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# THE MEMBRANE POTENTIAL OF RAT DIAPHRAGM MUSCLE FIBRES AND THE EFFECT OF DENERVATION

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SUMMARY

1. Resting membrane potentials of rat diaphragm muscles were measured *in vitro* after previous denervation for 0–10 days. In some experiments denervated muscles were incubated *in vitro* for 3 hr while in others they were cultured for 15–24 hr to allow adequate exposure to drugs before recording.

2. It was found that resting membrane potentials within 2·5 mm of the site of nerve section were significantly lower, within 3 hr, than resting membrane potentials measured more than 9 mm away from site of nerve section. This difference could be reduced or abolished by bathing preparations in solutions containing adrenaline (10 $\mu$M), noradrenaline (10 $\mu$M) or isoprenaline (10 $\mu$M) or dibutyryl cyclic AMP (10 $\mu$M–0·25 mM in the presence of 2 mM theophylline). Cyclic AMP (0·5 mM) was ineffective.

3. Application of solutions containing dibutyryl cyclic AMP for 3 hr also raised the resting membrane potential of muscles denervated 4–5 days previously. Culture studies showed that this effect was sustained when the time of incubation was 24 hr.

4. Incubating freshly denervated preparations with cycloheximide (22 $\mu$g/ml.) puromycin (10 or 50 $\mu$g/ml.) or actinomycin D (1 $\mu$g/ml.) did not prevent the development of the early (3 hr) fall in resting membrane potential despite a concomitant inhibition of RNA or protein synthesis. Culturing freshly denervated muscles in solutions containing cycloheximide (10 or 25 $\mu$g/ml.) which blocked 93% of protein synthesis, did not prevent the expected drop in resting membrane potential after 15 or 24 hr.

5. It was found that exposure to ouabain (1 or 5 mM) produced a rapid (15 min) fall in resting membrane potential in innervated and denervated preparations treated with dibutyryl cyclic AMP but not denervated

preparations. After 5 days denervation cyclic AMP levels in muscle were increased by about 40%.

6. It is suggested that upon denervation an electrogenic action of a $Na^+$-pump is blocked and that dibutyryl cyclic AMP and catecholamines are capable of stimulating this pump.

## INTRODUCTION

After some controversy (Li, Shy & Wells, 1957) the report that the resting membrane potential of mammalian muscle falls after denervation (Ware, Bennett & McIntyre, 1951) has been abundantly confirmed (Lüllmann, 1958; Klaus, Lüllmann & Muscholl, 1960; Locke & Solomon, 1967; Albuquerque & Thesleff, 1968; Albuquerque & McIsaac, 1970; Albuquerque, Schuh & Kauffman, 1971; Redfern & Thesleff, 1971). In rat muscle the depolarization apparently begins in the end-plate region and spreads outward to the musculo-tendinous junction, taking some 15–24 hr for completion (Albuquerque *et al.* 1971). Muscle fibres closest to the site of nerve section are first affected, the onset of the fall in resting membrane potential being delayed about 40 min for every mm of nerve stump remaining attached to the preparation (Locke & Solomon, 1967; Albuquerque *et al.* 1971). While there is some conflict about the exact time the resting membrane potential changes begin after denervation it is agreed that the fall is the earliest sign of denervation (Albuquerque, Warnick, Sansone & Onur, 1974; Thesleff, 1974) and so is of great interest to investigators.

The present investigation is concerned principally with the question, what muscle function is affected by denervation? One possibility, suggested by Locke & Solomon (1967), is that the active transport of $Na^+$ is impaired upon denervation. This hypothesis, based on the finding that the resting membrane potential of rat muscle fibres *in vivo* (about 80 mV) falls within 5 min to the denervated level (about 60 mV) after topical application of ouabain and that the resting membrane potential of the denervated muscle fibres *in vivo* is relatively insensitive to ouabain, implies that in mammalian innervated muscle about a quarter of the resting membrane potential is electrogenic in origin.

Abundant evidence for active Na transport contributing directly to the membrane potential of 'Na-rich' muscle fibres is available for both frog (Kernan, 1962; Cross, Keynes & Rybová, 1965; Frumento, 1965; Hashimoto, 1965; Adrian & Slayman, 1966; Harris & Ochs, 1966) and more recently for rat muscle (Dockry, Kernan & Tangney, 1966; Kernan, 1968; Akaike, 1975). In fresh rat muscle however the resting membrane can be explained as a diffusion potential set up by passive ionic movement in so

far as the resting membrane potentials measured are in agreement with the membrane potentials expected from the intracellular [$K^+$] of the muscle fibres (Kernan, 1963, 1972; Akaike, 1975). It is, however, relevant to the electrogenic hypothesis that Dockry *et al.* (1966) found that the magnitude of the active transport of $Na^+$ was decreased in rat muscle fibres within 2 hr of denervation as would be expected on the Locke & Solomon (1967) hypothesis.

