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ELECTROPHYSIOLOGY AND PHARMACOLOGY OF STRIATED MUSCLE FIBRES CULTURED FROM DISSOCIATED NEONATAL RAT PINEAL GLANDS

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SUMMARY

1. Striated muscle fibres were found in each of twenty consecutive pineal glands cultured from individual neonatal rats.

2. In subsequent experiments performed with dissociated cultures of pineal organs pooled from several litters, myotubes were first visible after about 1 week in culture.

3. During the next several weeks the myotubes increased in size, developed cross-striations, and began to twitch spontaneously.

4. The resting membrane potential increased with age in culture. All myotubes studied showed delayed rectification. Action potentials either occurred spontaneously or could be evoked if the membrane were sufficiently polarized. No spontaneous end plate potentials were seen.

5. Acetylcholine (ACh) produced a brief, monophasic depolarizing response. Noradrenaline, serotonin, melatonin, dopamine, and γ-aminobutyric acid (GABA) had no effect on the resting membrane potential when applied iontophoretically.

6. The ACh response was reversibly blocked by 10^{-6} M-tubocurarine and irreversibly blocked by 10^{-6} M-α-bungarotoxin. Atropine (10^{-4} M) reduced the amplitude and shortened the time course of the ACh response, and 10^{-3} M-atropine produced complete but reversible inhibition.

7. We conclude that pineal muscle fibres are electrophysiologically and pharmacologically similar to skeletal muscle fibres *in vitro*. Although the pineal gland has undetectable levels of ACh, pineal muscle develops ACh receptors but not noradrenaline, serotonin, melatonin, dopamine, or GABA receptors mediating electrophysiological responses, although these latter substances (except dopamine) are found in the pineal.

INTRODUCTION

Striated muscle fibres have been observed within the pineal organs of several mammalian species including man (Hayano, Sung & Hill, 1976). Serially sectioned pineal glands have been examined by light and electron microscopy, and in the few pineals where muscle fibres have been seen, they have been sparse and have lacked regular cross-striations typical of skeletal muscle. The muscle has tended to occur in clusters about parenchymal blood vessels and has not appeared innervated (Hayano

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et al. 1976). Nothing is known of the function or origin of these muscle fibres, although it has been suggested that the muscle may be carried into the pineal during early embryonic development (Hayano *et al.* 1976).

Several groups of investigators have reported the occurrence of striated muscle fibres in cultured pineal glands (Nathanson, Binkley & Hilfer, 1977; Quay, 1965; Rowe, Neale, Alvins, Giroff & Schrier, 1977), but the physiological and pharmacological properties of these muscle fibres have not been described.

We have observed abundant multinucleated muscle fibres in dissociated rat pineal glands in culture. Because it is thought that the pineal does not contain acetylcholine (ACh) (Schrier & Klein, 1974), we sought to determine if cultured pineal muscle fibres possess ACh receptors as do striated muscle fibres outside the central nervous system (skeletal muscle) or if they exhibit electrophysiological responses to substances known to occur in the pineal (Wurtman & Moskowitz, 1977). An approach to learning about a possible function of these muscle fibres includes determining the chemosensitivity of the myotubes.

We describe in this paper the incidence and the electrophysiological and pharmacological characteristics of striated muscle fibres cultured from dissociated rat pineal glands. We compare these results with the reported properties of isolated twitch-type muscle preparations and dissociated embryonic skeletal muscle in culture.

