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THE EFFECTS OF CALCIUM AND MAGNESIUM ON MAMMALIAN MUSCLE FIBRES

By D. H. PAUL

From the Department of Physiology, King's College, London, W.C. 2, and the Department of Zoology and Comparative Physiology, University of Birmingham

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During an investigation into the neuromuscular block produced by magnesium in the rat diaphragm, it was found that although calcium reversed the effect of magnesium on neuromuscular transmission, it failed to restore the twitch height to its original level (Paul, 1957). A considerable number of workers have found that calcium and magnesium depress the excitability of amphibian muscle (Engbaek, 1948; Jenerick & Gerard, 1953; del Castillo & Engbaek, 1954), but little work appears to have been done on mammalian muscle, most of the earlier work being concerned with the anaesthetic action of magnesium in whole animals. However, Nothman (1921) reported that high blood calcium concentrations reduced the excitability of human skeletal muscle, and Maaske & Gibson (1939) found that magnesium reduced the twitch height of directly stimulated and denervated dog muscle, owing, they concluded, to an increase in threshold.

The effects of calcium and magnesium on an isolated mammalian muscle, namely, the rat phrenic-nerve–diaphragm preparation, are described in the present paper. An account of part of this work has been previously submitted to the University of London in part fulfilment of the requirements for the degree of Doctor of Philosophy.

METHODS

A modified version of the technique introduced by Bülbring (1946) and subsequently developed by McDowall, Miechowski & Shafei (1949) and Hadju & McDowall (1949) was employed. Diaphragm muscles from albino rats were dissected complete, together with both phrenic nerves, and placed in oxygenated Krebs's solution, in which all further handling was carried out. A rectangular segment was cut out, as this preserved as many fibres intact as possible, damage being confined to the two parallel cuts made along the line of the fibres. The entry of the phrenic nerve was included in this segment. The preparation was attached to a standard plastic diaphragm electrode (Bell, 1953) by the central tendon, and the costal margin was attached to the recording system. The nerve was laid over a pair of platinum electrodes, and the plastic holder was suitably wired to allow independent direct stimulation of the muscle, again through platinum electrodes.

The preparation was immersed in a 50 ml. organ bath filled with Krebs's solution of composition (mM): Na^+ 145, K^+ 5·9, Ca^{2+} 2·6, Mg^{2+} 1·2, Cl^- 128·9, $H_2PO_4^-$ 1·2, SO_4^{2-} 1·2, HCO_3^- 25·0, glucose 5·5 (Krebs & Henseleit, 1932). To increase the concentration of an ion during an experiment, suitable amounts of a molar solution of its chloride were added to the bath. The resulting increase in volume was kept to a maximum of 1·5 ml. or less throughout the experiment. Aeration was carried out with a 95 % O_2 + 5 % CO_2 gas mixture, bubbled through a plastic tube plugged with fluted glass. The gas bubbles were made to impinge on the tissue to ensure adequate oxygenation (Creese, Scholes & Whalen, 1958), and they also served to mix the contents of the bath. The pH of the aerated solution was 7·4. The bath was warmed by a heated water jacket and kept at 37–38° C. The nerve was also immersed and was stimulated below the surface of the bath fluid.

Stimulation was performed by means of square pulses of a constant voltage, which was set to give a maximal twitch response for each individual muscle in normal Krebs's solution. Impulse duration was 0·2 msec for nerve and 2 msec for direct stimulation. Stimulation frequency was 15–16/min, at which rate a preparation normally continued to respond for at least 8 hr.

In most cases contractions were recorded with a simple isotonic lever. Dimensions were: muscle attachment to fulcrum 2 cm; fulcrum to writing point 18 cm; total weight of unloaded lever 1·25 g.

