligated just below the sucker and its ganglion. This distal end was connected to a force-displacement transducer (Grass FT-3) which permitted a 1 mm movement for each 50 g of muscle tension. A chart recorder (Brush, Mark II) was used to record mechanical tension. The set-up is shown in Fig. 2.

A pair of platinum wire electrodes was gently lowered onto the surface of the horizontally positioned tube foot. The level of the bathing fluid in the muscle chamber was adjusted to about 1 mm above the upper surface of the preparation. Electrode separation was 1·5 mm. The electrodes were connected to a square wave stimulator whose output was also recorded on the second channel of the chart recorder.

Drugs were added to the bath with a 1 ml hypodermic syringe. This permitted rapid mixing. For most experiments, fresh, natural seawater was used as the bathing medium. In experiments on the effects of an increased potassium concentration, an artificial seawater (ASW) was used. It had the following composition: NaCl 433,

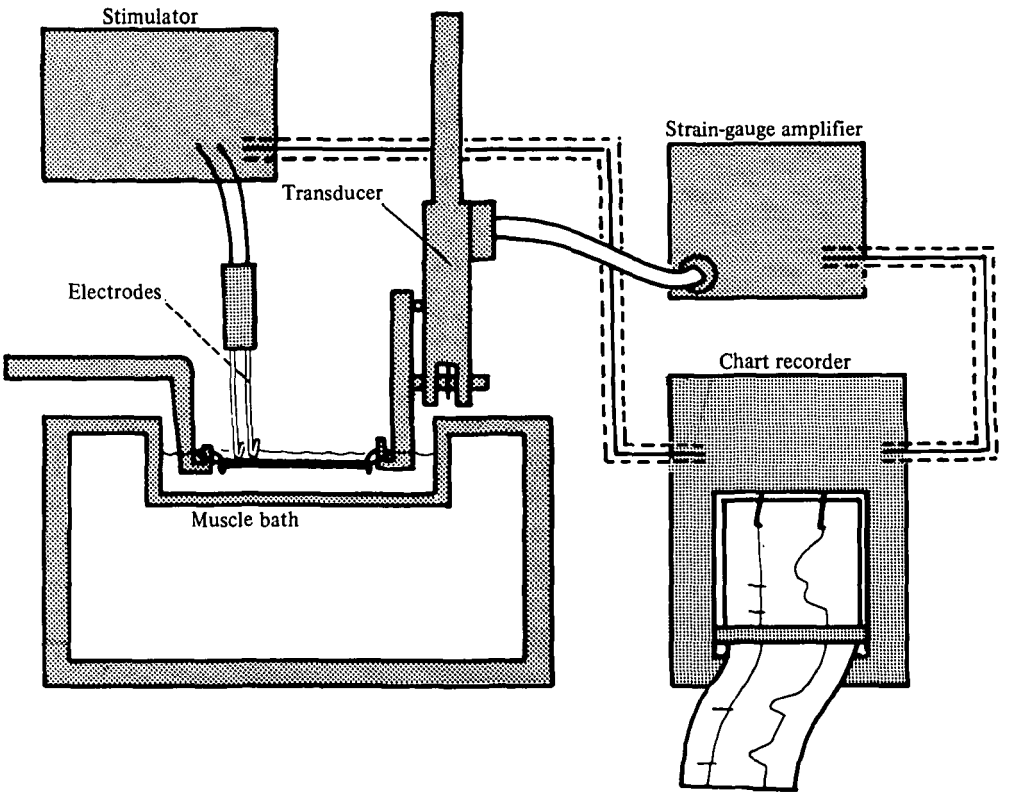

Fig. 2. Diagrammatic representation of experimental arrangement used for stimulation and tension recording.

KCl 8, $MgCl_2$ 50, $CaCl_2$ 10 mM. The 'high-K medium' was made up as follows: NaCl 406, KCl 35, $MgCl_2$ 50, $CaCl_2$ 10 mM.