METHODS

Tissue culture techniques

Pineal glands were removed from 2-day-old rats and were cleaned of adhering dura and blood vessels. Routinely, pineal glands from six litters were pooled and dissociated by incubation with 5·0 ml. of a solution of trypsin, collagenase, and chick serum in Ca^{2+}-, Mg^{2+}-free Hanks' (Coon, 1966) containing deoxyribonuclease 0·1 mg/ml. (Sigma, St Louis, Mo.) for 75 min at 37 °C in an atmosphere of 95 % air–5 % CO_2. The dissociation medium plus glands was then mixed with 5·0 ml. ice-cold modified Ham's F12 (Coon & Weiss, 1971) containing 10 % fetal bovine serum (Flow Labs, Rockville, Md.) and gentamicin sulphate (Sigma, 5 ug/ml), and was triturated vigorously for 30 sec. The resultant cell suspension was centrifuged at room temperature for 5 min at 1500 *g*. The pellet was washed once with ice-cold culture medium and then resuspended in culture medium with added glutamine (0·4 mg/ml.) and ascorbic acid (0·2 mg/ml.) to a final cell density of 10^6 cells/ml. Portions of 0·2 ml. of the suspension were plated in the centre of 35 mm plastic culture dishes (Costar, Cambridge, Mass.) and incubated overnight at 34 °C in 95 % air–5 % CO_2. Culture medium, 1·5 ml., with added glutamine and ascorbic acid was then added to each dish. The medium was changed every second day. Conditions for the dissociation of individual pineal glands were the same except that the entire procedure for each gland was executed in the well of a Costar microlitre dish. The resultant cell suspensions from each gland were cultured in separate 35 mm dishes.

Electrophysiological and pharmacological techniques

Culture dishes were transferred directly or in a perfusion chamber to the stage of an inverted phase contrast microscope. Acid-base balance was maintained by passing water-saturated 10 % CO_2 over the surface of the culture medium. Most experiments were performed at room temperature. Under direct visualization and aided by a Leitz micromanipulator, muscle fibres were penetrated by single-barrelled, fibre-filled, glass electrodes filled with either 3 M-KCl or 3 M-CH_3COOK and having resistances of 50–100 MΩ. Recordings were made using a high-impedance unity gain electrometer with negative capacitance feed-back. Using the bridge circuit of the electrometer, current was passed through the same electrode. Current was monitored using an operational amplifier that converts current to voltage and clamps the bath at virtual ground. Measurements were made from photographs of a Tektronix 565 oscilloscope display and from the record of a Gould Brush 240 penwriter.

Ionophoretic application of drugs was made using a control module designed to maintain applied voltage and vary time in order to pass a predetermined total charge (Willis, Myers & Carpenter, 1978). This method aids in eliminating electroosmotic flux which is largely a function of applied voltage. Single-barrelled electrodes with resistances of 50–100 MΩ (when filled with KCl) and five-barrelled electrodes with resistances of about 50 MΩ were used. Backing currents of 1–3 nA were routinely used. In order to minimize the extent to which the duration of the ionophoretic current artifact extended into the voltage response, the fastest duration setting was always used. During experiments, as various charge settings were selected, the ionophoretic pulse ranged from about 10-500 msec. A given pulse rarely varied by more than 50 msec, suggesting that, in practice, electrode tip impedance did not change a great deal.

Solutions of drugs to be ionophoresed were made up fresh from stock for each experiment. Except for melatonin, the drugs were dissolved in distilled water and ejected as cations. Because melatonin is uncharged and poorly soluble in water, the drug was dissolved in a small amount of dimethylsulphoxide and then further diluted in distilled water. NaCl was added to a concentration of 0·2 M to serve as a charge carrier. The melatonin was then ejected by electroosmosis. Alternatively, melatonin was applied by bringing a leaky, blunt-tipped electrode near the cell. This latter technique was also used for a few cells to apply serotonin and noradrenaline.

Concentrations of ionophoretically applied drugs were: ACh hydrochloride, 0·5 or 1 M; L-noradrenaline hydrochloride, 1 M; dopamine hydrochloride, 1 M; 5-hydroxytryptamine (5-HT, serotonin) hydrochloride, 0·5 M, or creatinine sulphate, saturated solution; and γ-amino-*n*-butyric acid (GABA), 1 M. The final concentration of melatonin was 0·1 M.

Drugs applied by perfusion were dissolved in culture medium and applied via a Holter perfusion pump at about 1 ml./min. The concentrations of perfused drugs were: (+)-tubocurarine, 10^{-6} M; α-bungarotoxin, 10^{-6} M; atropine sulphate, 10^{-4} and 10^{-3} M.

All drugs were obtained from commercial sources except α-BT, which was supplied by Drs G. Tobias and M. Donlon of this institute.