An alternative hypothesis is that there is a fall in $K^+$ activity in muscle due to sequestering of $K^+$ by newly formed protein (Albuquerque & McIsaac, 1970; Albuquerque *et al.* 1971; Guth, 1975). There are well-documented changes in protein metabolism in muscle upon denervation (Gutmann, 1963). In particular, in some muscles such as rat diaphragm, denervation induces an increased synthesis of protein which goes on for some 10 days (Buse, McMaster & Buse, 1965; Harris & Manchester, 1966). It could be predicted from this hypothesis that inhibition of RNA or protein synthesis would prevent the fall of resting membrane potential upon denervation and indeed a single injection of actinomycin D or cyclohexmide delayed the onset of the post-denervation fall in testing membrane potential in mouse leg muscles (Grampp, Harris & Thesleff, 1972).

We have investigated the two hypotheses, sequestration of $K^+$ by protein synthesis and the turning off of an electrogenic pump, by measuring resting membrane potentials of rat diaphragm muscles *in vitro*, after previous denervation from 3 hr to 6 days. We have been concerned with the time of onset of the resting membrane potential fall after denervation, the sensitivity of this fall to RNA and protein synthesis inhibitors and the effect of inhibitors and stimulators of the Na pump in muscle upon the resting membrane potential of denervated and innervated muscle. A brief report of preliminary findings was presented to the Otago Medical School Research Society (Bray, Hawken & Hubbard, 1974).

## METHODS

*Preparation of left hemidiaphragms for incubation.* Female albino rats of the Wistar strain weighing 180–210 g were used in all experiments. For experiments involving 3 hr incubation the ventral portion of the left diaphragm taken from rats, killed by decapitation, was pinned to 1·3 times its resting length on a Perspex frame. The phrenic nerve was sectioned within 0·5 mm of its entrance into the muscle. The preparation was then incubated for 3 hr in 50 ml. Liley bicarbonate-buffered solution (Liley, 1956) gassed with 95 % $O_2$–5 % $CO_2$ and at 37° C. The solution was prepared immediately before use and changed every hour.

*Electrophysiological measurements.* To determine the resting membrane potential of muscle fibres, the left hemidiaphragm was pinned out in a Perspex recording chamber mounted on a microscope stage. The chamber was perfused with Liley

solution, equilibrated with 95% $O_2$–5% $CO_2$ at room temperature (18–20°) at a flow rate of 3–4 ml./min. The diaphragm was illuminated by transmitted light and visualized with a Zeiss binocular dissecting microscope at 40× magnification. The membrane potentials were recorded with 3 M-KCl filled glass micro-electrodes with resistances between 8–16 MΩ. The recordings were amplified in the usual manner and displayed on an oscilloscope and on a paper recorder. Any electrodes that showed a tip potential greater than 1–2 mV were discarded. Penetrations were made adjacent to the intramuscular nerve and accepted only if the potential change was 'instantaneous' and miniature end-plate potentials, m.e.p.p., could be recorded. In short-term denervation experiments recordings were made within 2·5 mm of nerve section, and at a distance, greater than 9·0 mm from nerve section.

*Long-term denervation experiments.* Left hemidiaphragms were denervated for 4–5 days before recording membrane potentials by cutting the left phrenic nerve in the neck close to the brachial plexus as described by Miledi & Slater (1970). Resting membrane potentials were recorded in the same manner as in short-term denervation experiments the criteria being 'instantaneous' potential change in fibres adjacent to the visible remains of the intramuscular nerve, within 5 mm of its point of entry.

*Cultured muscle.* The methods of preparation and maintenance were in all particulars the same as those used by Purves & Sakmann (1974) with the following minor alterations. The left phrenic nerve was cut 0–10 days before removal of the left hemidiaphragm. Strips of muscle were 3–6 mm wide. Strips were pinned to 1·3 times their resting length with the thoracic surface up. The culture fluid was Trowell T8 (Trowell, 1959) with 32 mg/l. insulin and the other supplements described by Purves & Sakmann (1974). Recording conditions were identical to those described for the other experiments except that Trowell T8 solution was used instead of Liley solution.

*Chemicals.* The following chemicals were added to the incubation solution: L-noradrenaline D-bitartrate (Sigma), L-adrenaline (Sigma), L-isoprenaline D-bitartrate (Sigma), dibutyryl cyclic AMP ($N^6$,$O^2$-dibutyryl adenosine 3′,5′-cyclic monophosphate, monosodium salt; Sigma), cyclic AMP (adenosine 3′,5′-cyclic monophosphoric acid; Sigma) 5′ AMP (adenosine monophosphate, sodium salt; Sigma), theophylline (Calbiochem), prostaglandins, $PGE_1$ and $PGE_2$ (Upjohn, kindly supplied by Dr J. E. Pike), puromycin hydrochloride (Sigma), cycloheximide (Sigma) and actinomycin D (Sigma), ouabain octahydrate (Strophanthin-G; Sigma).