Estimations of potassium and sodium were made with an EEL flame photometer. Tissue samples were blotted dry, weighed, and dried at 120° C for 15–20 hr. After cooling in a desiccator the samples were reweighed and dissolved in 50 % (v/v) nitric acid at 100° C on a water-bath. The resulting solution was diluted to 50 ml. for analysis. The solutions used for the soaking experiments were prepared with the concentrations already adjusted. In these experiments increases in concentration of ions were not accompanied by dilution of the solution.

RESULTS

Effects of calcium and magnesium on the twitch height

Additions of either calcium or magnesium to the bath depressed the twitch height of an active preparation. Figure 1 shows the effect of increasing the bath concentration of calcium or magnesium by 4 mM. The preparation had been curarized to block neuromuscular transmission with a bath concentration of 2×10^{-5} D-tubocurarine, and was stimulated directly. This was necessary to show the effect of magnesium on the muscle fibres as distinct from its blocking action on neuromuscular transmission (Liley, 1956; Paul, 1957).

Preparations readily recover from a depression induced by calcium or magnesium when washed with normal Krebs's solution. Both depression and recovery take place more rapidly with calcium than with magnesium. As the hydrated calcium ion is rather smaller than the hydrated magnesium ion, this might be attributable to a difference in diffusion rate into the tissue. The relative ionic radii, based on potassium as unity, have been given by Conway (1945) as Ca^{2+} 2·51, and Mg^{2+} 2·64.

Progressive increases in the calcium or magnesium concentrations produce corresponding decreases in the twitch height, until the total depression is of the order of 30 % with calcium and 50 % with magnesium. The

majority of preparations reach maximum depression with 20 mM excess calcium or magnesium.

Most of these experiments were performed on curarized preparations, but the calcium depression could be obtained equally well on indirectly stimulated preparations. Several reports have been published of neuromuscular blocks produced by excess calcium in amphibian nerve–muscle preparations (Locke, 1894; Mines, 1908; Coppée, 1946; Hunt & Kuffler, 1950), but this could not be repeated for rat muscle. Excess calcium never abolished the response completely.

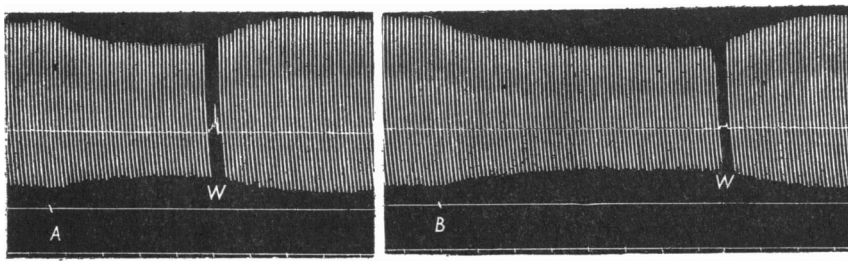

Fig. 1. Depression due to increase of Ca^{2+} and Mg^{2+}. The preparation had been curarized. *A*, Ca^{2+} concentration increased by 4 mM. At *W* preparation washed with normal Krebs's solution. *B*, Mg^{2+} concentration increased by 4 mM. At *W* preparation washed with normal Krebs's solution. Time trace shows 1 min intervals.

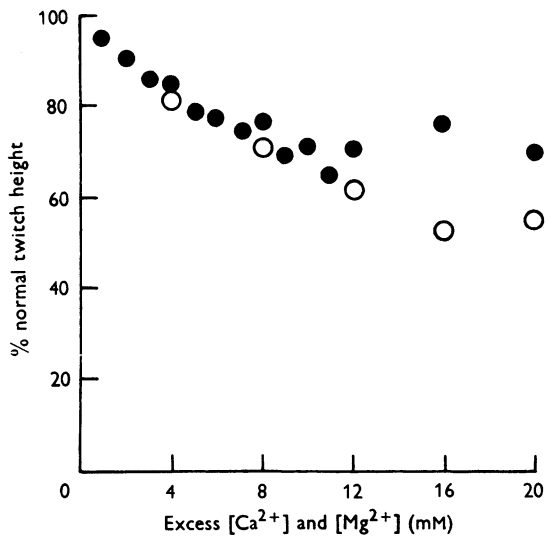

Fig. 2. Progressive depression of twitch height with increase of Ca^{2+} or Mg^{2+} until maximum depression was reached. Each point represents the mean value of at least four observations. Ca^{2+} and Mg^{2+} concentrations are plotted as mM excess over the normal concentration. Twitch height is plotted as a percentage of the initial twitch height. Ca^{2+} ●; Mg^{2+} ○.