RESULTS

Morphology

The incidence of muscle fibres in individual pineal glands was determined by separately culturing each of twenty consecutive dissociated neonatal rat pineals. Striated muscle fibres were found in each of the cultured pineal glands. Subsequent studies were performed on cultures of pineal glands pooled from several litters. The dissociation procedure yielded a mixture of cell types, and individual myoblasts could not be easily identified as such during the first week of culture. After about 1 week, fusion of myoblasts to form spindle-shaped or cylindrical multinucleated myotubes allowed identification. During the second week of culture, the myotubes increased in size and many developed spontaneous twitching. After several weeks in culture, many fibres had cross-striations visible under phase contrast and contrast interference optics. Large contracting muscle fibres were maintained in culture for over 4 months, the extent of our observation. Experiments, however, were limited to cultures of 1–4 weeks of age.

Electrical membrane properties

Although not studied systematically, the resting membrane potential was related to age in culture. Generally the potential ranged from −35 to −45 mV during the first week after fusion (second week of culture). By the second week after fusion (third week of culture), the range was increased to −45 to −65 mV.

Current–voltage relations were determined in over twenty-five cells with resting membrane potentials from −45 to −65 mV. Every cell showed a linear relationship from −100 to about −35 mV; further depolarization led to a delayed increase in

conductance (delayed rectification) that inactivated with time (Fig. 1). The input resistance could be computed by determining the slope of the current–voltage plot in the hyperpolarizing quadrant. As with the resting membrane potential, the value of input resistance was related to age in culture and to fibre size, but these relationships were not studied in detail. The range of values of input resistance was 1–50 MΩ

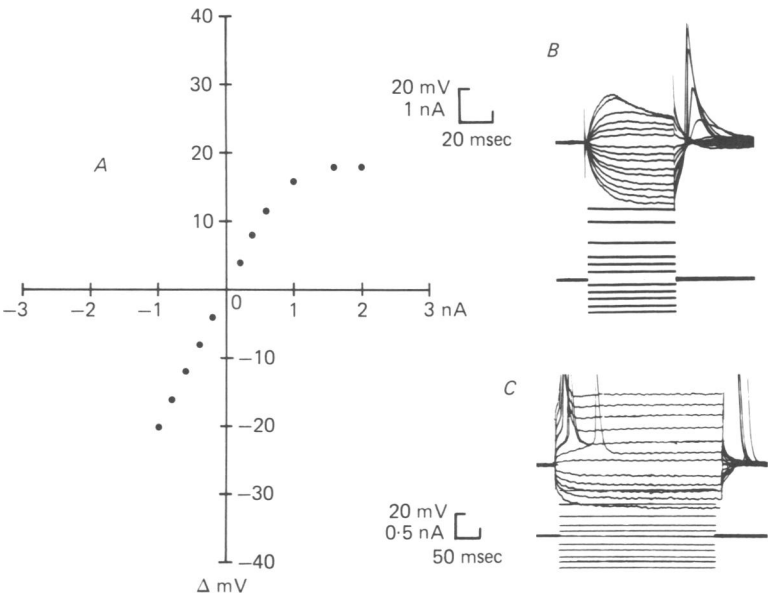

Fig. 1. Current–voltage relations in pineal gland myotubes. *A* and *B* are from same myotube with a resting membrane potential of −50 mV. *C* is from a myotube with a potential of −60 mV. In *B* the upper trace shows change in membrane potential in response to different rectangular current pulses (lower trace) of constant duration. Responses were measured at the end of the current pulse. In *A* these relations are displayed graphically. The origin of the plot represents the resting membrane potential. Note the onset of delayed rectification after the membrane potential is depolarized to about −35 mV. *C*, inactivation of delayed rectification during long depolarizing current pulses. Upper trace voltage, lower trace current.

Using both depolarizing and hyperpolarizing (anodal break) current pulses, action potentials could be evoked from all cells having stable resting membrane potentials. Myotubes with values below −50 mV usually required hyperpolarization to a more negative potential before action potentials could be evoked (Fig. 2*A*). Many cells gave rise to spontaneous action potentials that, similar to evoked potentials, generally were not seen at resting potentials below −50 mV. When cells with lower potentials were hyperpolarized by passage of steady inward current, spontaneous action potentials were often elicited (Fig. 2*B*).