*Assay of protein and RNA synthesis in hemidiaphragms.* Cycloheximide or puromycin was used to block protein synthesis and actinomycin D to block RNA synthesis in hemidiaphragms prepared and incubated as described above. The degree of inhibition of protein synthesis was assessed by measuring the incorporation of L-[4,5-$^3$H]leucine (specific activity 58 c/m-mole) into trichloroacetic acid insoluble protein. To eliminate non-specific binding of tritiated leucine, the protein was dissolved in 0·5 M-NaOH containing an excess of 'cold' leucine, the solution was neutralized and protein was reprecipitated by adding an equal volume of 10% trichloroacetic acid. The degree of inhibition of RNA synthesis was determined by measuring the incorporation of [5-$^3$H]uridine (specific activity 30 c/m-mole) into the RNA fraction prepared as described by Hutchison & Munro (1961). Briefly, the tissue was extracted with cold 5% trichloroacetic acid containing an excess of uridine and then with chloroform–methanol (2:1). The residue was incubated in 0·3 M-NaOH for 2 hr to selectively degrade RNA. Protein and DNA were reprecipitated with trichloroacetic acid and the acid-soluble fragments of RNA were recovered in the supernatant on centrifugation. To determine the amount of radioactivity, the samples were neutralized and 1 ml. was added to 10 ml. Triton scintillation fluid

(30% Triton X-100; 0·3% 2,5′-diphenyloxazole; 0·01% 1,4-bis-[2,5 phenyloxazolyl]benzene in toluene). The samples were counted in a Packard Tri-carb liquid scintillation spectrometer at an efficiency of 33–35%. The protein content of the samples was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

*Assay of cyclic AMP in hemidiaphragms.* Left hemidiaphragms of rats weighing 200–220 g were denervated by cutting the left phrenic nerve in the neck close to the brachial plexus; the right hemidiaphragms of these same rats served as controls. Five days after denervation the rats were decapitated and the intact diaphragm, which was removed in less than 1 min, was plunged into liquid nitrogen. Frozen muscle was dissected separately from both left and right sides of the diaphragm and placed in 3 ml 5% trichloroacetic acid –10% methanol at below 0° C. A small quantity of [$^3$H]cyclic AMP was added at this stage to check the final recovery of cyclic AMP. The samples were disrupted by sonication with an ultrasonic probe (Wave Energy Systems; Newtown, Pa.) and centrifuged. The pellet was retained for protein estimation while the supernatant was extracted 5 times with 2 vol. ether and applied to a Dowex-1-formate column (1 cm × 1 cm). The column was washed with 10 ml. water and cyclic AMP was eluted with 1 M formic acid as described by Bannai & Sheppard (1974). The eluate was freeze-dried and redissolved in 0·5 ml. buffer. Recovery of the added [$^3$H]cyclic AMP was approximately 70%. The amount of cyclic AMP in 10 $\mu$l sample was determined by the cyclic AMP protein binding method of Gilman (1970).

## RESULTS

### *Decline of resting membrane potential in vitro following denervation*

In the original report of a fall of resting membrane potential within 2 hr of denervation (Albuquerque *et al.* 1971) motor nerves were sectioned *in vivo* and muscles removed just before recording resting membrane potentials *in vitro*. As Table 1, row 2 indicates, we have found that the

TABLE 1. Mean resting membrane potentials of rat hemidiaphragm muscle fibres *in vitro*, measured within 2·5 mm (near) and greater than 9 mm from nerve section (far)

| | No. of preparations | Near, mean ± S.E. of mean (mV) | Far, mean ± S.E. of mean (mV) | Difference (Far − Near) |
|---|---|---|---|---|
| Recorded within 30 min of removal from the animal | 3 | 79·4 ± 0·5 ($n$ = 75) | 78·4 ± 0·5 ($n$ = 77) | −1·0 (n.s.) |
| Recorded after 3 hr incubation | 7 | 68·8 ± 0·4 ($n$ = 163) | 75·7 ± 0·4 ($n$ = 162) | +6·9 ($P$ < 0·001) |
| Albuquerque *et al.* (1971) (Near: 1·5–2·49 mm) (Far: 5·5–6·5 mm) | 4 | 68·5 ± 0·7 ($n$ = 32) | 77·4 ± 0·5 ($n$ = 31) | +8·9 |

The total number of fibres sampled in each condition is denoted by $n$. The significance of the difference between the means has been tested by Student's $t$ test. A probability of $P > 0·05$, is considered to be non-significant (n.s.).

fall in resting membrane potential develops if the muscles are *in vitro* from the time of nerve section. The fall in resting membrane potential of the 'near' fibres (within 2·5 mm of nerve section) upon incubation of denervated muscle (Table 1) cannot be attributed solely to the incubation, for the fall is of the same order, as Table 1 row 3 shows, as that reported by Albuquerque *et al.* (1971), who recorded resting membrane potentials 15–30 min after removal of muscles from their animals.

The slightly lower resting membrane potential of the 'far' fibres (more than 9 mm from the site of nerve section) after 3 hr incubation compared to *in vivo* experiments can presumably be attributed to the inadequacies of the bathing medium even though surface fibres were used (Creese, 1954; Kernan, 1963).