The following drugs were used: acetylcholine chloride (Merck); atropine sulphate (Sigma); chloralhydrate (Sigma); chloretone (1,1,1,-trichloro-2-methyl-2-propanol; Sigma); D-tubocurarine chloride (Schuchardt); 5-hydroxytraptamine creatine sulphate (Calbiochem); mytolon (Sterlin-Winthrop Research Institute) and physostigmine (Eserine) (Burroughs & Wellcome Co.). Stock solutions of the drugs (10^{-3} g/ml ASW) were stored in the refrigerator. Chloretone and chloralhydrate were stored as a 1% solution in ASW at room temperature.

All experiments were carried out with bath temperature of 14–16 °C.

For electron microscopy tube feet were fixed in 2·3% glutaraldehyde in 0·1 M-NaCl, buffered with 0·2 M-phosphate buffer (pH 7·3), and post-fixed in 2% osmium tetroxide buffered at pH 7·3 in 1·25% sodium bicarbonate. Following dehydration and staining *en bloc* with 1% uranyl acetate in 70% alcohol, they were embedded in Epon. Thin sections were stained with lead citrate (Reynolds, 1963) and viewed in a Zeiss EM 9s electron microscope.

Tube feet of *Echinus esculentus* were used for cholinesterase localization. They were fixed at room temperature for 15 min in phosphate-buffered glutaraldehyde and incubated for 1–1·5 h on ice in a mixture consisting of thiolacetic acid and lea

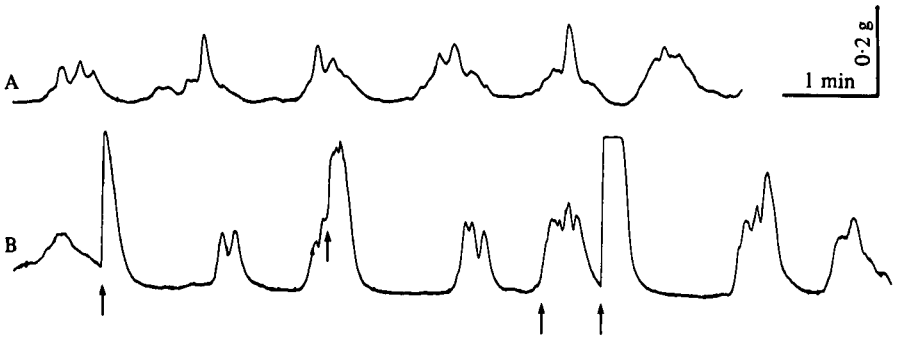

Fig. 3. (A) Spontaneous contractions of isolated tube foot. This preparation was exceptional in the regularity of the contractions. (B) Contractions evoked by mechanical stimuli (arrows) are superimposed on the spontaneously occurring contractions. Note that in this case the stimuli alter the spontaneous rhythm.

citrate in cacodylate buffer according to the method of Barrnett (1962). Specimens were then postfixed in collidine-buffered 2% osmium tetroxide and dehydrated and embedded as described above. Thin sections were double stained in uranyl acetate and lead citrate.

RESULTS

General observations

Immediately after cutting, the tube feet shorten to about 20–25% of their original length. When placed in a shallow dish of seawater they bend occasionally and, in general, curl up. When the rim of the sucker is touched the sucker bends towards the stimulus site. Touching the side of the stem of the tube foot results in a momentary shortening and bending of the stem towards the stimulated side.

Cutting off the sucker and its ganglion is a major stimulus which causes overall contraction. Relaxation occurs only after several minutes. Touching the side of the tube foot again results in a contractile response similar to that seen before.

If extended tube feet are first ligated and then cut off just proximal to the ligature, they remain inflated and extended for a time, gradually shortening over a period of many minutes, due presumably to exudation of luminal fluid across the tube foot wall. Ligated and freshly cut tube feet bend in response to mechanical stimulation, they also make a quick contractile response. It appears that all parts of the tube foot wall are mechanically excitable and that such excitation results in muscle contraction. A connection with the basal part of the tube foot or with the radial nerve is not necessary for these responses to occur.

Tension measurements

When freshly set up in the apparatus, the tube feet were gently stretched until the transducer registered a tension of about 0·1 g. Particularly during the first minutes, many preparations were spontaneously active (whether or not the sucker and ganglion were present). They periodically generated tensions up to about 0·25 g. An example is shown in Fig. 3*a*. This activity gradually subsided.

