J. Physiol. (1974), **240**, *pp.* 517–533
With 4 plates and 6 text-figures
Printed in Great Britain

THE ENTRY OF LABELLED CALCIUM INTO THE INNERVATED REGION OF THE MOUSE DIAPHRAGM MUSCLE

BY R. H. EVANS

From the Department of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD

(*Received 26 June 1973*)

SUMMARY

1. A method is described for measuring the distribution of ^{45}Ca between the junctional and non-junctional regions of the mouse diaphragm muscle.

2. Muscles which were incubated in Ringer solution containing labelled calcium accumulated the tracer at the junctional region when acetylcholine or carbachol was present. Denervation did not prevent this accumulation at the normally innervated region.

3. Histochemical evidence indicated that, in the presence of acetylcholine or carbachol, calcium entered diaphragm muscle fibres at the junctional region.

4. Accumulation of calcium occurred at all regions of muscle fibres depolarized by potassium. In the presence of acetylcholine or carbachol, accumulation occurred at the junctional region in the absence of muscle fibre post-junctional membrane depolarization.

5. It is suggested that the desensitized muscle fibre post-junctional membrane has an increased calcium permeability.

INTRODUCTION

Acetylcholine causes staining of calcium at motor end-plates of skeletal muscle fibres, as has been shown with the dyes alizarin red S (Csillik & Sávay, 1963; Lièvremont, Czajka & Tazieff-Depierre, 1968) and glyoxal bis (2-hydroxyanil) (Meunier, 1972). Lièvremont *et al.* (1968) also reported that stimulation of the phrenic nerve in the presence of the anticholinesterase neostigmine, caused staining in the junctional region of the mouse diaphragm, that such staining was suppressed in the presence of tubocurarine, and that the stained area spread outside the junctional region in denervated muscle. Furthermore, acetylcholine (ACh) and other neuromuscular depolarizing drugs increased the uptake of labelled calcium into

frog sartorius muscle (Ahmad & Lewis, 1961, 1962) and depolarized denervated rat diaphragm muscle (Jenkinson & Nicholls, 1961). The purpose of the present study was to examine whether ACh specifically alters the permeability of the muscle fibre post-junctional membrane to calcium. A combination of radioisotope, histochemical and electrophysiological techniques have been used to investigate such an action of ACh and carbachol at the motor end-plates of mouse diaphragm muscle.

METHODS

Preparation of diaphragms

Adult laboratory strain mice of either sex, body weight 25–35 g were decapitated and the whole diaphragms together with the rib cage were removed. Before use the diaphragms were incubated for 60 min at 37° C in Ringer solution. Unless stated otherwise the Ringer solution had the following composition (mM): NaCl, 137; KCl, 5; NaH_2PO_4, 1; $CaCl_2$, 5; $MgCl_2$, 1; $NaHCO_3$, 12; glucose, 11. The Ringer solution was gassed with 95 % O_2, 5 % CO_2 at all times. In some experiments a high potassium Ringer solution of the following composition was used (mM): KCH_3SO_4, 134; NaCl, 5; $CaCl_2$, 5; $KHCO_3$, 12; glucose, 11. Ringer solutions which contained more than 5 mM calcium became turbid. When such solutions were centrifuged, and the calcium in the clear supernatant was measured by atomic absorption spectrophotometry, it was found that the calcium concentration was not significantly different from the expected value.

Diaphragms were denervated as follows. Mice were anaesthetized with chloroform and the left phrenic nerve was pulled through a small incision in the thorax by means of a glass hook. The nerve was cut with scissors close to its entry into the muscle and approximately 3 mm of nerve was removed. Eighteen days later the diaphragms were removed for experiment.

To show the effects of phrenic nerve stimulation on entry of ^{45}Ca to the muscles diaphragms were incubated in Ringer solution which contained 2·5 mM calcium and no magnesium because the presence of magnesium had been shown to decrease the accumulation of calcium (*vide infra*, Text-fig. 4). The muscles were stimulated through the left or the right phrenic nerve which was mounted across two stainless-steel wire electrodes. Square pulses (up to 3·0 V, 0·6 msec duration) at a frequency of 50 Hz were passed through the electrodes. This rate of stimulation caused tetanic contracture on the stimulated side of the diaphragms, but when the stimulus rate was decreased at the end of each experiment, single twitches could still be elicited. This method of stimulation caused some stretching of the unstimulated side of the muscles. This stretching did not appear to have influenced the results, because the calcium entry into the non-innervated segments of the unstimulated side of these muscles was similar to the calcium entry into the non-innervated segments of unstimulated muscles. In the presence of eserine (10^{-5} M) contracture of stimulated muscles was maintained for only a few seconds. After the period of stimulation the muscles were incubated in tracer-free Ringer solution for 15 min and then placed in acetone as described below.

Measurement of ^{45}Ca

At the beginning of each experiment, groups of three whole diaphragms were placed in 25 ml. Ringer solution which contained $^{45}CaCl_2$ in trace amounts (10^6 cpm of $^{45}CaCl_2$, specific activity 15–25 mCi/mg Ca.ml. Ringer solution). To terminate

the experiment the diaphragms were rinsed briefly in tracer-free Ringer solution, blotted, and placed in acetone (50 ml./diaphragm). After three changes of acetone over a period of 2 hr, the diaphragms were placed in xylene which made the intramuscular nerve branches visible. At this stage the diaphragm muscle was dissected free of the rib cage, and the muscle zones on each side of the diaphragm were divided with a scalpel blade into innervated and non-innervated segments (see Pl. 1) under × 16 magnification, in the manner described for the rat diaphragm by Hebb, Krnjević & Silver (1964). The innervated and non-innervated muscle segments were placed separately in 20 ml. polyethylene scintillation counting vials and the vials were heated at 65° C for 30 min in a water-bath to remove all xylene. The dried muscle samples were then weighed on a torsion balance. The weights of the innervated and the non-innervated segments of a single diaphragm were usually about 3–5 mg respectively and the ratio of wet weight of tissue/dry weight for muscles dried in this way was $4{\cdot}00 \pm 0{\cdot}03$ S.E. of mean. The weighed muscle samples were replaced in the polyethylene vials, 0·3 ml. 11·3 N-HCl was added to each muscle sample and the samples were digested for 20 min at 65° C. Ethanol (2 ml.) followed by 10 ml. phosphor (2,methoxyethanol, 3 l.; toluene, 7 l.; 2,5-diphenyloxazole, 40 g; 1:4-di-2(5-phenyloxazolyl)-benzene, 1 g) was added to each vial and the radioactivity of the ^{45}Ca was determined by liquid scintillation counting at an efficiency of 76 %. Standards which consisted of a known volume of the ^{45}Ca Ringer solution were prepared for each experiment and counted in the same way as the muscle samples. The difference between the efficiency of counting of the muscle samples and the counting of the ^{45}Ca standards, approximately 4 %, was not corrected for.