TABLE 2. Mean resting membrane potentials measured at end-plate regions within 2·5 mm of the site of nerve section in rat diaphragm muscles exposed to RNA and protein synthesis inhibitors *in vitro* for 3 hr.; *n* is number of fibres sampled

| Drug | Resting membrane potentials, mean ± S.E. of mean (mV) | No. of preparations | % inhibition of protein or RNA synthesis (mean ± S.E. of mean) | No. of preparations |
|---|---|---|---|---|
| Control | 70 ± 0·7 ($n$ = 95) | 5 | — | — |
| Actinomycin D (1 μg/ml.) | 68 ± 0·7 ($n$ = 97) | 5 | 51 ± 14 | 4 |
| Puromycin | | | | |
| 10 μg/ml. | 68 ± 0·7 ($n$ = 98) | 5 | 64 ± 8 | 4 |
| 50 μg/ml. | 67 ± 1 ($n$ = 50) | 3 | 74 ± 6 | 4 |
| Cycloheximide 33 μg/ml | 70 ± 0·7 ($n$ = 100) | 5 | 88 ± 3 | 4 |

*The effects of protein synthesis inhibitors*

It has been claimed that protein and RNA synthesis inhibitors will delay or prevent the fall of resting membrane potential upon denervation (Grampp *et al.* 1972) although actinomycin D cannot reverse the fall (Grampp *et al.* 1972; Muchnik, Ruarte & Kotsias, 1973).

As Tables 2 and 3 indicate, we found no significant effect of cycloheximide upon the fall of resting membrane potential in denervated preparations, when examined after 3 hr *in vitro* (Table 2) or in preparations denervated and immediately cultured for 15–24 hr (Table 3). Actinomycin D and puromycin were likewise without effect upon the development of the fall in resting membrane potential *in vitro* (Table 2).

It might be argued that the drugs were not used in adequate concentrations. The argument cannot be sustained in view of our finding (Table 2)

that the inhibition of RNA synthesis was $51 \pm 14\%$ ($n = 4$), comparable with that reported by Grampp *et al.* (1972) in mouse muscle and the inhibitions of protein synthesis (Table 2 cycloheximide) were also comparable with those found by Grampp *et al.* (1972). The inhibition of protein synthesis achieved with cycloheximide (10 $\mu$g/ml.) in the culture experiments (Table 3) was 93% ($n = 2$).

TABLE 3. Resting membrane potentials of muscle fibres from diaphragm strips (from an innervated muscle) after 15 or 24 hr in organ culture with or without cycloheximide. The number of fibres sampled is denoted by $n$

| Drug concentration ($\mu$g/ml.) | Term of incubation (hr) | Mean ± S.E. of mean (mV) |
|---|---|---|
| 0 | 15 | $59{\cdot}1 \pm 0{\cdot}9$ ($n = 18$) |
| 25 | 15 | $64{\cdot}0 \pm 1{\cdot}2$ ($n = 12$) |
| 0 | 24 | $61{\cdot}5 \pm 0{\cdot}8$ ($n = 25$) |
| 25 | 24 | $59{\cdot}4 \pm 1{\cdot}1$ ($n = 20$) |
| 0 | 24 | $59{\cdot}7 \pm 0{\cdot}7$ ($n = 39$) |
| 10 | 24 | $60{\cdot}8 \pm 0{\cdot}6$ ($n = 50$) |

TABLE 4. Effects of catecholamines and prostaglandins on the denervation induced depolarization of rat hemidiaphragm muscle fibres. The hemidiaphragms were incubated at 37° C in the presence of catecholamines or prostaglandins for 3 hr before recording resting membrane potentials. Results are expressed as in Table 1

| | No. of preparations | Near, mean ± S.E. of mean (mV) | Far, mean ± S.E. of mean (mV) | Difference, far − near |
|---|---|---|---|---|
| Control | 7 | $68{\cdot}8 \pm 0{\cdot}4$ ($n = 163$) | $75{\cdot}7 \pm 0{\cdot}4$ ($n = 162$) | +6·9 |
| Noradrenaline (10 $\mu$M) | 5 | $74{\cdot}3 \pm 0{\cdot}4$ ($n = 126$) | $75{\cdot}4 \pm 0{\cdot}4$ ($n = 122$) | +1·1 ($P < 0{\cdot}05$) |
| Adrenaline (10 $\mu$M) | 9 | $73{\cdot}9 \pm 0{\cdot}4$ ($n = 225$) | $75{\cdot}2 \pm 0{\cdot}4$ ($n = 213$) | +1·3 ($P < 0{\cdot}025$) |
| Isoprenaline (10 $\mu$M) | 4 | $74{\cdot}8 \pm 0{\cdot}4$ ($n = 100$) | $76{\cdot}9 \pm 0{\cdot}4$ ($n = 99$) | +2·1 ($P < 0{\cdot}001$) |
| Prostaglandin $E_1$ (1 $\mu$M) | 3 | $64{\cdot}1 \pm 0{\cdot}7$ ($n = 68$) | $73{\cdot}7 \pm 0{\cdot}6$ ($n = 60$) | +9·6 ($P < 0{\cdot}001$) |
| Prostaglandin $E_2$ (1 $\mu$M) | 3 | $66{\cdot}7 \pm 0{\cdot}6$ ($n = 56$) | $73{\cdot}6 \pm 0{\cdot}6$ ($n = 51$) | +6·9 ($P < 0{\cdot}001$) |

### *The electrogenic hypothesis*

The failure of our attempts to find some evidence for the $K^+$ sequestration theory encouraged us to try to stimulate the presumed electrogenic pump in 3-hr-denervated preparations using our *in vitro* assay system

(Table 1), by exposing muscles to catecholamines and prostaglandins which are known to elevate cyclic AMP levels in the rat diaphragm (Craig, Rall & Larner, 1969; Butcher & Baird, 1968) and to stimulate ion fluxes in rat muscle (Dockry *et al.* 1966).