Under the microscope, the presence or absence of innervation of the myotubes could not be reliably ascertained because the muscle fibres were usually surrounded by and covered with unidentified background cells. At no time, however, were excitatory or inhibitory post-synaptic potentials seen that would suggest innervation.

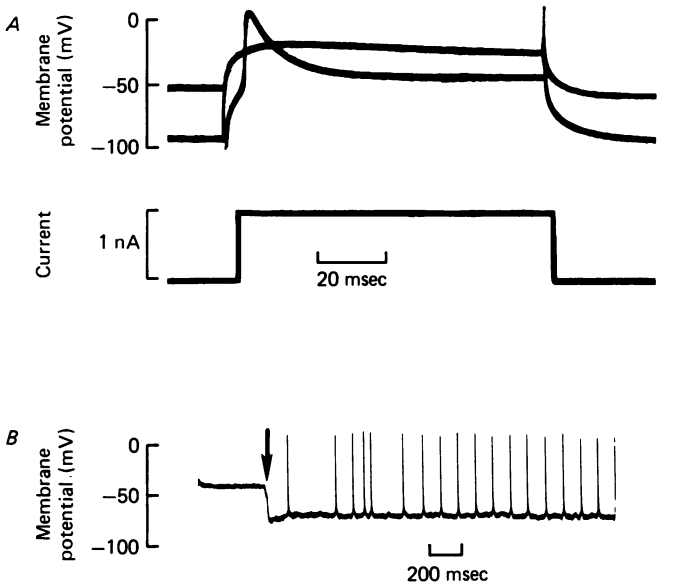

Fig. 2. Dependence of action potential generation on resting membrane potential. *A*, at a resting membrane potential of −50 mV, a current pulse evokes a passive response. After hyperpolarization to −90 mV, the same size current pulse elicits a spike. *B*, after hyperpolarization of the myotube membrane from −45 to −75 mV, spontaneous action potentials, not seen at a resting potential, are generated. Arrow marks onset of direct current.

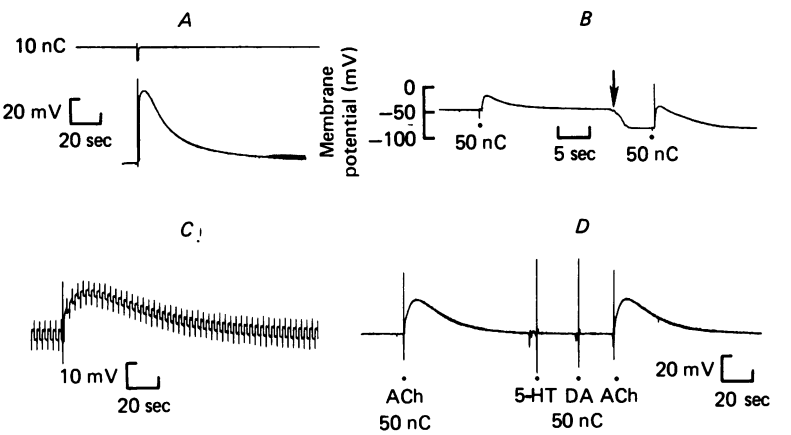

Fig. 3. Depolarizing response of myotubes to ACh. *A*, a typical response to a single pulse of ACh. Upper trace current expressed as charge, lower trace voltage. *B*, ACh depolarizing response increases in amplitude and gives rise to an action potential after the membrane is hyperpolarized from a resting membrane potential −50 to −75 mV by passing steady inward current (indicated by arrow). Dot marks time of application of ACh. *C*, membrane resistance is monitored by passing brief constant hyperpolarizing current pulses across the cell membrane. During the ACh response the resistance falls. *D*, using a multibarrelled ionophoretic pipette, 50 nC pulses (applied at dot) of ACh, serotonin (5-HT), and dopamine (DA) are ejected at different times. The myotube responds only to ACh. Negative responses to melatonin, noradrenaline, and γ-amino-butyric acid are not shown.