The effect of potassium on depressions due to calcium and magnesium

The effect of calcium on a preparation is different when the experiment is carried out in Krebs's solution with an increased potassium concentration, but the effect of excess magnesium is not altered. After the potassium concentration has been raised an increase in calcium concentration produces first a decrease in twitch height (as in normal Krebs's solution), followed by a slow increase in twitch tension which may take 10 min or more to reach its maximum. Figure 3 shows the response of a preparation to four successive increases of calcium concentration, each of 4 mM, after the potassium concentration had previously been increased by 4 mM.

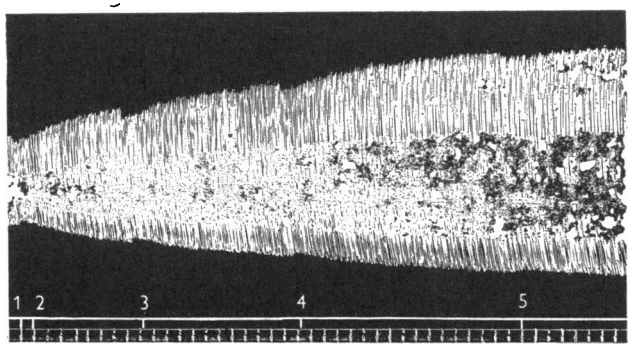

Fig. 3. The effect of successive 4 mM increases of Ca^{2+} concentration after the potassium concentration had been increased by 4 mM. At signals 2, 3, 4, and 5 the calcium concentration was increased by 4 mM. The signal is displaced to the right by the distance between 1 and 2. The twitch height had been depressed 44 % by the potassium and was increased finally to 103 % normal by the addition of Ca^{2+}. Time trace: 1 min intervals.

By using two preparations from the same animal the twitch heights at various levels of calcium and magnesium concentration both before and after an increase in potassium concentrations can be obtained. Figures 4 and 5 show the twitch height, as a percentage of the original twitch height, plotted against the calcium and magnesium concentrations, respectively, both before and after the potassium concentration was raised by 4 mM. The magnesium depression was similar in both cases, but the calcium depression was reversed with increased potassium.

Potassium has been shown to have a twofold effect on the rat diaphragm, depending on the concentration; at low concentrations the twitch height is increased, and at higher concentrations the twitch height is depressed (Hadju, Knox & McDowall, 1950). The absolute potassium concentration at which augmentation of the twitch is superseded by depression varies considerably from preparation to preparation, so that in the present

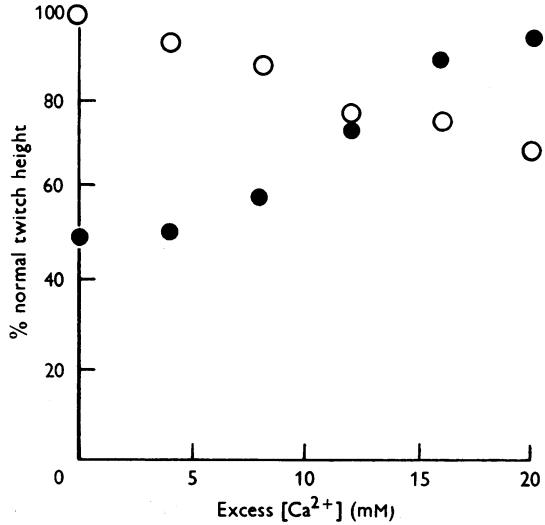

Fig. 4. Effect of increasing Ca^{2+} concentration before and after the potassium concentration had been increased by 4 mM. ○, normal Krebs's solution; ●, plus 4 mM potassium.