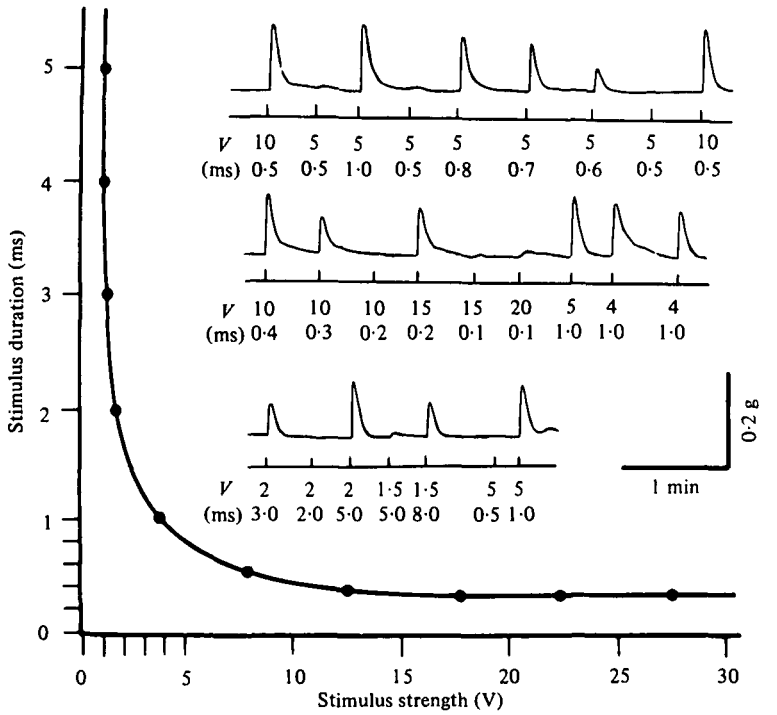

Fig. 4. Strength–duration curve representing stimulus thresholds as measured in a typical preparation. The inset shows actual records and corresponding stimulus parameters.

When the wall of the tube foot is gently touched with the tips of fine foreceps a transient tension develops which reaches a peak within 1 to 3 s and then subsides. Responses of this type are shown in Fig. 3*b*. They can be elicited repeatedly and occur also in quiescent preparations. The magnitude of individual responses varied; maximal tensions never exceeded 1 g and generally were below 500 mg.

Electrical stimuli cause transient contractions. A single pulse (minimal duration 0·1 ms) is sufficient to elicit what looks like a twitch contraction. The resulting effect depends both on the strength and on the duration of the applied stimuli as is shown in Fig. 4. When thresholds are plotted (duration versus strength of applied stimulus) typical chronaxy curves are obtained (see Fig. 4).

Observation through the microscope showed that contraction occurs not only in the vicinity of the electrodes but also in more distal parts. With stronger stimuli, larger parts of the tube foot are involved in the contractile response.

In several experiments the tube foot was slit lengthwise to expose its inner, endothelial surface. The electrodes could thus be brought much closer to the muscle fibers. Higher stimulating currents were then required to elicit contractile responses.

In other experiments the nerve was removed by making two longitudinal cuts to either side of it and peeling it off the preparation, or by cutting out a strip of the tube foot wall which contained the nerve. The responsiveness of the preparations to electrical (or mechanical) stimulation was not altered. The parts distal from the electrodes were observed to contract as well as in the presence of the nerve.

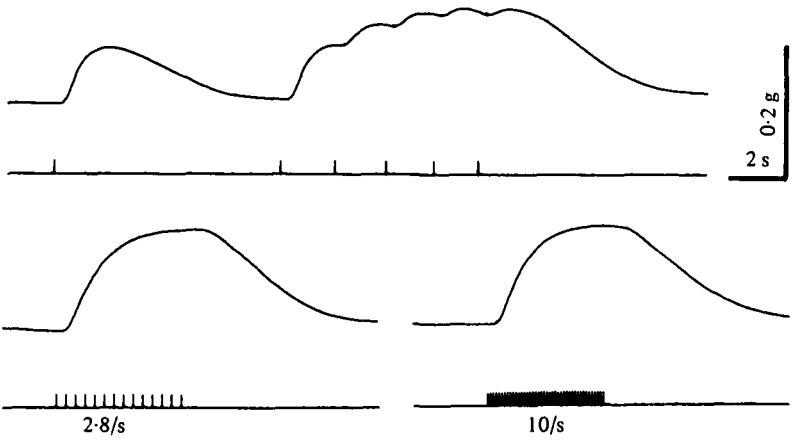

Fig. 5. Contractile responses of an isolated tube foot to supra-threshold electrical stimuli (duration 1 ms). Two-channel recording; upper channel = mechanical tension, lower channel = applied stimuli.