*Catecholamines.* As Table 4 indicates, incubation of diaphragms for 3 hr in the presence of catecholamines maintained the resting membrane potential of fibres 'near' to nerve section at the value close to fibres 'far' from nerve section (see Table 1). While the difference in resting membrane potential between 'near' and 'far' fibres in normal Liley buffer was 6·9 mV, in the presence of 10 $\mu$M noradrenaline, 10 $\mu$m adrenaline or 10 $\mu$M isoprenaline the difference was only 1·1, 1·3, and 2·1 mV respectively. Furthermore the mean resting membrane potential of fibres 'far' from nerve section after 3 hr incubation in catecholamines was not significantly greater than the resting membrane potential of 'far' fibres of diaphragms incubated in normal bathing solutions, perhaps implying that the catecholamines were more effective in the presence of denervation. Prostaglandins, $PGE_1$ and $PGE_2$, at concentrations of 1 $\mu$M did not prevent the fall in resting membrane potential under these conditions (Table 4).

TABLE 5. The effects of dibutyryl cyclic AMP and theophylline on the denervation induced depolarization of rat hemidiaphragm muscle fibres. Results are expressed as in Table 1

| | No. of preparations | Near, mean ± S.E. of mean | Far, mean + S.E. of mean | Difference, far − near (mV) |
|---|---|---|---|---|
| Cyclic AMP (0·5 mM) + theophylline (2 mM) | 1 | 69·9 ± 0·9 ($n$ = 19) | 79·9 ± 1·2 ($n$ = 19) | +10·0 ($P < 0·001$) |
| Dibutyryl cyclic AMP (10 $\mu$M) + theophylline (2 mM) | 1 | 71·1 ± 1·0 ($n$ = 23) | 77·0 ± 0·8 ($n$ = 25) | +5·3 ($P < 0·01$) |
| Dibutyryl cyclic AMP (0·1 mM) + theophylline (2 mM) | 1 | 73·0 ± 1·2 ($n$ = 23) | 75·8 ± 1·1 ($n$ = 25) | +2·8 (n.s.) |
| Dibutyryl cyclic AMP (0·25 mM) + theophylline (2 mM) | 3 | 77·7 ± 0·6 ($n$ = 60) | 77·8 ± 0·8 ($n$ = 64) | +0·1 (n.s.) |

*Cyclic AMP.* The effect of catecholamines encouraged us to try to raise cyclic AMP levels directly by incubating preparations with dibutyryl cyclic AMP in the presence of 2 mM theophylline to inhibit cyclic nucleotide phosphodiesterase. As Table 5 shows, cyclic AMP itself had no effect in raising the resting membrane potential of the denervated (near) portion

of the diaphragm, perhaps due to an inability to penetrate membranes. The dibutyryl derivative of cyclic AMP did produce a significant dose-dependent increase in resting membrane potential in the 'near' portion of the diaphragm. At the higher dose level (0·25 mM) the effect of denervation upon resting membrane potential appeared to be completely reversed. The effects of larger amounts of dibutyryl cyclic AMP (0·5 mM) were also examined. Spontaneous contraction of muscle fibres occurred which prevented reliable measurement of resting membrane potentials. It was also

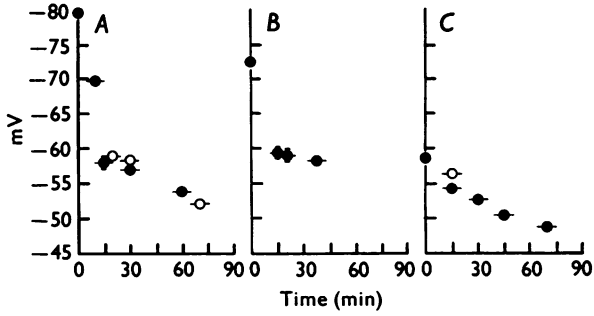


Fig. 1. Time course of the fall in muscle fibre resting membrane potential after incubation in ouabain. *A*, innervated muscle; *B*, denervated muscle incubated for 24 hr with dibutyryl cyclic AMP; *C*, denervated muscle. Ouabain concentrations were 1 mM (●) and 5 mM (○). Each point represents the mean ± S.E. of mean of twenty or more observations from one to four experiments. Horizontal bars indicate time period over which recordings were made. Ordinate, resting membrane potential; abscissa, incubation time in ouabain. Note that in *A* the 15 min point represents observations in 1 and 5 mM ouabain.

observed that there was a great increase in m.e.p.p. frequency and in the incidence of 'giant' potentials which presumably were sufficiently large enough to evoke muscle action potentials (Liley, 1957).