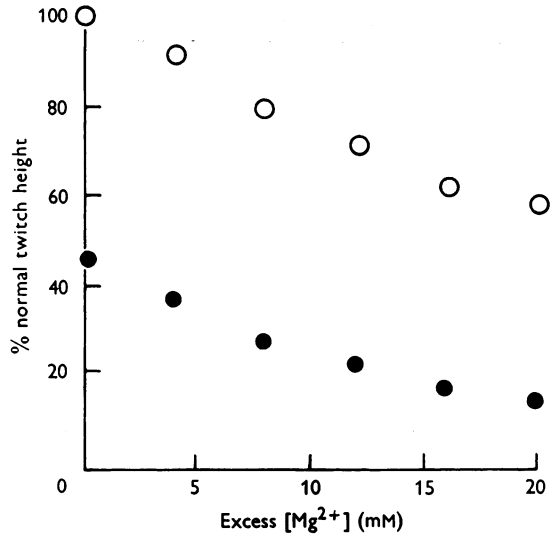

Fig. 5. Effect of increasing Mg^{2+} concentration before and after the potassium concentration had been increased by 4 mM. ○, normal Krebs's solution; ●, plus 4 mM potassium. This preparation was from the same animal as that in Fig. 4.

experiments increasing the potassium concentration by 4 mM usually resulted in depression but sometimes in a maintained increase in twitch height. In the latter case increasing the calcium concentration produced its typical response of depression followed by augmentation. The final increase in twitch height exceeded that produced by the potassium alone.

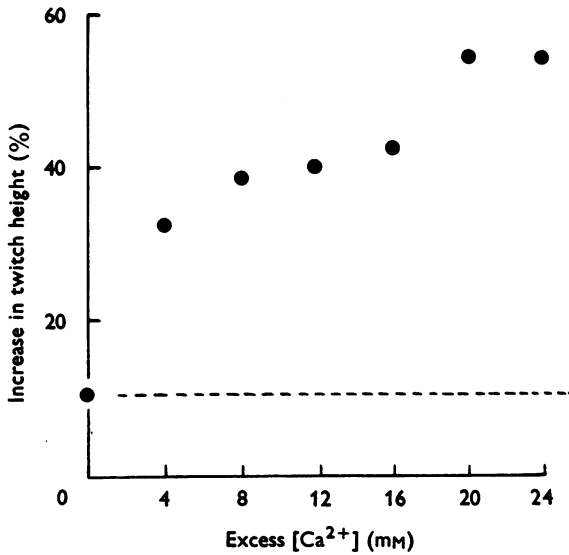

Fig. 6. Effect of increase of Ca^{2+} concentration after the potassium concentration had been increased by 4 mM, but had not produced any depression. Broken line indicates level to which twitch height was increased by the potassium. Ordinate gives percentage increase over initial twitch height.

Effects of calcium on post-tetanic potentiation

As post-tetanic potentiation has been ascribed to the accumulation of potassium ions in the extracellular muscle spaces (Brown & von Euler, 1938), experiments were carried out to see whether calcium affected this action. A muscle was tetanized for 10 sec at a frequency of 5 impulses/sec. A low frequency was used to avoid fatiguing the muscle unduly, as the procedure was repeated several times. The amount of potentiation after the tetanus was measured during the time taken for the twitch height to return to normal. Figure 7 shows the relationship between potentiation and time after a tetanus, for various concentrations of calcium. The higher the calcium concentration, the longer the potentiation lasts, and the greater is the degree of potentiation. An increase in magnesium concentration of 4 mM did not produce a detectable alteration of post-tetanic potentiation. These results clearly resemble the action of potassium on the calcium depression described above.