Ionophoretic responses

Ionophoresis of ACh produced a brief, monophasic depolarizing response (Fig. 3*A*), the amplitude of which was dose dependent until saturation occurred. Depending on the level of the resting membrane potential, the ACh response could bring the cell to threshold for action potential generation (Fig. 3*B*). When the membrane resistance was monitored by passing brief hyperpolarizing current pulses across the cell membrane, a fall in resistance was seen during the depolarizing re-

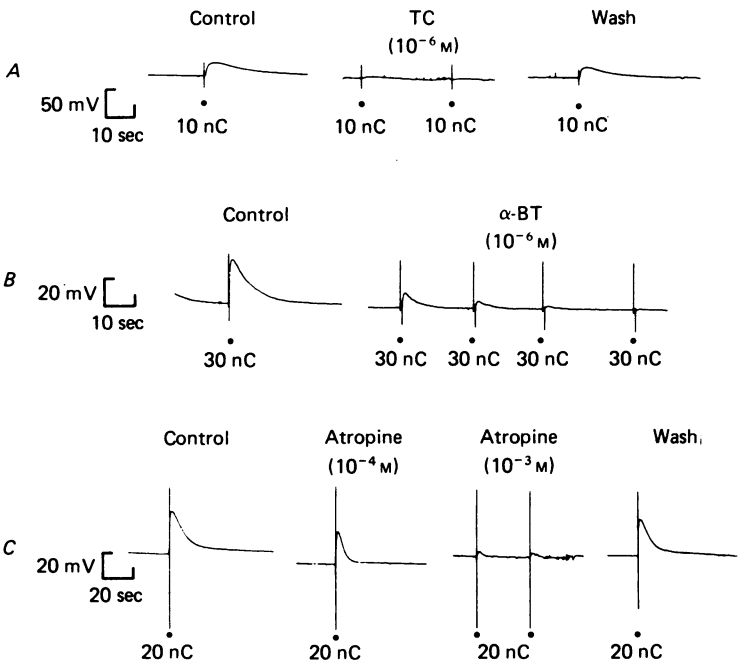

Fig. 4. Effects of pharmacological antagonists on the ACh response. In all traces, the dot marks the time of ACh ionophoretic pulse. *A*, TC completely blocks the ACh response. Recovery of the ACh response is seen after a 30 min wash. *B*, α-BT inhibits the ACh response which is not restored after a 45 min wash. *C*, atropine (10^{-4} M) reduces the amplitude and shortens the time course of the ACh response. Atropine (10^{-3} M) blocks the ACh response. Recovery is shown after the 45 min wash.

sponse to ACh (Fig. 3*C*). Repositioning the ionophoretic electrode every 25–50 μm over the muscle surface showed that the ACh response varied in sensitivity from about 0·5–60 mV/nC and was widespread in distribution over the fibre.

Application of noradrenaline, serotonin, dopamine, GABA, and melatonin to the myotube membrane produced no change in the resting potential (Fig. 3*D*). A minimum of ten cells was sampled for each drug, and several sites over the muscle surface were tested. Ionophoretic pulses of 10 to over 1000 nC were applied; the electrode was often moved away from the cell between pulses to check for desensitization.

The effect of antagonists on the response to ACh

Several classes of ACh antagonists were tested on the ionophoretically evoked response. Blockade of the ACh response was achieved by perfusing 10^{-6} M-tubocurarine into the recording chamber. This inhibition was reversed by washing out the tubocurarine by perfusion with fresh medium (Fig. 4*A*). α-bungarotoxin (10^{-6} M) produced complete inhibition of the ACh response. Attempting to reverse the blockade by perfusing with toxin-free medium for over 45 min was unsuccessful (Fig. 4*B*). Atropine at a concentration of 10^{-4} M did not block the ACh response, but the amplitude of the response was attenuated and the time course shortened. Total blockade was achieved after perfusion of 10^{-3} M-atropine, and this inhibition was reversible after washout with fresh medium (Fig. 4*C*).

DISCUSSION

Our results suggest that myogenic cells of unknown embryonic origin occur commonly and may be a normal constituent in the neonatal rat pineal gland. When allowed to differentiate in culture, the myotubes are electrophysiologically similar to embryonic skeletal muscle *in vitro* and isolated twitch-type skeletal muscle.