*Effects of ouabain.* As Fig. 1*A* indicates, in the presence of 1 mM (filled circles) or 5 mM (open circles) ouabain, mean resting membrane potentials of innervated muscle dropped within 15 min to the level characteristic of denervated preparations (Fig. 1*C*, time 0). While Locke & Solomon (1967) observed little effect of ouabain on denervated preparations we did see (Fig. 1*C*) a small fall in resting membrane potential within 15 min, in denervated preparations. In both denervated and innervated preparations (Fig. 1*C* and *A*) there was a further fall in resting membrane potential after the initial change presumably associated with blockage of active transport.

As Fig. 1*B* shows preparations in which the mean resting membrane potential had been raised by treatment with 1 mM dibutyryl cyclic AMP

in the presence of 2 mM theophylline were also affected by 1 mM ouabain and dropped within 15 min to a level characteristic of denervated preparations (compare Fig. 1*B* time 15 min with *C* time 0), confirming that dibutyryl cyclic AMP stimulated an electrogenic pump.

*Long-term denervation.* It might be thought that the effects of cyclic AMP delayed a fall in resting membrane potential but could not reverse the effects of denervation as was reported for the effects of actinomycin D on denervated mouse muscle (Grampp *et al.* 1972). We therefore denervated rat diaphragms *in vivo* and after 3–9 days the diaphragms were removed from the animal and examined. Three days after section of the left phrenic nerve at the level of the brachial plexus the muscle fibres of the left diaphragm were depolarized by about 20 mV and remained so for at least 6 days.

As Table 6 indicates, the application of dibutyryl cyclic AMP was capable of raising the resting membrane potential of 4- or 5-day denervated muscles by 8 mV from the control level of 58–60 mV. It should be noted that whereas 40% of the fibres in the treated diaphragms had resting membrane potentials greater than or equal to 70 mV, no fibres in the diaphragms recorded before incubation had resting membrane potentials greater than 67 mV. Butyric acid, theophylline and 5′ AMP did not increase resting membrane potential but 5′ AMP in the presence of theophylline induced a small but significant increase in resting membrane potential (Table 6). Butyric acid actually induced a small depolarization.

*Resting membrane potential in cultured diaphragms.* We were able to analyse the effects of denervation upon resting membrane potentials in more stable conditions by culturing diaphragms for 24 hr after previous denervation (4–10 days). Culturing muscles had the further advantage that our drugs could be applied for longer time periods. As Table 7 (control) indicates the mean resting membrane potentials in cultured muscles were slightly higher than in denervated muscles immediately after dissection (Table 6, control) as previously reported by Purves & Sakmann (1974) possibly due to the presence of insulin.

As Table 7 indicates dibutyryl cyclic AMP was capable of raising the resting membrane potential of cultured muscle fibres indicating a long continued effect of the substance. Greater concentrations of dibutyryl cyclic AMP could be employed in the culture situation than was possible after short term denervation and as Table 7 (1 mM dibutyryl cyclic AMP) indicates it did appear the effects of the drug were dose-dependent as in the short-term denervation experiments (Table 5). Furthermore, as Table 7 indicates, the effects of dibutyryl cyclic AMP were not affected by the presence of cycloheximide in levels known to block protein syn-

thesis in rat diaphragms and cycloheximide itself had no effect upon resting membrane potentials in cultured preparations as was inferred from the experiments after 15 and 24 hr incubations of freshly denervated preparations (Table 3).

TABLE 6. The effects of dibutyryl cyclic AMP and theophylline on the resting membrane potential of muscle fibres denervated 4 or 5 days previously. Membrane potentials (mV) were recorded within 30 min of removal from the animal (Before) and after 3 hr incubation (After)

| | No. of preparations | Before (mean + S.E. of mean) (mV) | After (mean + S.E. of mean) (mV) | Difference, after − before (mV) |
|---|---|---|---|---|
| Control: Liley solution | 3 | 58·7 ± 0·36 ($n$ = 60) | 57·4 ± 0·40 ($n$ = 60) | −1·3 ($P < 0·02$) |
| Dibutyryl cyclic AMP (0·5 mM) + theophylline (2 mM) | 4 | 60·2 ± 0·63 ($n$ = 80) | 68·4 ± 1·02 ($n$ = 80) | +8·2 ($P < 0·001$) |
| 5′-AMP (0·5 mM) + theophylline (2 mM) | 3 | 59·4 ± 0·48 ($n$ = 63) | 62·5 ± 0·44 ($n$ = 63) | +3·1 ($P < 0·001$) |
| 5′-AMP (0·5 mM) | 3 | 58·1 ± 0·48 ($n$ = 61) | 58·3 ± 0·49 ($n$ = 59) | +0·2 (n.s.) |
| Theophylline (2 mM) | 3 | 59·4 ± 0·47 ($n$ = 61) | 59·2 ± 0·43 ($n$ = 60) | −0·2 (n.s.) |
| Butyric acid (0·5 mM) | 1 | 59·1 ± 0·88 ($n$ = 20) | 52·7 ± 0·68 ($n$ = 20) | −6·4 ($P < 0·001$) |