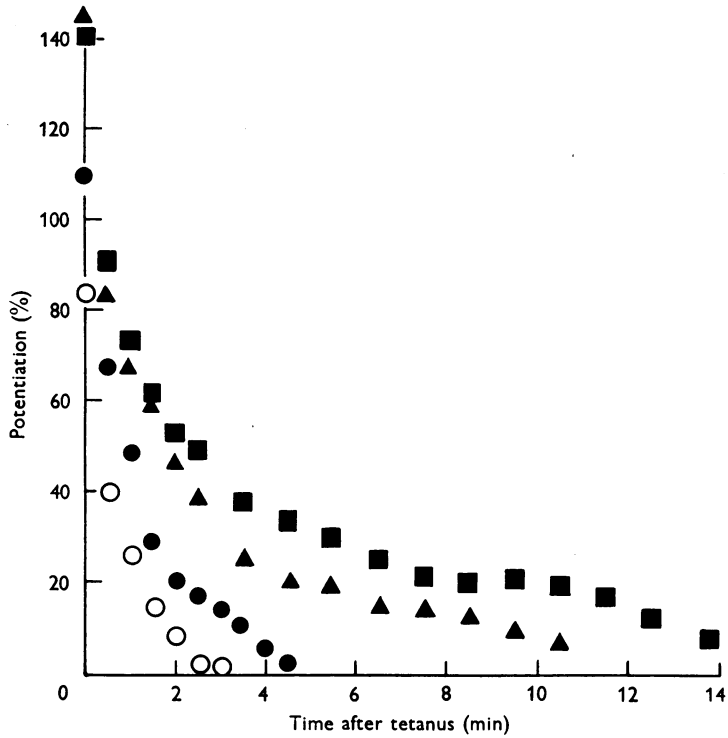

Fig. 7. Effect of Ca^{2+} on post-tetanic potentiation. Potentiation is plotted as percentage increase in twitch height over the initial level. ○, normal Krebs's solution; ●, plus 4 mM-Ca^{2+}; ▲, plus 8 mM-Ca^{2+}; ■, plus 12 mM-Ca^{2+}.

Effect of calcium on the potassium and sodium content of muscle

As the potassium ion is known to affect the twitch height of the rat diaphragm (Hadju *et al.* 1950) experiments were carried out to see whether calcium had any effect on the tissue content of this ion. Potassium movements often compensate for movements of sodium, and that ion was also estimated at the same time.

Diaphragms were divided into four sections, and one section from each muscle was soaked in one of the following solutions: normal Krebs's solution, and Krebs's solution containing 2, 3, or 5 times normal calcium concentration. The muscles were soaked for 1·5 hr at 37·5° C, and then analysed for potassium and sodium content. The results are given in Table 1. No statistically significant difference was found between muscles soaked in normal Krebs's solution and those soaked in high-calcium solutions.

In a further series of experiments segments from each diaphragm were

soaked for 1 hr in normal Krebs's solution, in solutions with twice normal Ca^{2+}, with twice normal K^+, and in a solution with twice normal Ca^{2+} as well as twice normal K^+. The results are shown in Table 2. Doubling the potassium concentration produced a fall in tissue potassium content of about 7–8 m-equiv/kg wet tissue. The sodium concentration increased by a similar amount. Both the decreased tissue potassium and the increased tissue sodium were significantly different from the normal value (for K^+, $P = 0{\cdot}02$–$0{\cdot}01$; for Na^+, $P = 0{\cdot}05$–$0{\cdot}02$). Assuming a constant value of 0·28 for extracellular space (see Creese *et al.* 1958) and the values for water content of the tissues given in the table, the fall in tissue potassium

TABLE 1. Tissue content of sodium and potassium with different concentrations of Ca^{2+} in the soaking solution. Results are expressed as mean ± S.E. of mean, with number of observations in brackets. In no case is the value for increased Ca^{2+} significantly different from the control. The water content of the tissues is also given. Tissues were soaked for 1·5 hr