From records taken at high chart speeds (125 mm/s) it was possible to determine the latency between stimulus and onset of contraction. The shortest delay measured was 100 ms, but delays were mostly in the range of 140–170 ms. Measurements made on four different preparations yielded a mean of 162 ms ± 9·2 S.E. (n = 17).

Repetitive stimulation caused summated contractions, but in fresh preparations tension never increased to more than about twice the tension produced by a single stimulus. The same maximum is reached with stimulation at 3/s and at 30/s (Fig. 5).

As the preparations aged, twitch tension became smaller and summation correspondingly greater so that in later phases of the experiments it was not uncommon to find tetanic tensions up to four times larger than twitch tension.

Anaesthesia

When chloretone or chloralhydrate were added to the muscle bath (final concentrations of 0·03% or 0·1%), responses to touch ceased within a few seconds and responses to electrical stimulation declined progressively until, after not more than 30 s, they too failed, even at five times the current strength previously used. The anaesthesia was found to be readily and completely reversible. Full responses reappeared within 10 min after washing out the anaesthetic.

Effect of cholinergic drugs

ACh concentrations of 10^{-6} g/ml or higher produced sustained contraction. Maximal tension was reached with concentrations of about 10^{-5} g/ml. There was little desensitization and even 5 min after onset of the effect, electrical stimulation produced superimposed tension increments. Maximal tensions produced by ACh were usually similar to the maximal tensions reached during repetitive electrical stimulation (in the absence of ACh).

Carbachol was effective in the same range of concentrations as ACh but tensions

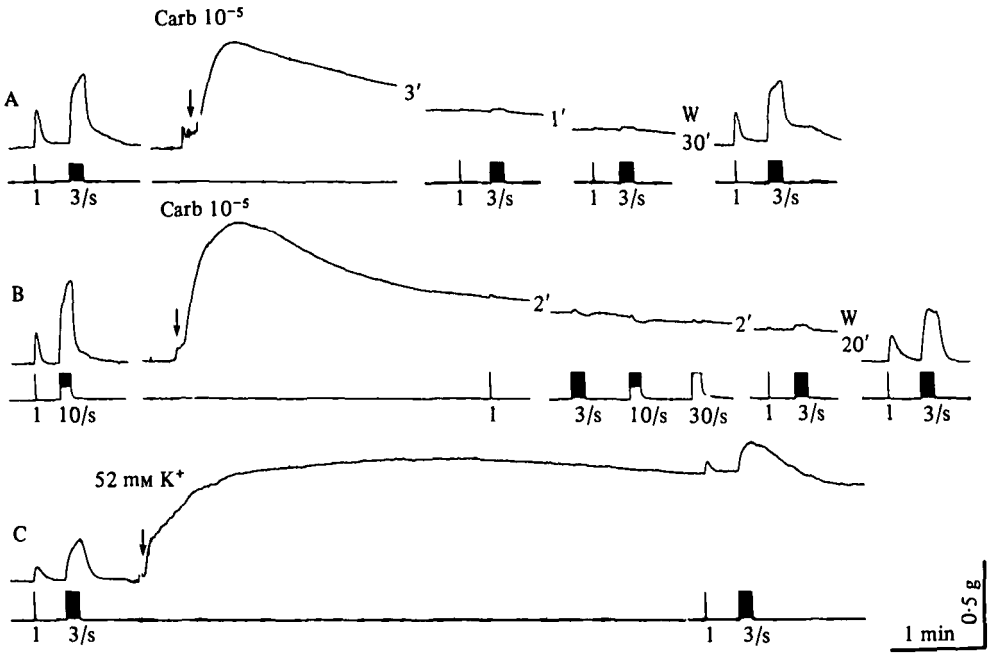

Fig. 6. Desensitization to carbachol (Carb) reversibly abolishes responsiveness to electrical stimulation (A, B). During sustained contraction due to elevation of K^+ concentration of bathing medium electrical stimulation remains effective. W = washing. Because of limited frequency response of the recording system stimulus records appear fused at higher stimulation frequencies.