TABLE 7. Mean resting membrane potentials of muscle fibres cultured for 24 hr after denervation *in vivo* 4–6 days before

| Medium | No. of preparations | Mean ± S.E. of mean (mV) |
|---|---|---|
| Control | 6 | 63·24 ± 0·38 ($n$ = 133) |
| 0·5 mM dibutyryl cyclic AMP + 2 mM theophylline | 4 | 70·94 ± 0·45 ($n$ = 81) ($P < 0·001$)* |
| 1 mM dibutyryl cyclic AMP + 2 mM theophylline | 4 | 72·30 ± 0·32 ($n$ = 151) ($P < 0·001$)* |
| Cycloheximide 50 μg/ml. | 1 | 65·66 ± 0·87 ($n$ = 29) ($P < 0·02$)* |
| Cycloheximide + 1 mM dibutyryl cyclic AMP + 2 mM theophylline | 2 | 73·12 ± 0·58 ($n$ = 41) ($P < 0·001$)† |

* Compared with control by Student's $t$ test.
† Compared with cycloheximide by Student's $t$ test.

In view of the fact that there are three histological types of muscle fibre in rat diaphragm (Gauthier & Padykula, 1966) and there are changes in their distribution after 7 days denervation (Yellin, 1974), we considered the possibility that the actions demonstrated in Table 7 were exerted on a particular group or groups of muscle fibres. Fig. 2 shows the spread of

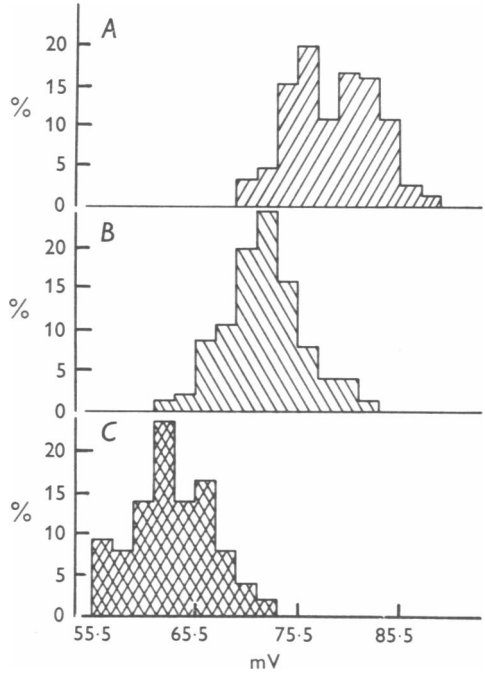


Fig. 2. Distribution of resting membrane potentials from rat diaphragm muscle fibres. All recording made *in vitro*. *A*, innervated fibres, $n = 133$; *B*, $n = 151$, and *C*, $n = 133$, fibres denervated then incubated for 24 hr in organ culture, with or without 1 mM dibutyryl cyclic AMP respectively. Ordinate, percentage of fibres; abscissa, resting potential magnitude in classes of 2 mV.

resting membrane potentials in innervated preparations (Fig. 2*A*), denervated preparations treated with dibutyryl cyclic AMP (Fig. 2*B*) and denervated (Fig. 2*C*) preparations. It is obvious that the resting membrane potentials are approximately normally distributed in each type of preparation indicating that the effect of the drug (Table 7) was presumably exerted on all fibre groups equally.

*Muscle cyclic AMP levels.* A simple explanation of the post-denervation fall in resting membrane potential might be that post-denervation there is a fall in muscle cyclic AMP levels. As Table 8 indicates, cyclic AMP levels in each half of innervated diaphragms were much the same but

after denervation of the left halves their cyclic AMP level increased by some 40%. The levels in the muscle fibres of the innervated side did not appear to be affected by denervation. It has been reported elsewhere (Carlsen, 1975) that denervation increases muscle cyclic AMP levels.

TABLE 8. Cyclic AMP levels (pmole/mg protein) in normal and denervated rat hemidiaphragms. Student's *t* test was used to evaluate *P*

| | No. of preparations | Left hemidiaphragm (mean) | Right hemidiaphragm (mean) | Difference, left − right |
|---|---|---|---|---|
| Normal hemidiaphragms | 2 | 5·68 (6·08, 5·27) | 5·57 (5·73, 5·40) | 0·11 |
| Hemidiaphragms of rats in which the left phrenic nerve was sectioned 4–5 days previously | 4 | 8·13 ± 0·58 (S.E. of mean) | 5·69 ± 0·21 (S.E. of mean) | 2·44 ($P < 0·01$) |

## DISCUSSION

The present results (Tables 2 and 3) serve to exclude $K^+$ sequestration by protein synthesis as a general cause of the fall in resting membrane potential upon denervation. In any case the presence of RNA and protein synthesis inhibitors served only to delay and could not reverse the fall in resting membrane potential (Grampp *et al.* 1972; Muchnik *et al.* 1973).