Soaking solution	Tissue concentrations (m-equiv/kg wet tissue) Na	K	Water content (% wet weight)
Normal Krebs's solution (control)	69·2 ± 3·5 (8)	52·0 ± 1·9 (8)	76·4 ± 0·03 (8)
$2 \times Ca^{2+}$	65·4 ± 2·7 (9)	54·4 ± 1·7 (9)	76·0 ± 0·25 (9)
$3 \times Ca^{2+}$	71·8 ± 3·9 (7)	50·8 ± 2·6 (7)	76·4 ± 0·17 (7)
$5 \times Ca^{2+}$	69·6 ± 6·0 (5)	55·5 ± 3·1 (5)	76·6 ± 0·20 (5)

TABLE 2. Effects of increasing Ca^{2+} and K^+ concentrations on the tissue content of potassium and sodium. Results as in Table 1. The tissues were soaked for 1 hr

Soaking solution	Tissue concentrations (m-equiv/kg wet tissue) Na	K	Water content (% wet weight)
Normal Krebs's solution (control)	65·4 ± 1·7 (17)	71·2 ± 1·4 (17)	77·1 ± 0·1 (17)
$2 \times Ca^{2+}$	68·0 ± 3·7 (18)	71·8 ± 1·0 (18)	76·7 ± 0·1 (18)
$2 \times K^+$	73·4 ± 2·9 (20)	64·1 ± 2·3 (20)	77·6 ± 0·1 (20)
$2 \times Ca^{2+}$ and $2 \times K^+$	73·1 ± 2·9 (15)	63·7 ± 3·0 (15)	78·0 ± 0·1 (15)

can be interpreted as a fall in intracellular potassium concentration of about 13–14 m-equiv/l. fibre water. The intracellular sodium rises by a similar amount. This effect of twice normal potassium was not altered by doubling the calcium concentration.

A few experiments with a mechano-electric transducer confirmed that the effects of the solutions used for the soaking experiments resembled the results recorded with the isotonic lever.

DISCUSSION

Both calcium and magnesium have a considerable depressive effect on rat-diaphragm muscle fibres. As the depression appears within seconds of adding the ions to the bath the action almost certainly takes place at the

fibre membrane. Numerous observations have been made of a similar action of these ions on amphibian muscle, and it has been demonstrated that high calcium and magnesium concentrations decrease the excitability of the fibre membrane by raising the threshold to stimulation (Chao, 1935; Ashkenaz, 1938; Carleton, Blair & Latchford, 1938; Jenerick & Gerard, 1953; del Castillo & Engbaek, 1954). Weidmann (1955) has observed a similar effect on mammalian Purkinje fibres, finding that a greater degree of depolarization is required for excitation when the calcium or magnesium concentration is raised. A similar effect of calcium on nerve is well known (literature reviewed by Brink, 1954).

The decrease in excitability with increased calcium concentration is not so marked in the rat diaphragm as in frog muscle. Several observations of neuromuscular blocks with high calcium concentrations have been made (Locke, 1894; Mines, 1908; Coppée, 1946), but the experiments of Hunt & Kuffler (1950) using the frog sartorius preparation have demonstrated that the end-plate potential could still be obtained when the muscle, under the influence of high calcium, had ceased to respond to stimulation. The apparent block was due to a big decrease in excitability of the fibre membrane. Engbaek (1948) was able to show that magnesium in high concentrations also prevented the spread of excitation from the end-plate in frog muscle. In the rat diaphragm complete loss of excitability with high calcium or magnesium was never seen, the maximum depression ever recorded being 65% with 16 mM excess magnesium. Maaske & Gibson (1939) were also unable to depress completely the response of denervated dog muscle with magnesium.