On Pl. 1 it can be seen that the innervated segments (N) of the diaphragm muscles contained junctional and non-junctional regions of muscle fibres. To correct for this it was assumed that the accumulation of ^{45}Ca in the non-junctional region occurred evenly throughout the muscle. A value for the ^{45}Ca which entered the non-junctional region of the innervated segments was obtained by multiplying the radioactivity per mg of non-innervated segments by the weight of the innervated segments for each muscle. Subtraction of this value from the total radioactivity of the innervated segments gave the amount of ^{45}Ca specifically accumulated at the junctional region. This result was expressed as n-mole calcium per diaphragm as shown by eqn. (1).

$$\text{n-mole Ca accumulated at the junctional region} = \left[\text{cpm innervated segments} - \frac{\text{cpm non-innervated segments} \times \text{Wt. innervated segments}}{\text{Wt. non-innervated segments}} \right] \times \frac{\text{n-mole Ca in standard}}{\text{cpm standard}}. \quad (1)$$

The value specified by eqn. (1) was found to be fairly constant under given experimental conditions (see Results). However, the amount of calcium accumulated in the non-innervated segments varied from muscle to muscle depending on the weight of muscle included in this portion. For this reason the calcium of the non-innervated segments was expressed in n-mole/mg dry muscle.

In some experiments diaphragms were incubated for 15 min in tracer-free Ringer solution after the period in ^{45}Ca. This had the effect of accentuating any differences which may have occurred between the entry of ^{45}Ca to the innervated and non-innervated regions of the muscle (*vide infra*, Efflux of ^{45}Ca). The technique for determination of the ^{45}Ca entry was checked by comparison with muscles which were freeze dried after removal from the Ringer solution. The total amount of ^{45}Ca which entered these muscles did not differ significantly from the amount which entered muscles dried in acetone and xylene as described above, but the amount measured

in the junctional region of the freeze dried muscle was much more variable probably because of the greater difficulty in separating innervated and non-innervated segments from these muscles.

Electrical recording

Transmembrane potentials were recorded with a Devices heated stylus pen recorder with glass electrodes of 20–40 MΩ resistance. Miniature end-plate potentials (m.e.p.p.s) were recorded on photographic film with a Telford oscilloscope camera.

Histochemical methods

Diaphragms were incubated for 15 min in calcium and magnesium-free Ringer solution after the standard incubation in carbachol. This procedure served to remove calcium and magnesium from the interfibre spaces. Then the muscles were cut from the rib cage and frozen. Transverse sections 6 μm thick were cut from the frozen muscle and dried in air at room temperature. Alternate serial sections were stained either for cholinesterase, to locate junctional regions, or for the presence of calcium ions.

Cholinesterase was stained by a modification of the method described by Koelle & Friedenwald (1949). Sections were immersed in Ringer solution which contained 0·1 % $CuSO_4.5H_2O$, 0·2 % glycine and 5 mM acetylthiocholine iodide adjusted to pH 6·5 with a few drops of a 10 % solution of 2-amino-2-methylpropan-1-ol. After 15 min in this solution at room temperature the sections were rinsed in distilled water and placed in a 1 % solution of yellow ammonium sulphide at pH 9 for 5 sec, followed by a rinse in distilled water and immersion in 70 % ethanol.

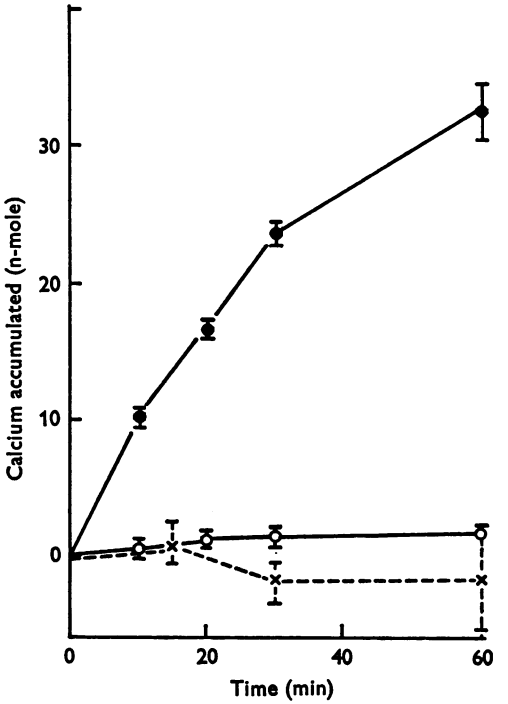
The calcium stain was prepared by mixing 16 ml. 0·4 % glyoxal bis (2-hydroxyanil) dissolved in methanol with 7·2 ml. 5 % NaOH (Kashiwa & Atkinson, 1963). Slides bearing the sections to be stained were dipped into this solution and placed in air so that the sections were covered by a thin film of staining solution. After 2 min the sections were dipped once more into the staining solution and placed in air for a further 2 min, they were then rinsed in 70 % ethanol. Cytoplasmic counterstains, such as fast green, caused the calcium stain to fade very quickly; because of this 0·25 % methylene blue in 70 % ethanol was used to counterstain the sections. Methylene blue has the disadvantage, in this case, of staining the nuclei as well as the cytoplasm. The counterstained sections were dehydrated in acetone, cleared in xylene and mounted under cover glasses with DPX. It was necessary to photograph the slides soon after their preparation because the calcium stain faded overnight. Treatment of calcium stained sections with alcoholic Na_2CO_3/KCN solution, in order to remove the stain from divalent ions other than calcium (Kashiwa & Atkinson, 1963), made no difference to the appearance of the sections so this stage was omitted from the routine staining procedure.

RESULTS

Effect of ACh and carbachol

Carbachol (0·1 mM) caused a progressive accumulation of ^{45}Ca at the junctional region of diaphragms (Text-fig. 1) as specified by eqn. (1). There was no increase in the accumulation of ^{45}Ca at the junctional region in the absence of carbachol or in the non-innervated segments in the presence of carbachol. Carbachol at 5×10^{-6} M caused a significant accumulation of calcium, and the maximum effect was produced at 10^{-4} M (Text-fig. 2).

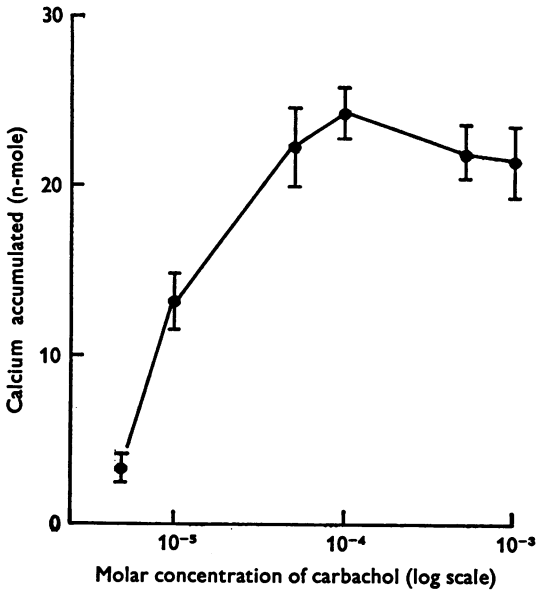
ACh was less effective than carbachol; a tenfold greater concentration of ACh (1 mM) was required to produce maximal accumulation of ^{45}Ca at the junctional region. Choline chloride was about as effective as ACh. In some of the experiments 1 mM-ACh was used as the stimulus but in other experiments 0·1 mM carbachol was used because it produced less variable results.