reached were generally higher and sometimes surpassed those caused by electrical stimulation (see Fig. 7). With carbachol, desensitization was conspicuous. Within 5 min tension fell to its resting value. Desensitized preparations failed to respond to electrical stimulation. This was not due to fatigue: preparations subjected to a saline with elevated K-concentration maintained near maximal tension for many minutes and even retained the ability to produce additional tension on electrical stimulation as is illustrated in Fig. 6.

Preparations desensitized with carbachol did not respond to ACh. Failure to respond to added ACh coincided with failure to respond to electrical stimulation.

Anaesthetized preparations which no longer responded to electrical stimulation contracted when ACh or carbachol was added to the bath. The tension developed was similar to that generated in unanaesthetized preparations when the same concentration of ACh or carbachol was added.

As reported earlier (Florey *et al.* 1975) it is possible to peel off the epidermis and most if not all of the nerve plexus. In preparations that had been set up for an hour or more it was particularly easy to strip off the outer layer. Such skinned preparations gave faster and more powerful contractile responses to ACh and to carbachol, but the response to an elevated K-concentration was reduced. Evidently, much of the high-potassium effect on intact preparations is mediated by excitation of peripheral, presumably sensory and/or motor elements.

Eserine (2×10^{-6} g/ml) sensitized the preparations to ACh and caused a c

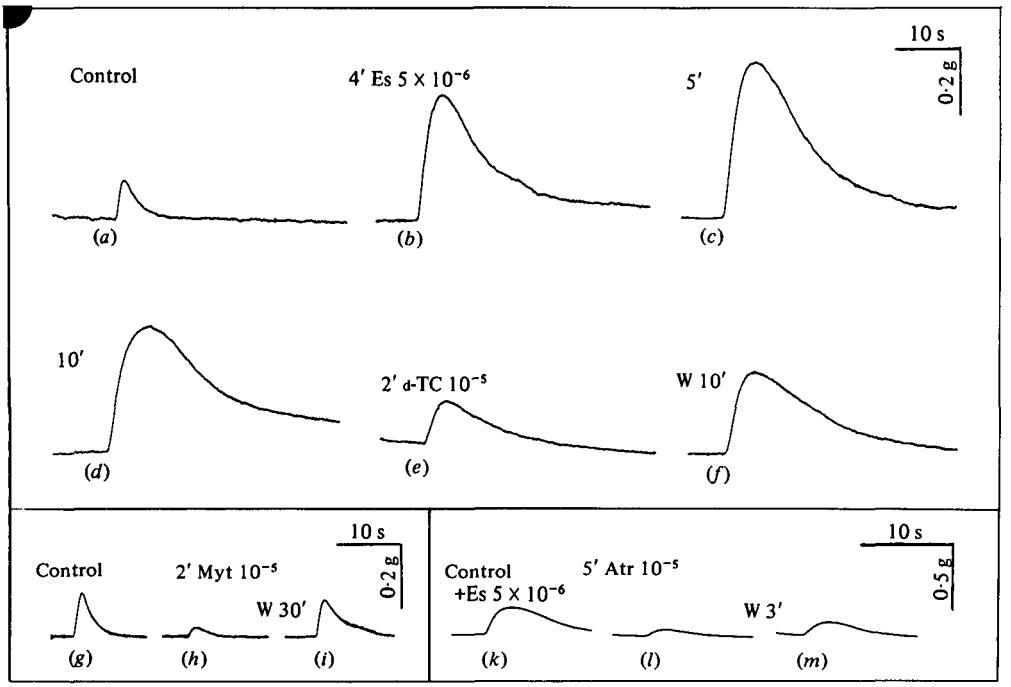

Fig. 7. Twitch-like contractions produced by constant stimuli are enhanced by eserine (records (*a*)–(*f*)) and antagonized by mytolon (records (*g*)–(*i*)) and by atropine (records (*k*)–(*m*)). Atr = atropine, Es = esterine, Myt = mytolon, W = washing. All concentrations in g/ml. All recordings are from the same preparation.

spicuous enhancement of both magnitude and duration of tension response to electrical stimuli. Examples are shown in Fig. 7. There was no increase of the carbachol effect. In contrast to ACh, carbachol is not hydrolyzed by cholinesterase.