This difference in susceptibility between frog and rat muscle may be due to the smaller factor by which the calcium concentration was raised in these present experiments. Coppée (1946) used 16 times Ringer's concentration of calcium to produce a block, and this is equivalent to a concentration of 27·8 mM. A comparable increase, i.e. 16 times Krebs's concentration, would be 39·6 mM calcium, but the calcium concentration of Krebs's solution cannot be raised much above 30 mM without the salt being precipitated, so that the required concentration cannot be reached. The observed difference between the effects of equal amounts of calcium and magnesium at high concentrations (Fig. 2) may be accounted for by a similar argument. As the normal calcium concentration is more than twice that of magnesium, equal amounts of these ions do not increase the respective concentrations by the same factor, that for magnesium being twice that for calcium.

The effect of an increase in the potassium concentration on the response to calcium resembles the observations of Niedergerke (1956), that calcium enhances the tension developed by an isolated frog's heart when the fibres

have been partly depolarized by potassium. In the rat diaphragm also calcium enhances the tension developed after the fibres have been subjected to an increased potassium concentration. From the usual Nernst equation the theoretical decrease in membrane potential due to a doubling of external potassium concentration would be about 18 mV. The accompanying fall in the intracellular potassium concentration by 13 m-equiv/l. is not what would be expected to occur on *a priori* grounds. The membrane potential might be expected to decrease, and the intracellular potassium concentration to show a slight increase. A possible explanation of the observed fall in concentration could be that the lowering of the membrane potential by potassium increases the leakiness of the membrane to sodium, and loss of potassium would then compensate for the increased entry of sodium.

Calcium itself does not produce any alteration in the potassium and sodium contents, and its augmenting action with increased potassium concentrations is not duplicated by magnesium. This leads to the conclusion that a specific action of the calcium ion is involved. Apparently potassium sensitizes the muscle to calcium, revealing an action that is masked under normal circumstances. The experiments on post-tetanic potentiation suggest that a similar effect is obtained when the potassium concentrations inside and outside the cell are varied in a more physiological way.

Antagonisms between potassium and calcium in a variety of tissues have been described (Ringer, 1883; Mines, 1908, 1912; Libet & Gerard, 1939). However, in the present case a simple antagonism between calcium and potassium affecting the twitch height is not operating. Calcium does not simply reverse the effect of potassium.

Micro-injection of calcium into muscle fibres causes the fibrils to shorten in the region of the injection (Heilbrunn & Wiercinski, 1947; Niedergerke, 1955). The activity of squid giant nerve fibres has been shown to be accompanied by an influx of calcium into the axoplasm (Hodgkin & Keynes, 1957), and calcium is also released to the exterior as a result of muscular activity (Heilbrunn, 1956). The recent experiments of Bianchi & Shanes (1959) on the entry of labelled calcium into active muscle have enabled the authors to support the prediction that entry of calcium into muscle fibres underlies the development of a contractile response. These reports strongly suggest that activity is accompanied by movements of calcium, and that contraction may depend on calcium entering the muscle fibre. If calcium is concerned with contraction in this way the effect of potassium might be to increase the movement of calcium into the cell during activity.

SUMMARY

1. The effects of calcium and magnesium ions on mammalian muscle fibres have been studied with the rat phrenic-nerve–diaphragm preparation.

2. Both calcium and magnesium depress the twitch height of a contracting diaphragm by affecting the muscle fibres directly. The depression reaches a maximum of 30 % with calcium and 50 % with magnesium when the concentration of either is increased by 16–20 mM.

3. With twice normal extracellular [K], calcium produces a rise in twitch height while magnesium still depresses it. A fall in tissue potassium content of about 7 m-equiv/kg wet weight also occurs in high [K], together with a corresponding rise in tissue sodium content.

4. Calcium has no effect on potassium and sodium contents.

5. Post-tetanic potentiation is increased both in magnitude and duration by increased calcium concentration. This again suggests that with a small increase in the extracellular potassium concentration the muscle fibres are able to develop more tension than normal in the presence of high calcium concentrations.

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