Text-fig. 1. Time course of accumulation of calcium by mouse diaphragm muscle. Diaphragms were incubated in ^{45}Ca Ringer solution at 37° C with 0·1 mM carbachol, followed by washout for 15 min in tracer-free Ringer solution before immersion in acetone. ●——●, n-moles of calcium per diaphragm accumulated in the junctional region. ○——○, n-moles of calcium per mg dry muscle accumulated in the non-innervated segments. ×----×, accumulation of calcium by the junctional region in the absence of carbachol. Each point is the mean result of six muscle ±2 S.E. of mean.

It is important to note at this stage that when $Na_2{}^{35}SO_4$ was used as a tracer, carbachol did not cause a specific accumulation of ^{35}S at the junctional region as it did for ^{45}Ca. Furthermore, the ratio of ^{35}S which entered 1 g muscle in 30 min/^{35}S contained in 1 ml Ringer solution, was calculated to be 0·233 and this ratio was considered to represent the volume of the extracellular space. From this value it can be calculated that with a Ringer concentration of 5 mM calcium, equilibration of this with the extracellular

space should yield an entry of calcium into the non-innervated segments of 4·66 n-mole calcium per mg dry muscle. In the experiment illustrated by Text-fig. 1 the muscles were given a 15 min washout period in tracer-free Ringer solution which had the effect of specifically decreasing the ^{45}Ca content of the non-innervated segments. When muscles were not subjected to such a tracer-free washout period after 30 min incubation with ^{45}Ca, the mean calcium entry to the non-innervated segments in different groups of muscles varied from 5·5 to 7·5 n-mole per mg dry muscle. These values indicate that some of the calcium entered the muscle fibres.



Text-fig. 2. Effect of carbachol on the accumulation of calcium at the junctional region of the mouse diaphragm. The diaphragms were incubated for 30 min in ^{45}Ca Ringer solution at each concentration of carbachol. Each point represents the mean result of six muscles ± 2 S.E. of mean.

Effect of calcium and magnesium concentrations

The relationship between the concentration of calcium in the Ringer solution and the amount of calcium which entered the junctional region of the muscle during 30 min incubation at 37° C in the presence of 1 mM-ACh is shown in Text-fig. 3. Calcium showed a concentration dependent entry at the junctional region with a probable saturation at 5 mM. At higher calcium levels the amount of tracer which entered the non-innervated segments of the muscle was more variable and this increased the error of the estimation.

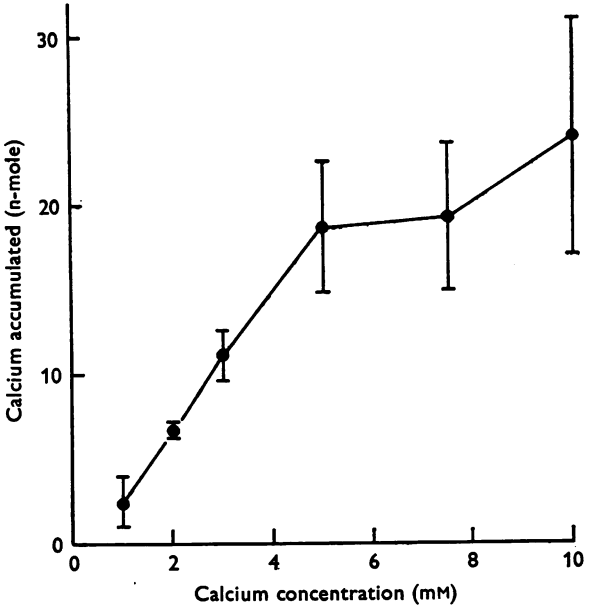
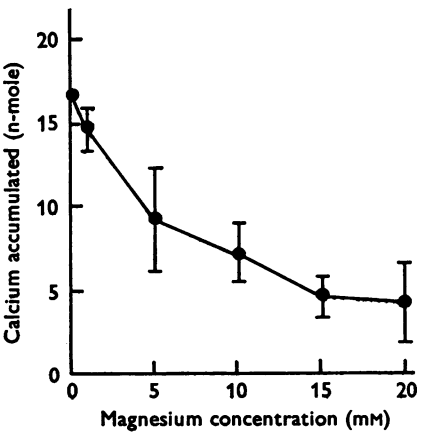


Fig. 3. Effect of calcium concentration of the Ringer solution on the accumulation of calcium at the junctional region of mouse diaphragm muscles. The diaphragms were incubated for 30 min at 37° C in ^{45}Ca Ringer solution with 1 mM-ACh. Each point represents the mean result of at least six muscles (n-moles of calcium per diaphragm ± 2 S.E. of mean).

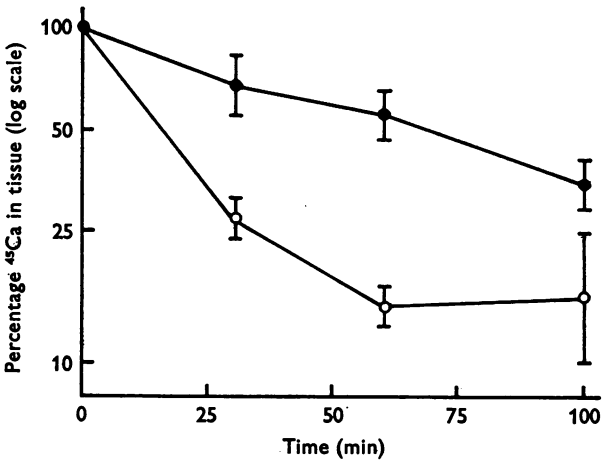


Text-fig. 4. Effect of magnesium concentration of the Ringer solution on the accumulation of calcium at the junctional region of mouse diaphragm muscle. The diaphragms were incubated for 30 min at 37° C in ^{45}Ca Ringer solution containing 1 mM-ACh and 5 mM-$CaCl_2$. Each point represents the mean of three muscles ± 2 S.D.

When the magnesium chloride concentration of the 5 mM calcium Ringer solution was increased (Text-fig. 4), the amount of calcium accumulated at the junctional region in the presence of 1 mM-ACh was decreased.

Efflux of ^{45}Ca from diaphragms

In order to measure efflux of ^{45}Ca from diaphragm muscles, groups of six diaphragms were loaded with ^{45}Ca for 30 min in the presence of 1 mM-ACh and subsequently placed in calcium-free Ringer solution. Measurement of the ^{45}Ca which remained in the muscles showed that the efflux from the junctional region could be described approximately by a single



Text-fig. 5. Efflux of ^{45}Ca from mouse diaphragm muscle. The diaphragms were incubated for 30 min at 37° C in ^{45}Ca Ringer solution which contained 1 mM ACh. They were then placed in calcium-free Ringer solution and removed for analysis at the intervals shown. The ^{45}Ca present in the muscles is expressed as a percentage of the amount present at the beginning of the washout period. ●——●, ^{45}Ca in the junctional region of the muscle, mean exponential rate constant for efflux 0·0113 min^{-1}, S.D. 0·0013. ○——○, ^{45}Ca in the non-innervated segments of the muscle. Each point represents the mean of six muscles ± 2 S.E. of mean.

exponent with a half time of 65 min (Text-fig. 5). The tracer in the non-innervated segments was mostly present in the extracellular space and 50 % of this radioactivity was lost from the non-innervated segments in 15 min (Text-fig. 5). Addition of 0·1 mM carbachol, 1·5 mM caffeine or 11 mM-$MgCl_2$ to the calcium-free washout solution had no significant effect on the amount of ^{45}Ca lost from the junctional region in 60 min.