Of the cholinergic blocking agents, we had previously found that mytolon and atropin and to a lesser extent D-tubocurarine greatly reduce the contractile effects of ACh (Florey *et al.* 1975). The same drugs strongly antagonize the contractile responses to electrical stimulation. Examples are shown in Fig. 7. D-tubocurarine was the least effective of the three blocking agents.

In several experiments 5-hydroxytryptamine (5-HT) was applied to intact and skinned preparations. In no case did we observe a contraction, nor a relaxation, nor an antagonism to ACh, even with concentrations as high as 10^{-5} g/ml.

DISCUSSION

The results of our experiments make it quite clear that the stem of the sea urchin tube foot contains all the functional elements necessary for a reflex control of the tube foot musculature. Mechanical stimulation (= touching) of the epidermis elicits a tension development that can be as large as that achieved by maximal ionic (= high K^+), chemical (ACh) or electrical stimulation. This implies the presence of sensory elements in the epidermis which respond to mechanical stimulation and which can

excite those neural elements responsible for the activation of the musculature. Such sensory cells have been observed in the epithelium of the base of the spines of *Echinus esculentus* (Weber & Grosmann, 1977). In the pedicellariae of the same species, Cobb (1970) found that all epithelial cells are ciliated and can be regarded as sensory cells. In the tube feet of *Echinus* and other echinoid species we failed to observe sensory cells: the epithelial cells seen in all our electron micrographs have microvilli but no cilia. We were confident that the tube foot epithelial cells did not constitute a sensory epithelium (Florey & Cahill, 1977). On reinvestigation using scanning electron microscopy (unpublished data), we have found that in the tube foot epithelium of *S. franciscanus* ciliated cells occur in small, sparse clusters. The much more common unciliated epithelial cells do not extend cell processes into the nerve plexus but instead reach through the plexus towards the basal lamina where they are attached (Florey & Cahill, 1977). We have not yet determined whether or not the nerve terminals observed within the plexus, especially on the basal lamina, are in part or entirely derived from sensory cells.

It is interesting that even very brief (0·2 ms) electrical pulses give rise to a twitch-like contraction. This indicates electrical excitability. The sharp thresholds, which can be fitted into a chronaxy diagram (Fig. 4), indicate that the excitation is of the all-or-nothing type. The rather long delay between stimulus and contractile response (on the average 162 ms) implies that the electrical stimulus does not affect the muscle directly. Further evidence for this is the fact that electrode placement close to the muscle layer is far less effective than placement close to the nerve plexus. The experiments with anaesthetics are particularly telling: the musculature of anaesthetized tube feet responds fully to chemical stimulation by ACh or carbachol. Evidently, the muscle cells are not anaesthetized.

All our results are compatible with the assumption that electrical (and mechanical) stimulation excites nerve elements which, in turn, excite the muscle. The following pharmacological findings provide strong evidence that transmission of excitation from nerve elements to muscle is by way of release of ACh as chemical transmitter.

(1) Earlier experiments (Florey *et al.* 1975) established that ACh occurs in conspicuously high concentration in the outer layer of the tube foot wall which contains the nerve plexus. It was later shown that nerve terminals located on the outer surface of the connective tissue layer contain clear vesicles (Florey & Cahill, 1977), a fact compatible with the assumption that these terminals are cholinergic.

(2) The musculature responds to low concentrations of ACh with contraction.

(3) When the ACh-receptors are desensitized by carbachol, mechanical and electrical stimulation fail to elicit contractile responses.

(4) Cholinergic blocking agents antagonize both the action of applied ACh (see Florey *et al.* 1975) and the effect of electrical stimulation.