The alternative hypothesis, an electrogenic component to the resting membrane potential, removed by denervation receives support from our results. The proposed mechanism was first described by Hodgkin (1951), who pointed out that if the $Na^+$ pump was switched off by a suitable metabolic inhibitor then the inward movement of $Na^+$ would rapidly depolarize the affected cell. A redistribution of $K^+$ and $Cl^-$ would follow causing a secondary slower depolarization.

The effects of ouabain (Fig. 1) are those of the 'suitable metabolic inhibitor' described by Hodgkin (1951). Furthermore, catecholamines and dibutyryl cyclic AMP could restore resting membrane potential to or close to the innervated level in denervated preparations even after 6 days denervation (Tables 4–7). This hyperpolarization was electrogenic in that the effect of dibutyryl cyclic AMP was antagonized by ouabain (Fig. 1*B*).

The effect of catecholamines on the resting membrane potential of denervated diaphragm muscle fibres is probably mediated by cyclic AMP. Adrenaline has been shown to raise cyclic AMP levels severalfold in the rat diaphragm *in vitro* (Craig *et al.* 1969) and dibutyryl cyclic AMP in the present experiments increased the resting membrane potential of denervated fibres by 8 mV. Lentz (1972, 1974) observed that cyclic AMP, in combination with an inhibitor of phosphodiesterase, was capable of

maintaining the cholinesterase activity and end-plate structure in cultured muscles of the newt (*Triturus viridescens*) and therefore suggested that cyclic AMP may play a role in the mechanism of trophic action. The simplistic explanation that a neurotrophic factor maintains muscle resting membrane potential by raising the cyclic AMP content, is not borne out by the present observation that the cyclic AMP content is actually higher in denervated muscle fibres (Table 8; see also Carlsen, 1975). However, the fact that total muscle cyclic AMP rises following denervation does not necessarily eliminate it as a crucial factor in $Na^+$-pump stimulation in innervated muscle. The apparent paradox might be explained by compartmentalization of cyclic AMP. From the present experiments it appears possible that the hypothetical trophic factor and dibutyryl cyclic AMP effect the same cellular mechanism, that is, an electrogenic $Na^+$-pump.

It might be objected that resting membrane potential in rat muscle, as in frog muscle can be completely explained by a $K^+$ diffusion hypothesis and that the membrane potential, $E_m$, can be accurately predicted from the intracellular $[K^+]$ in adequately maintained preparations *in vivo* and *in vitro* (Kernan, 1963; Akaike, 1975). These results, however, depend on assuming an intracellular $K^+$ activity coefficient of 1. The physical state of intracellular $K^+$ is currently a matter of dispute but in frog muscle there is evidence that the activity coefficient of $K^+$ is certainly less than 1 and possibly as low as 0·7 (Armstrong & Lee, 1971; Kernan & MacDermott, 1975). If the same is true of rat muscle the reported agreement of $E_m$ and $K^+$ equilibrium potential, $E_K$ can only be fortuitous.

Other investigators have also found that the diffusion hypothesis cannot completely explain the resting membrane potential of rat muscle. For instance, Lüllmann (1958) reported a divergence between measured resting membrane potential in rat muscle *in vitro* and the resting membrane potential expected from ionic gradients in the same tissue, which became greater with the prolongation of incubation. At that time it was assumed that changes in membrane permeabilities after denervation could explain the results. The presumed $K^+$ permeability changes were found (Klaus *et al.* 1960) but it is now known that the changes in membrane resistance after denervation do not take place until 72 hr after denervation (Albuquerque *et al.* 1971).

The much greater effect of catecholamines upon resting membrane potential in denervated muscles than in innervated muscle (Table 4, compare 'far' and 'near') appears to be a new finding. It has been known for some time that adrenaline, noradrenaline and isoprenaline caused a hyperpolarization of innervated and denervated mammalian muscle but, perhaps because the records were of 'demarcation potentials' rather than resting membrane potential, no particular difference was noted between effects

in the denervated and innervated state (Brown, Goffart & Vianna Dias, 1950; Bowman & Raper, 1965, 1966). Studies on resting membrane potential in preparations *in vitro* which had an attached nerve have shown inconstant effects of catecholamines on innervated muscle. For instance, Kuba (1970) reports that adrenaline and isoprenaline raised resting membrane potential by 3–4 mV while noradrenaline had no significant effect. Krnjević & Miledi (1958) had previously reported that adrenaline had no effect on resting membrane potential in rat diaphragm.

Our results imply that the lack of effect of catecholamines upon resting membrane potential of innervated muscle is a consequence of the innervation. Small increases in resting membrane potential might be expected if the muscle fibres were a little depolarized due to denervation (Table 1, 'near'). The extent of the denervation in any particular investigation would be a function of the length of the attached nerve stump, the distance of the muscle fibres from which recordings were made from the site of denervation and the relationship of the site of recording to the end-plate region, effects being expected first at the end-plate (Albuquerque *et al.* 1971).

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