Diaphragms which were loaded with tracer, as described above, and then placed in the usual Ringer solution which contained 5 mM calcium but no tracer, lost 39 % of the tracer from the junctional region in 60 min. Addition

of 0·1 mM carbachol to the washout solution, in this case, significantly reduced the loss of tracer from the junctional region to 23% ($P < 0·01$). ACh (1 mM) produced a similar effect to carbachol on efflux of tracer into Ringer solution which contained calcium.

Entry of ^{22}Na

Some diaphragms were incubated for 30 min in Ringer solution which contained $^{22}NaCl$ as a tracer and the ^{22}Na which entered the muscle was measured by the method which has already been described for ^{45}Ca. Carbachol, in contrast to the effect with ^{45}Ca, had no significant effect on the entry of ^{22}Na into the junctional region of the muscle, and a 15 min washout period in tracer-free Ringer solution caused the loss of 85% of the radioactivity of the ^{22}Na from both regions of the muscle. At the end of the 30 min incubation with ^{22}Na the ratio of radioactivity per gram of wet tissue compared to that contained in 1 ml. Ringer solution was calculated to be 0·233, which indicates that the ^{22}Na entered the muscle in amounts consistent with the volume of the extracellular space.

TABLE 1. Entry of labelled calcium into nerve stimulated mouse diaphragm muscles (mean ±S.E. of mean, number of estimations in parentheses). The phrenic nerve on the left or right side of each muscle was stimulated with pulses of 1·5 or 3·0 V, 0·6 msec duration at 50 Hz for 15 min. Thus, the unstimulated side of each diaphragm acted as a control. The $^{45}CaCl_2$ tracer and when applicable the eserine were added 5 min before the start of stimulation. After the period of stimulation the muscles were incubated in tracer-free Ringer solution for 15 min (see text for further details)

	Non-stimulated side		Stimulated side	
		Eserine 10^{-5} M		Eserine 10^{-5} M
Ca in junctional region, n-moles/ hemidiaphragm	$-0·12 \pm 0·11$ (8)	$3·16 \pm 0·26$ (11)	$0·00 \pm 0·17$ (7)	$3·51 \pm 0·16$ (12)
Ca in non-innervated segments n-mole/ mg dry muscle	$0·57 \pm 0·08$ (8)	$0·79 \pm 0·07$ (10)	$1·59 \pm 0·11$ (8)	$0·73 \pm 0·05$ (11)

The effect of motor nerve stimulation

Carbachol and ACh both caused accumulation of calcium at the junctional region of diaphragm muscles. To see if ACh released from nerve terminals would cause a similar accumulation of calcium, diaphragms were stimulated electrically in the presence of ^{45}Ca as described under Methods. The figures on the top line of Table 1 show that electrical stimulation of the phrenic nerve did not cause accumulation of calcium at the junctional region. Eserine (10^{-5} M) caused a significant accumulation of calcium at

the junctional region, but phrenic nerve stimulation did not increase this any further. Lièvremont *et al.* (1968) found that neostigmine (2×10^{-7} M) caused calcium staining at the junctional region of nerve stimulated muscles, but in the present experiments, this level of neostigmine had no discernible effect on the entry of ^{45}Ca into nerve stimulated or unstimulated muscles. However, 10^{-5} M neostigmine caused an accumulation of 2·67 n-mole calcium/hemidiaphragm (S.E. of mean 0·29, $n = 8$) at the junctional region and stimulation, in this case, increased this value to 3·59 n-mole calcium/hemidiaphragm (S.E. of mean 0·15, difference in means significant at the $P < 0.05$ level).

TABLE 2. The effect of denervation on the accumulation of labelled calcium in the junctional region of mouse diaphragm muscles (n-moles of calcium per hemidiaphragm ±S.E. of mean). Four muscles were incubated in ^{45}Ca Ringer solution for 30 min at each concentration of ACh

ACh concn. (mM)	Left hemidiaphragm (denervated)	Right hemidiaphragm
0	0	0
0·1	4·29 ± 0·23	0
1·0	7·77 ± 0·93	10·69 ± 0·30

TABLE 3. The effect of denervation on the entry of labelled calcium into the non-innervated segments of mouse diaphragm muscles (n-moles of calcium/mg dry muscle ±S.E. of mean). Other details as for Table 2

ACh concn. (mM)	Left hemidiaphragm (denervated)	Right hemidiaphragm
0	7·67 ± 0·10	7·53 ± 0·38
0·1	10·19 ± 0·15	6·36 ± 0·12
1·0	9·04 ± 0·16	6·02 ± 0·10

The amounts of calcium which entered the non-innervated segments of the muscle are compared on the lower line of Table 1. It can be seen that electrical stimulation in the absence of eserine caused a marked increase in the entry of tracer to the non-innervated segments of the muscle. The presence of eserine suppressed this effect. Bianchi & Shanes (1959) have shown that activity of frog muscles can be correlated with an increased influx of calcium. The above results for calcium which entered the non-innervated segments confirm that this is true also of mouse diaphragm muscle. The suppression of this effect in the eserine treated muscles confirms the observation that neuromuscular blockade persisted in the presence of 10^{-5} M eserine.

The effect of denervation

To see if the accumulation of calcium was confined either to the intramuscular nerve (or nerve terminals) or associated with the post-junctional muscle fibre membrane, it was decided to examine the effect in muscles which were denervated as described under methods. Tables 2 and 3 show the respective entries of ^{45}Ca into the junctional region and the non-innervated segments of these muscles in the presence of two concentrations of ACh. It can be seen from Table 2 that ACh still caused specific entry of calcium into the junctional region of the muscle despite the absence of nerve terminals. Thus it is fair to suggest that a large proportion, at least 73% of the calcium at the junctional region was accumulated independently of the presence of nerve terminals. The higher level of accumulated calcium at the junctional region of innervated muscle may be significant, and it is reasonable to suppose that this difference could be accounted for by the activity of nerve terminals. However, a more likely explanation of the lower calcium entry into the junctional region of denervated muscle (Table 2) is that this may reflect a lowered ACh receptor density at the junctional region of the denervated muscle. For instance, Albuquerque & Thesleff (1968) have observed that the highest 'spot' sensitivity to ACh at the junctional region of rat leg muscles was lower than that found at the end-plates of innervated muscles.