The morphology of the myotubes as seen under phase contrast and contrast interference microscopy is similar to that reported for cultured embryonic skeletal muscle (Fambrough & Rash, 1971; Fischbach, Fambrough & Nelson, 1973; Harris, Marshall & Wilson, 1973; Konigsberg, 1963). Several groups of investigators who have examined light and electron microscopic sections of pineal glands of several mammalian species have reported that striated muscle fibres are rare and structurally atypical (Hayano *et al.* 1976). The difference between our findings and those of the above-mentioned authors cannot be emphasized since we did not use electron microscopy, but it may reflect the response of muscle cells to dissociation and culture and/or the age of the animals selected for study.

The passive electrical membrane properties of the cultured pineal myotubes were similar to those described for isolated twitch-type skeletal muscle fibres (Adrian, Chandler & Hodgkin, 1970) as well as skeletal myotubes in culture (Fischbach, Nameroff & Nelson, 1971; Harris *et al.* 1973; Ritchie & Fambrough, 1975; Spector & Prives, 1977). In these pineal muscle fibres the active electrical membrane properties studied were characteristic of embryonic skeletal muscle in culture (Fischbach *et al.* 1971; Harris *et al.* 1973; Kano, 1975; Kidokoro, 1975; Spector & Prives, 1977). That no calcium-mediated action potentials (Kano, 1975) were seen may reflect the maturity of the myotubes selected for study (Spector & Prives, 1977).

Ionophoresis of ACh gave rise to depolarizing responses of relatively low sensitivity (0·5–60 mV/nC) and widespread distribution. Variations within this range of sensitivities were apparent from one patch of membrane to another. This non-homogeneity of sensitivity suggests clustering of ACh receptors (Sytkowski, Vogel & Nirenberg, 1973), yet no 'hot spots' of high sensitivities were found in the range of hundreds to several thousands of mV/nC as reported for other non-innervated muscle preparations (Betz & Osborne, 1977; Fambrough & Rash, 1971; Fischbach & Cohen, 1973; Fischbach *et al.* 1973). In part this may be due to species differences since it is

reported that rat myotubes show less ACh receptor clustering than do chick myotubes as measured by labelled α-bungarotoxin autoradiography (Fambrough, Hartzell, Rash & Ritchie, 1974). However, Askansas, Engel, Ringel & Bender (1977) have found diffuse non-junctional distribution of ACh receptors in aneurally cultured human, rat, and chicken skeletal muscle. They believe variations in the geometry of the muscle fibre surface can explain what others consider to be biologically significant 'hot spots'. Another explanation for the relatively low ACh sensitivity is that it may be a normal property of striated muscle fibres in the pineal.

The complete inhibition of the pineal muscle ACh response by tubocurarine and α-bungarotoxin and the thousandfold lesser sensitivity to atropine are in accordance with the known pharmacology of 'nicotinic' ACh receptors on skeletal muscle (Beránek & Vyskočil, 1967, 1968; Harris *et al*, 1973; Katz & Miledi, 1973*a*, *b*; Vogel, Sytkowski & Nirenberg, 1972). The ACh response in the pineal muscle fibres was blocked by 10^{-6} M-tubocurarine (lower concentrations not tested). This sensitivity is similar to innervated and denervated rat diaphragm preparations (Beránek & Vyskočil, 1967). In cultured chick myotubes, however, Harris *et al.* (1973) have reported that 3×10^{-5} M-tubocurarine was required for inhibition of the ACh response. The variability among these reported tubocurarine sensitivities may be due to species differences. The atropine concentration of between 10^{-4} and 10^{-3} M required to inhibit the ACh response in our preparation is in agreement with the atropine sensitivities reported for other striated muscle systems (Beránek & Vyskočil, 1967, 1968; Harris *et al.* 1973; Katz & Miledi, 1973*b*).

The effect of atropine on the time course and amplitude of the ACh response in comparison to the effect of tubocurarine is similar to that reported by Beránek & Vyskočil (1967, 1968). It has been shown by voltage clamp and membrane noise analysis that tubocurarine and α-bungarotoxin competitively block access of ACh to the receptor, thereby reducing the frequency but not the time course or conductance of the elementary event (Katz & Miledi, 1972, 1973*a*). Atropine, however, acts as an uncompetitive inhibitor by altering elementary channel kinetics (Katz & Miledi, 1973*b*; Adler & Albuquerque, 1976). The life span of the elementary current is reduced but the elementary conductance is unchanged (Feltz, Large & Trautmann, 1977).