(5) Inhibition of cholinesterase by eserine not only potentiates the action of applied ACh but also enhances and prolongs the contractile response to electrical stimulation.

(6) The muscle tension elicited by application of ACh or carbachol can be as large as that elicited by maximal mechanical or electrical stimulation. The effect of ACh, therefore, can fully account for the action of stimulated nerve elements; no other transmitter need be involved. Indeed, other potential transmitter substances

ke 5-HT, noradrenaline, histamine, L-glutamine or L-aspartate, are without effect on the muscle (Florey *et al.* 1975 and this investigation).

Since no nerve fibres can be seen to cross the connective tissue layer and no synapses are found on the muscle cells, the conclusion seems inevitable that chemical transmission from cholinergic nerve terminals to muscle cells involves transfer of transmitter across the connective tissue layer. Considering that this layer is many microns thick, this conclusion is not easy to accept (Cobb, 1978). Although the connective tissue layer of the tube foot can be as thin as 4–5 μm (see Fig. 1), the following analysis of the problem makes it clear that chemical transmission even across 20 or 25 μm of connective tissue is feasible.

Low voltage electrical stimulation sets up a local contraction, presumably due to a localized excitation of nerve elements. The effectiveness of very brief stimulation pulses means that transmitter release is nearly instantaneous. We may further assume that the release is confined to a minute site, involving perhaps only one or a few terminals. Release may thus be regarded as approaching instantaneous release from a point source and can be treated like the release of ACh from a micropipette as used in iontophoresis of ACh onto muscle fibres.

Based on the mathematical description of diffusion from a point source (Carslaw & Jaeger, 1947; del Castillo & Katz, 1955; Curtis, Perrin & Watkins, 1960), it is possible to derive simple equations which permit calculation of diffusion time and of the concentrations reached when a substance is instantaneously released from a point source (Curtis, 1964):

The time T required for the maximal concentration, C_{max}, to be reached at distance d is given by

$$T = \frac{d^2}{6D10^8} \text{ s} \qquad (1)$$

and C_{max} can be obtained from

$$C_{max} = \frac{10^3\, Q \exp(-1{\cdot}5)}{8\,(\pi DT)^{1{\cdot}5}} = \frac{10^3\, Q \exp(-1{\cdot}5)}{8\,[\pi D(d^2/6D10^8)]^{1{\cdot}5}} = \frac{10^3\, Q \exp(-1{\cdot}5)}{8\,(\pi d^2/6{\cdot}10^8)^{1{\cdot}5}} \text{ M}, \qquad (2)$$

where Q is the number of moles released and D is the diffusion constant. In experiments on frog muscle Dreyer & Peper (1974) obtained for ACh a diffusion constant D of $1{\cdot}2 \times 10^{-5}$ cm^2/s.

Using these equations it can be shown that if $d = 5$ μm, T is 3·5 ms, and if $d = 20$ μm, T is 55·6 ms. This time interval is still short compared to the shortest measured delay between stimulus and onset of contractile response (160 ms). For a C_{max} of 10^{-6} M (required to elicit a modest contraction) Q must be $1{\cdot}7 \times 10^{-18}$ mole if $d = 5$ μm, and $1{\cdot}1 \times 10^{-16}$ M if $d = 20$ μm. For comparison: an amount of 10^{-17} mole corresponds to that released from two motor endplates on vertebrate striated muscle in response to a single stimulus (see Kuffler & Nicholls, 1977).

The calculations make it clear that, given a long delay between stimulus and contractile response (more than 160 ms), diffusion time is not a limiting factor; it is even less so when the peak of the contractile response is considered since this occurs 1 s or more after the stimulus.

Chemical long-distance transmission by ACh is possible only if there is no cholinesterase present in the connective tissue. Our histochemical investigation showed

that cholinesterase is absent from the connective tissue; the enzyme is detectable only on the surface of muscle fibres and in epithelial cells.

On the basis of ultrastructural evidence Cobb (1970) concluded that motor control of sea urchin tube foot ampullae is by way of chemical transmission across a connective tissue sheath. We now provide evidence that such a mechanism is also operative in the tube foot itself, and that the transmitter is ACh.

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