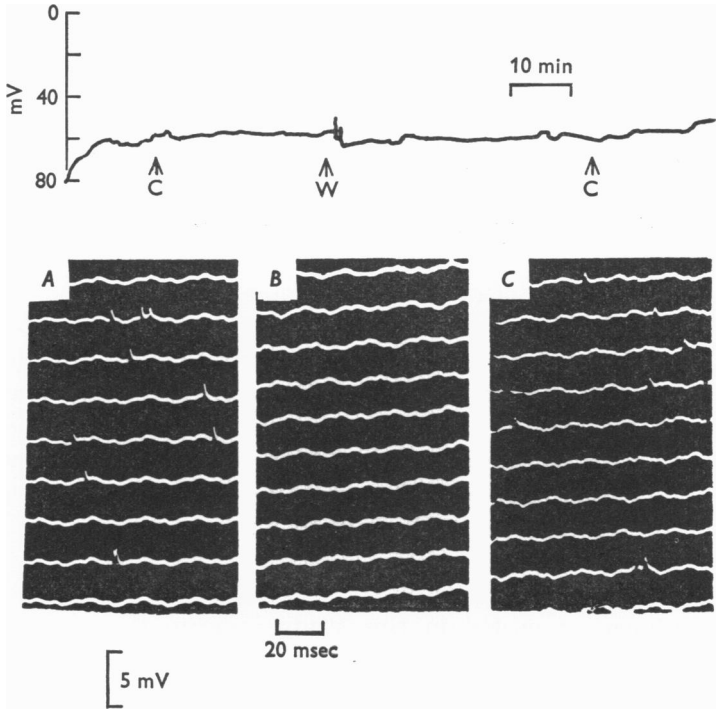
The observation that ACh, at a lower concentration (0·1 mM) than was required to produce calcium entry at the junctional region of innervated muscle (1 mM), increased the entry of calcium into the non-innervated segments of denervated muscles (Table 3) is not surprising in view of the supersensitivity to ACh which is found in the extrajunctional region of denervated muscle (Axelsson & Thesleff, 1959).

Entry of ^{45}Ca into potassium depolarized muscles

The experiments reported in this study indicate that calcium was accumulated at the junctional region of muscle fibres in the presence of depolarizing drugs. It was important to distinguish whether this accumulation of calcium was caused by drug receptor combination or whether it was caused by ion permeability changes which were secondary to the effect of carbachol. To distinguish between these possibilities diaphragms were incubated in potassium Ringer solution as described in the Methods section and the effect of carbachol was examined on these potassium depolarized muscles.

When diaphragms were equilibrated with potassium Ringer solution for 60 min and then incubated with ^{45}Ca under the standard conditions, ^{45}Ca entry into the non-innervated segments was significantly increased

up to a value of 29·2 n-mole calcium/mg dry muscle in 30 min (S.E. of mean 2·5) which compares with a value of 5·75 (S.E. of mean 0·23) for muscles which were incubated in the ordinary Ringer solution. In the potassium Ringer solution there was also an increased influx of ^{45}Ca into the junctional region of the muscle, which amounted to 18·8 n-mole calcium/diaphragm in 30 min (S.E. of mean 2·8). Addition of carbachol (0·1 mM) to the potassium Ringer solution caused no significant alteration in the distribution of ^{45}Ca in the diaphragm muscles, and in a similar way it was found that no significant effect was produced by 0·1 mM carbachol on the efflux of ^{45}Ca from the potassium depolarized muscles into calcium-free Ringer solution.



Text-fig. 6. Intracellular recordings from the post-junctional region of mouse diaphragm muscle fibres. The upper trace has been retouched and it is a slow d.c. recording (negative downwards). Carbachol (0·1 mM) was added to the muscle bath at C and washout of carbachol was commenced at W. The lower traces are a.c. recordings on photographic film (negative downwards) in order to show m.e.p.p.s. Record *A* is before addition of carbachol to the muscle bath, *B* is from the same fibre in the presence of 0·1 mM carbachol and *C* is from another fibre of the same muscle 15 min after washout of carbachol from the muscle bath.

Measurement of post-junctional muscle fibre membrane potentials

As already described depolarization alone produced an increased calcium influx to the muscle. This led to the possibility that carbachol could allow a local entry of calcium to occur simply by causing a local depolarization of muscle fibres at the junctional region. To check this, the effect of carbachol on the muscle membrane potential at the junctional region was measured as shown in Text-fig. 6. It can be seen from the upper record in the Figure that carbachol (0·1 mM) had no discernible effect on the muscle fibre membrane potential at the junctional region, although it completely abolished the m.e.p.p.s. ACh (1 mM) also had very similar effects.

Staining of accumulated calcium

The slow rate of efflux of the accumulated calcium from the junctional region of diaphragms (Text-fig. 5) suggested that the accumulated calcium was not superficially bound to fibres but had passed inside them. Pls. 2*A*, 3*A* and 4*A* show the typical appearance of transverse sections of muscles stained for calcium ions as described under Methods. The lower micrograph (*B*) of each plate is a serial section, stained for cholinesterase, which shows the same field as the upper micrograph. The muscle illustrated by Pl. 3 was incubated with 0·1 mM carbachol in the presence of 5 mM calcium and on Pl. 3*A* dark regions are seen which correspond to the cholinesterase stained regions on Pl. 3*B*. These darkly stained regions represent the red stain which results from combination of glyoxal bis(2-hydroxyanil) with calcium ions. On Pl. 2*A*, which is muscle incubated without carbachol, this staining does not occur. Also the staining was not seen in muscles which were incubated with carbachol in calcium-free Ringer solution (Pl. 4*A*). An examination of Pl. 3*A* shows that at the junctional region, fibres stained densely at the periphery and the stain decreased in intensity towards the interior of fibres. This indicates the presence of a gradient of calcium ions decreasing from the post-junctional muscle membrane towards the interior of the fibre. ACh (1 mM) again produced similar effects to carbachol. Staining of calcium inside the junctional region of muscle fibres has also been described by Marco, Mikiten & Nastuk (1969) who treated frog sartorius muscles with carbachol and used alizardin to identify calcium.

In connexion with the micrographs it is worth mentioning that fibres within the depth of the muscle appeared to be stained just as much as surface fibres. Thus the measurements of calcium entry are representative of all the muscle fibres and not just those on the surface of the diaphragm.

DISCUSSION

The results indicate that there was a specific entry of calcium at the junctional region of the mouse diaphragm which was caused by ACh or carbachol. The experiment which showed accumulation of calcium at the junctional region of denervated muscle and the photomicrographs (Pl. 3*A*) show that the calcium was accumulated inside muscle fibres at the post-junctional region. It should be noted in connexion with this point that glyoxal bis(2-hydroxyanil) will stain only free calcium ions (Kashiwa & Atkinson, 1963), so the pattern of staining shown by Pl. 3*A* reflects the distribution of free calcium ions which may be only a small fraction of the total calcium which is bound within the muscle cytoplasm. Takeuchi (1963) has suggested that the muscle fibre membrane at the junctional region may become slightly permeable to calcium ion during the action of ACh, because she observed a localized shortening at the end-plate region of frog muscle fibres treated with ACh in high calcium Ringer solution.