There was no electrophysiological response of the pineal myotubes to other substances known to be found in the pineal. The existence of presumably noninnervated striated muscle cells in an environment of undetectable levels of ACh and high levels of other chemicals does not appear to induce the formation of non-ACh receptors that mediate changes in membrane potential. This is consistent with studies in developmental neurobiology showing that chemosensitivity is coincident with differentiation in such a way that cells appear programmed to anticipate innervation and, as they differentiate, incorporate into their surface membranes receptors that can later mediate responses to appropriate neurotransmitters (Fambrough *et al.* 1974). Our results, however, do not exclude the possibility that receptors to substances such as taurine (Guidotti, Badiani & Pepeu, 1972) and peptides (Brownstein, 1977; Rossier, Vargo, Minick, Ling, Bloom & Guillemin, 1977) not tested in our experiments may be present on these fibres. Nor do they exclude the possibilities that sensitivity to chemicals other than ACh might develop later in post-natal life,

that conditions *in vivo* but not present in culture might permit development of such chemosensitivity, or that receptors to substances other than ACh exist that subserve functions not associated with changes in membrane potential.

Although pineal striated muscle fibres in culture appear like skeletal muscle *in vitro*, it seems unlikely that they are of functional importance in the pineal for two reasons: (1) despite their tendency to cluster about blood vessels, the muscle fibres in the post-natal animal are quite sparse and poorly developed in comparison to skeletal muscle; and (2) because these muscle fibres appear uninnervated and seem to have only ACh receptors, and because ACh is undetectable in the pineal, there is no obvious way these cells can function by way of conventional modes of synaptic transmission.

REFERENCES

ADLER, M. & ALBUQUERQUE, E. X. (1976). An analysis of the action of atropine and scopolomine on the end-plate current of frog sartorius muscle. *J. Pharmac. exp. Ther.* **196**, 360–372.

ADRIAN, R. H., CHANDLER, W. R. & HODGKIN, A. L. (1970). Voltage clamp experiments in striated muscle fibres. *J. Physiol.* **208**, 607–644.

ASKANSAS, V., ENGEL, W. K., RINGEL, S. P. & BENDER, A. N. (1977). Acetylcholine receptors of aneurally cultured human and animal muscle. *Neurology* **27**, 1019–1022.

BERÁNEK, R. & VYSKOČIL, F. (1967). The action of tubocurarine and atropine on the normal and denervated rat diaphragm. *J. Physiol.* **188**, 53–55.

BERÁNEK, R. & VYSKOČIL, F. (1968). The effect of atropine on the frog sartorius neuromuscular junction. *J. Physiol.* **195**, 493–503.

BETZ, W. & OSBORNE, M. (1977). Effects of innervation on acetylcholine sensitivity of developing muscle *in vitro*. *J. Physiol.* **270**, 75–88.

BROWNSTEIN, M. J. (1977). Biologically active peptides in the mammallian central nervous system. In *Peptides in Neurobiology*, ed. GAINER, H., pp. 145–182. New York: Plenum.

COON, H. G. (1966). Clonal stability and phenotypic expression of chick cartilage cells *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **55**, 66–73.

COON, H. G. & WEISS, M. C. (1971). A quantitative comparison of formation of spontaneous and virus produced viable hybrids. *Proc. natn. Acad. Sci. U.S.A.* **62**, 852–859.

FAMBROUGH, D., HARTZELL, H. C., RASH, J. & RITCHIE, A. K. (1974). Receptor properties of developing muscle. In *Trophic Functions of the Neuron*, ed. DRACHMAN, D. B., *Annals of the N.Y. Academy of Sciences*, **228**, 47–62.

FAMBROUGH, D. M. & RASH, J. (1971). Development of acetycholine sensitivity during myogenesis. *Devl Biol.* **26**, 55–68.

FELTZ, A., LARGE, W. A. & TRAUTMANN, A. (1977). Analysis of atropine action at the frog neuromuscular junction. *J. Physiol.* **269**, 109–130.