Carbachol failed to increase the efflux of accumulated calcium from the junctional region of the muscle into calcium-free Ringer solution. This indicated that the calcium which entered the muscle at the junctional region was bound within the muscle. However, carbachol and ACh decreased the efflux of accumulated calcium from the junctional region into 5 mM calcium Ringer solution. The latter effect may be explained if the route of efflux for calcium through the post-junctional region of the muscle fibre membrane was the same as that for influx. In this situation, efflux of tracer would be hindered in the presence of carbachol by the entry of calcium from the Ringer solution. The tracer-free calcium which entered the muscle could be accommodated by spare uptake sites within the muscle without displacing the tracer already bound. The presence of spare uptake sites is indicated by the upper curve in Text-fig. 1 which shows that uptake sites were not saturated after a 30 min period in 0·1 mM carbachol.

Lièvrmont *et al.* (1968) reported that nerve stimulation of muscles at 50 Hz for 15 min in the presence of neostigmine produced staining of calcium at the junctional region. In the present experiments neostigmine or eserine (10^{-5} M) caused a marked accumulation of ^{45}Ca at the junctional region of unstimulated muscles. Stimulation increased this accumulation by 34% in the presence of neostigmine, but the accumulation in the presence of eserine was unaltered by stimulation of the phrenic nerve. If the effect of eserine and neostigmine on calcium entry to the junctional region was produced by the spontaneous release of ACh from nerve endings then it is difficult to see why nerve stimulation in the presence of eserine did not increase this effect still further. A possible explanation for the absence of effect of nerve stimulation in the presence of eserine is, that this

level of eserine may have blocked conduction in fine nerve terminals, thus preventing the release of ACh by nerve impulses. Bullock, Nachmanson & Rothenberg (1946) have described nerve conduction block produced by eserine in squid giant axon, although the concentrations used were from 2 to 10 mM.

Potassium depolarization produced entry of calcium to both regions of the muscle with a greater entry at the junctional region. This suggested that depolarization of the post-junctional region could be a sufficient stimulus for entry of calcium. However, this could not have been the cause of the calcium entry in the presence of carbachol because carbachol produced no significant depolarization (Text-fig. 6). The greater effect of potassium on calcium entry at the junctional region indicated a potentially greater permeability to calcium in this region. This may be related to the greater surface area in this region because of the folded shape of the post-junctional muscle membrane. The absence of effect of these concentrations of ACh and carbachol on the muscle membrane potential of mouse diaphragm fibres at the junctional region is somewhat surprising and it contrasts with the effect of carbachol (0·27 mM) on the post-junctional membrane potential of frog sartorius muscle fibres (Nastuk & Parsons, 1970). In these frog muscles muscle post-junctional membrane depolarization of up to 50 mV occurred in response to carbachol, and these post-junctional membrane potentials returned to normal within a period of 10–30 min. Also, Nastuk & Parsons (1970) observed strong twitches in the frog muscles on adding carbachol. In the present experiments there was no visual evidence of movement of mouse diaphragm muscles in response to perfusion with 0·1 mM carbachol.

The disappearance of m.e.p.p.s in the presence of carbachol or ACh indicated either that the pre-junctional release of transmitter was inhibited or that muscle fibre post-junctional sensitivity was depressed. Inhibition of pre-junctional release seemed less likely because it has been shown, for instance, that compounds of the depolarizing type can potentiate transmitter release (Blaber, 1970). Therefore, it seems likely that these levels of ACh and carbachol caused a decrease in post-junctional sensitivity which was so rapid that the muscle fibre membrane potential at the junctional region was not altered for more than a few milliseconds. This conclusion is supported to some extent by the observations of Fatt (1950) and Thesleff (1955). Fatt (1960) found that end-plate potentials of frog muscles bathed in neostigmine were curtailed rapidly as the ACh concentration was increased up to 16·3 μmole/l. Thesleff (1955) found that blockade of transmission at frog motor end-plates occurred in the continued presence of ACh and neostigmine even though muscle membrane potentials at the junctional region had returned to normal. From this Thesleff

concluded that blockage was caused by continued desensitization of post-junctional receptors.

No difference in the distribution of labelled sodium within the muscle was produced by carbachol under similar experimental conditions to those which were used to measure the distribution of ^{45}Ca. This again supports the finding that no noticeable change in muscle fibre membrane potential at the junctional region occurred following the application of 0·1 mM carbachol to the muscle. Boyd & Martin (1956) have calculated the charge displacement during the end-plate potential of single cat muscle fibres to be equivalent to a net transport of about $1{\cdot}5 \times 10^{-15}$ mol of univalent ions within a time of 2 msec. It can be calculated that the amount of calcium which enters each mouse diaphragm muscle fibre in the presence of 0·1 mM carbachol is about 5×10^{-18} mol in 2 msec. Thus if the electrical properties of cat and mouse muscle fibres are fairly similar a measurable change in muscle fibre membrane potential would not be expected to occur from this rate of entry of calcium.

It seems reasonable to conclude that in the presence of ACh or carbachol calcium progressively enters mouse diaphragm muscle fibres at the post-junctional region when this region of the muscle fibre membrane is desensitized.

This work was supported by the National Fund for Research into Crippling Diseases.

I wish to thank Professors R. Miledi and J. F. Mitchell for their advice in the course of this work.

REFERENCES

AHMAD, K. & LEWIS, J. J. (1961). The effects of gallamine, carbachol, ryanodine and protoveratrine A and B upon flux of calcium-47 in frog skeletal muscle. *J. Pharm. Pharmac.* **13**, 383–384.

AHMAD, K. & LEWIS, J. J. (1962). The influence of drugs which stimulate skeletal muscle and of their antagonists on flux of calcium, potassium and sodium ions. *J. Pharmac. exp. Ther.* **136**, 298–304.

ALBUQUERQUE, E. X. & THESLEFF, S. (1968). A comparative study of membrane properties of innervated and chronically denervated fast and slow skeletal muscles of the rat. *Acta physiol. scand.* **73**, 471–480.

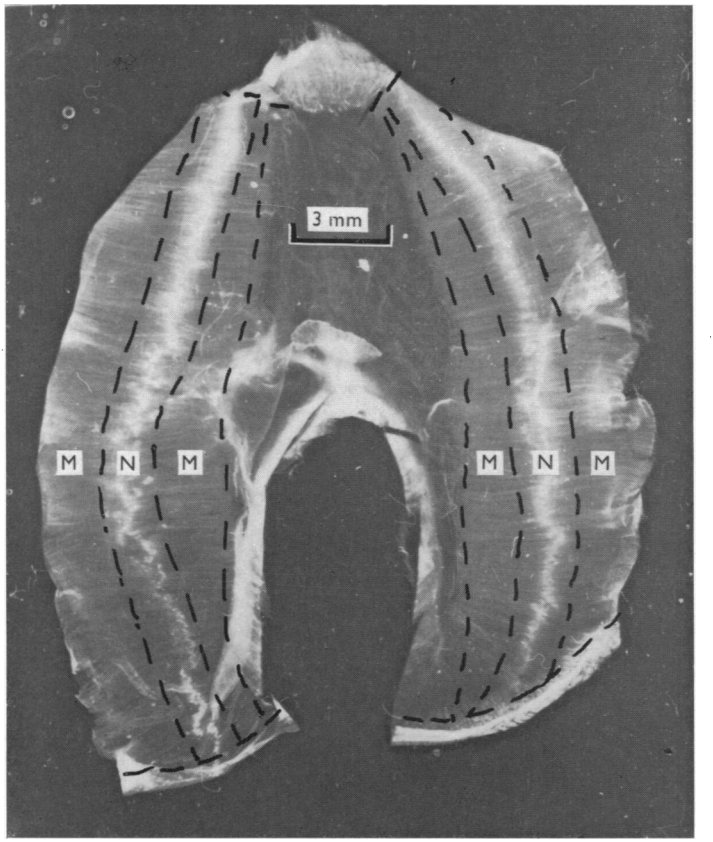
AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol.* **147**, 178–193.

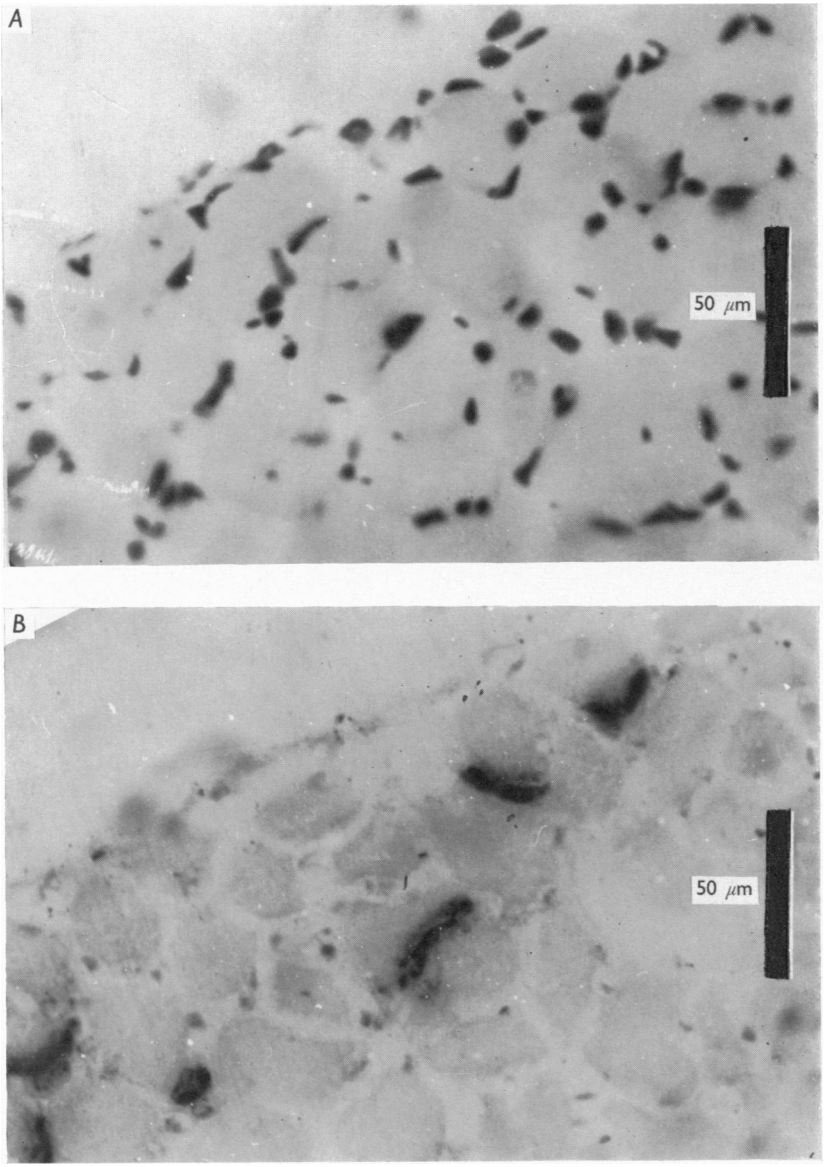
BIANCHI, C. P. & SHANES, A. M. (1959). Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. *J. gen. Physiol.* **42**, 803–815.

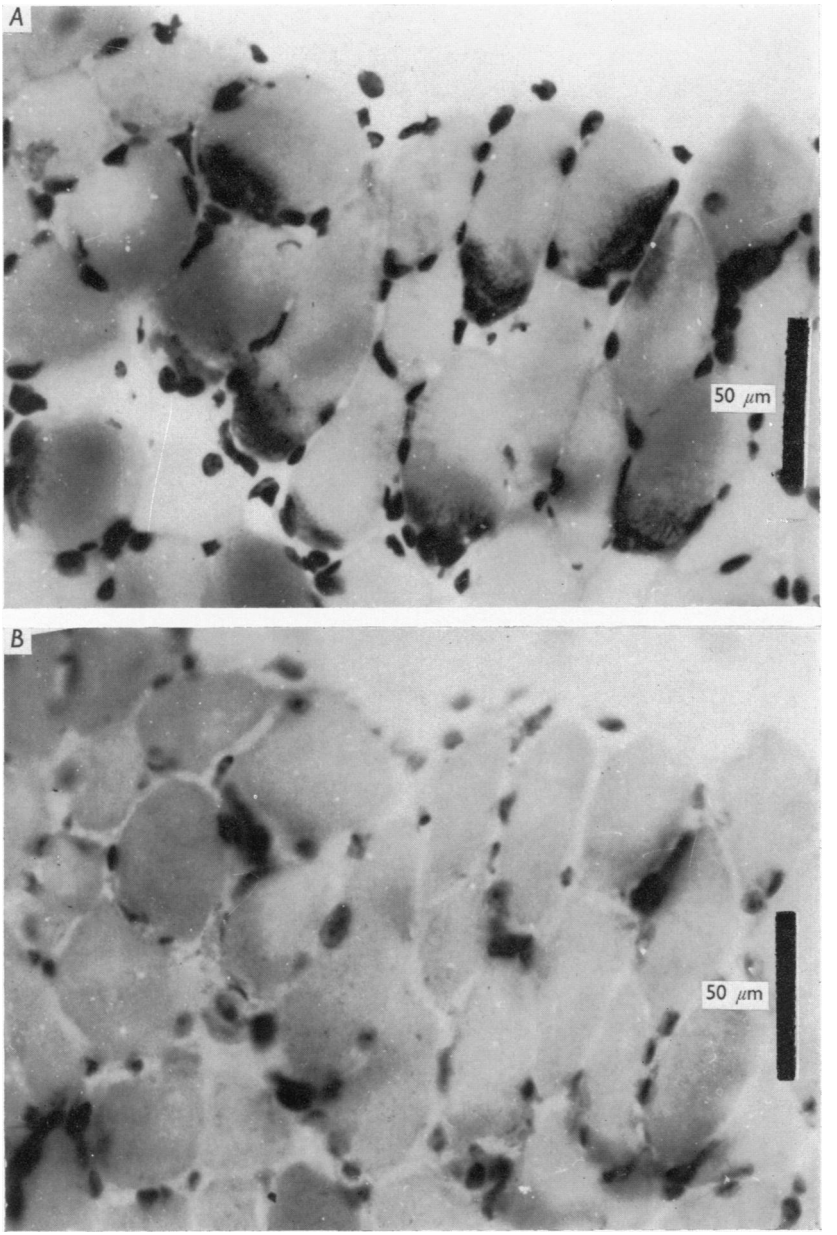
BLABER, L. C. (1970). The effect of facilitatory concentrations of decamethonium on the storage and release of transmitter at the neuromuscular junction of the cat. *J. Pharmac. exp. Ther.* **175**, 664–672.