FISCHBACH, G. D. & COHEN, S. A. (1973). The distribution of acetycholine sensitivity over uninnervated and innervated muscle fibers grown in cell culture. *Devl Biol.* **31**, 147–162.

FISCHBACH, G. D., FAMBROUGH, D. & NELSON, P. G. (1973). A discussion of neuron and muscle cell cultures. *Fedn Proc.* **32**, 1636–1642.

FISCHBACH, G. D., NAMEROFF, M. & NELSON, P. G. (1971). Electrical properties of chick skeletal muscle fibers developing in cell culture. *J. cell Physiol.* **78**, 289–300.

GUIDOTTI, A., BADIANI, G. & PEPEU, G. (1972). Taurine distribution in the brain. *J. Neurochem.* **19**, 431–435.

HARRIS, J. B., MARSHALL, M. W. & WILSON, P. (1973). A physiological study of chick myotubes grown in tissue culture. *J. Physiol.* **229**, 751–766.

HAYANO, M., SUNG, J. H. & HILL, E. G. (1976). Striated muscle in the pineal gland of swine. *J. Neuropath. exp. Neurol.* **35**, 613–621.

KANO, M. (1975). Development of excitability in embryonic chick skeletal muscle cells. *J. cell Physiol.* **86**, 503–510.

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.* **224**, 665–669.

KATZ, B. & MILEDI, R. (1973*a*). The binding of acetylcholine to receptors and its removal from the synaptic cleft. *J. Physiol.* **231**, 549–574.

KATZ, B. & MILEDI, R. (1973*b*). The effect of atropine on acetylcholine action at the neuromuscular junction. *Proc. R. Soc.* B **184**, 221–226.

KIDOKORO, Y. (1975). Developmental changes of membrane electrical properties in a rat skeletal muscle cell line. *J. Physiol.* **244**, 129–143.

KONIGSBERG, I. R. (1963). Clonal analysis of myogenesis. *Science, N.Y.* **140**, 1273–1274.

NATHANSON, M. A., BINKLEY, S. & HILFER, S. R. (1977). Cultivation of mammalian pineal cells: retention of organization and function in tissue culture. *In Vivo* **13**, 843–848.

QUAY, W. B. (1965). Histological structure and cytology of the pineal organ in birds and mammals. In *Progress in Brain Research*, ed. KAPPERS, J. A. & SCHADE, J. P., vol. 10, pp. 49–86. New York: Elsevier Publishing Co.

RITCHIE, A. K. & FAMBROUGH, D. (1975). Electrophysiological properties of the membrane and acetylcholine receptor in developing rat and chick myotubes. *J. gen. Physiol.* **66**, 327–355.

ROSSIER, J., VARGO, T. M., MINICK, S., LING, N., BLOOM, F. E. & GUILLEMIN, R. (1977). Regional dissociation of B-endorphin and enkephalin contents in rat brain and pituitary. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5162–5165.

ROWE, V., NEALE, E. A., ALVINS, L., GIROFF, G. & SCHRIER, B. K. (1977). Pineal cells in culture. *Expl Cell Res.* **104**, 345–356.

SCHRIER, B. K. & KLEIN, D. C. (1974). Absence of choline acetyltransferase in rat and rabbit pineal gland. *Brain Res.* **79**, 347–351.

SPECTOR, I. & PRIVES, J. M. (1977). Development of electrophysiological and biochemical membrane properties during differentiation of embryonic skeletal muscle in culture. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5166–5170.

SYTKOWSKI, A. J., VOGEL, Z. & NIRENBERG, M. W. (1973). Development of acetylcholine receptor clusters on cultured muscle cells. *Proc. natn. Acad. Sci. U.S.A.* **70**, 270–274.

VOGEL, Z., SYTKOWSKI, A. J. & NIRENBERG, M. W. (1972). Acetylcholine receptors of muscle grown *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **69**, 3180–3184.

WILLIS, J. A., MYERS, P. R. & CARPENTER, D. O. (1978). An ionophoretic module which controls electroosmosis. *JEPT* **6**, 34–41.

WURTMAN, R. J. & MOSKOWITZ, M. A. (1977). The pineal organ. *New Engl. J. Med.* **296**, 1329–1333, 1383–1386.