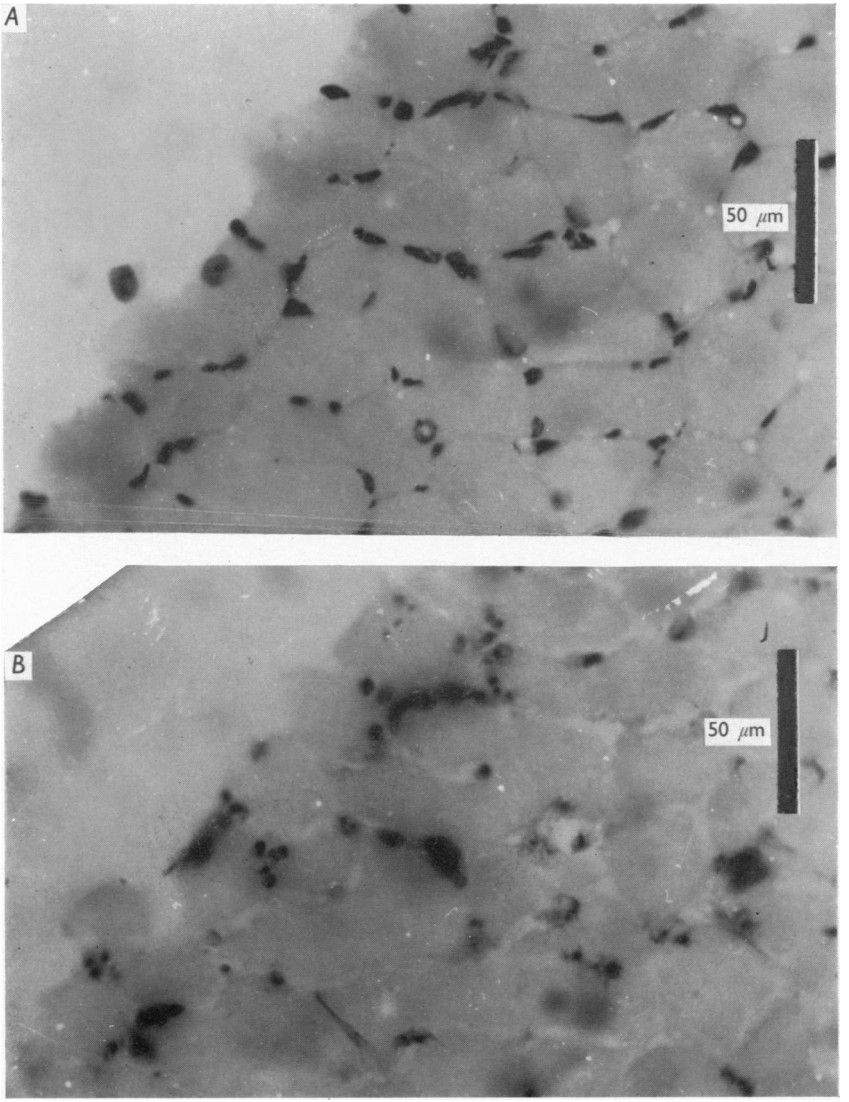
BOYD, I. A. & MARTIN, A. R. (1956). The end-plate potential in mammalian muscle. *J. Physiol.* **132**, 74–91.

BULLOCK, T. H., NACHMANSON, D. & ROTHENBERG, M. A. (1946). Effects of inhibitors of choline esterase on the nerve action potential. *J. Neurophysiol.* **9**, 9–22.


3 mm
M
N
M
M
N
M


A
50 μm
B
50 μm


A
50 μm
B
50 μm


A
50 μm
B
50 μm

CSILLIK, B. & SÁVAY, G. (1963). Release of calcium in the myoneural junction. *Nature, Lond.* **198**, 399–400.

FATT, P. (1950). The electromotive action of acetylcholine at the motor end-plate. *J. Physiol.* **111**, 408–422.

HEBB, C. O., KRNJEVIĆ, K. & SILVER, A. (1964). Acetylcholine and choline acetyltransferase in the diaphragm of the rat. *J. Physiol.* **171**, 504–513.

JENKINSON, D. H. & NICHOLLS, J. G. (1961). Contractures and permeability changes produced by acetylcholine in depolarised denervated muscle. *J. Physiol.* **159**, 111–127.

KASHIWA, H. K. & ATKINSON, W. B. (1963). The applicability of a new Schiff base, glyoxal bis(2-hydroxyanil), for the cytochemical localisation of ionic calcium. *J. Histochem. Cytochem.* **11**, 258–264.

KOELLE, G. B. & FRIEDENWALD, J. S. (1949). A histochemical method for localising cholinesterase activity. *Proc. Soc. exp. Biol. Med.* **70**, 617–622.

LIÈVREMENT, M., CZAJKA, M. & TAZIEFF-DEPIERRE, F. (1968). Etude in situ d'une fixation de calcium et de sa liberation à la jonction neuromusculaire. *C. r. hebd. Seanc. Acad. Sci., Paris* **267**, 1988–1991.

MARCO, L. A., MIKITEN, T. M. & NASTUK, W. L. (1969). Histochemical detection of calcium released at neuromuscular junctions of oscillating frog muscle fibers. *Fedn Proc.* **28**, 457.

MEUNIER, F. (1972). Localisation à l'appareil sous-neural du calcium apparaissant dans diverses conditions experimentales au niveau de la jonction neuromusculaire. *C. r. hebd. Seanc. Acad. Sci., Paris* **274**, 1818–1821.

NASTUK, W. L. & PARSONS, R. L. (1970). Factors in the inactivation of post-junctional membrane receptors of frog skeletal muscle. *J. gen. Physiol.* **56**, 218–249.

TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. *J. Physiol.* **167**, 141–155.

THESLEFF, S. (1955). The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. *Acta physiol. scand.* **34**, 218–231.

EXPLANATION OF PLATES

Micrographs *A* and *B* on Pls. 2, 3 and 4 show the same selected microscopic field of 6 μm serial sections stained for calcium ions and cholinesterase respectively.

PLATE 1

Photograph of alizarin red S stained mouse diaphragm after removal of the ribs with scissors. The places in which the diaphragm muscles were divided with a scalpel blade are indicated by the dashed lines. Two innervated segments (N), containing the junctional region, and four non-innervated segments (M) were obtained from each muscle.

PLATE 2

Sections from control muscle incubated in the normal Ringer solution with no drug added.

PLATE 3

Sections from muscle incubated with 0·1 mM carbachol for 30 min.

PLATE 4

Sections from control muscle pre-incubated with calcium-free Ringer solution for 30 min before incubation in calcium-free Ringer solution with 0·1 mM carbachol for 30 min.