J. Physiol. (1972), **223**, *pp.* 735–755
With 12 *text-figures*
Printed in Great Britain

THE EFFLUX OF CALCIUM FROM SINGLE CRAB AND BARNACLE MUSCLE FIBRES

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(*Received 13 January 1972*)

SUMMARY

1. The efflux of calcium, as the isotope ^{45}Ca, has been investigated from single muscle fibres from the barnacle *Balanus nubilus* and from the crab *Maia squinado*.

2. If the isotope was initially injected with sufficient calcium (5–65 mM) to cause a contraction, the efflux did not follow first order kinetics. There was an early rapid phase which reached a peak after 5–10 min and then declined slowly over a period of 50–150 min to a low residual value.

3. Injection of the isotope with the calcium-binding agent EGTA, so that the injected free calcium concentration was *ca.* 2×10^{-8} M, abolished the initial rapid loss of calcium. The efflux rose to give a steady value after 10–15 min and its magnitude was similar to the value of the residual efflux.

4. The rate constant for the low residual loss was *ca.* 7×10^{-4} min^{-1} for *Maia* and *ca.* 17×10^{-4} min^{-1} for *Balanus*. The rate constant predicted a calcium efflux of 0·4 p-mole/cm^2.sec for *Maia* and 1–2 p-mole/cm^2.sec for *Balanus* at 16–25° C based on the total fibre calcium concentration.

5. The residual calcium efflux was not affected by 0·5 mM ouabain or 0 potassium salines applied externally. It was stimulated, some 10–15 times in *Maia* and to a lesser extent in *Balanus*, by salines containing 600 mM potassium or 2–5 mM caffeine. The increased efflux was associated with a brisk contraction.

6. External application of salines containing 20, 40 or 60 mM potassium or 0·5 mM caffeine in *Maia* produced some stimulation of the residual efflux but no visible contraction.

7. Pre-treatment of *Maia* fibres with 40 mM potassium or 0·5 mM caffeine salines abolished the ability of the fibres to respond to higher concentrations of these agents. A depletion of a releasable calcium fraction by these subthreshold stimuli could explain this phenomenon.

8. Electrical stimulation, the injection of 50 mM calcium chloride or

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50 mM caffeine produced an elevated calcium efflux which was associated with a contraction.

9. Intracellular injections of EGTA only lowered the residual efflux by up to half its initial value. This suggests that calcium can be released rapidly within these muscle fibres and that the sarcoplasmic calcium concentration is not much altered from its normal value by the injection.

10. The experiments suggest that in *Maia*, changes in the calcium efflux reflect in magnitude, but not in time course, the internal calcium changes which can be observed with the calcium-sensitive protein, aequorin.

INTRODUCTION

It is now well established that calcium plays an important role in the control of contraction in muscle (see, for example, Cuthbert, 1970). This paper is concerned mainly with the injection of the radioactive isotope of calcium, ^{45}Ca, into the large single muscle fibres from the crab, *Maia squinado* and the barnacle, *Balanus nubilus*. After injection, the efflux of the ^{45}Ca from the single fibres has been studied under a wide variety of conditions with a view to finding correlations between the ^{45}Ca efflux and the internal calcium changes associated with contraction.

Certain aspects of this work have been discussed previously in various preliminary accounts (Caldwell, 1964; Ashley, Caldwell, Lowe, Richards & Schirmer, 1965; Ashley, 1967; Caldwell, 1970, 1972).

METHODS

Dissection and cannulation

Single muscle fibres were isolated from the carpopodite flexor of *Maia* and were generally 1–2 mm in diameter and 1–2 cm in length (Caldwell, 1958). The lateral and rostral depressor muscle fibres from *Balanus* were generally larger, often 1–3 mm in diameter and 2–3 cm long (Hoyle & Smyth, 1963). The fibres were ligatured and cannulated in the manner described by Caldwell & Walster (1963). A small glass or platinum weight was attached to the tendinous end of the fibres, which were able to contract isotonically.

Resting potentials

The resting potential was determined by inserting a fine capillary (100–150 μm diameter tip) containing 1 M potassium chloride into the fibre through the cannula. The potential difference was determined between this capillary and an indifferent silver–silver chloride electrode in the external saline. The resting potentials were generally in the range of -40 to -60 mV (see legends) and were not corrected for liquid junction potentials. Calculations suggest, however, that the true resting potential may be 3–11 mV more negative (Caldwell, 1969).

Microinjector

In the experiments on single *Maia* fibres, the all-glass microsyringe designed by Hodgkin & Keynes (1956) and modified by Caldwell & Walster (1963) was used. This injector produced in the range of 0·12–0·18 μl./cm movement of the barrel. In the experiments on *Balanus* fibres a commercially available syringe was used (Hamilton Co. Inc., Whittier, California), which had a total volume of 1 μl. and produced 0·14 μl./cm movement of the barrel relative to the plunger. In both syringes the terminal glass capillary that entered the muscle fibre had a tip diameter of 100–150 μm.

Isotopes

The isotope, a solution of calcium chloride containing *ca.* 0·5 mc ^{45}Ca/ml. and about 130 μg calcium/ml. at pH 6–8 was obtained from the U.K. Atomic Energy Authority, Amersham. A known volume of this solution was evaporated to dryness and redissolved in 50–100 μl. distilled water or 10 mM-Tris buffer (pH 7·1). Generally 0·12–0·36 μl. of the resulting solution was injected into the single muscle fibres. The concentration of the calcium injected was in the range 5–65 mM, which after dilution about 100 times within the fibre would increase the total internal calcium concentration by some 0·05–0·7 m-mole/kg wet wt. In some experiments the isotope was redissolved in 200 mM-EGTA instead of water, and in this case the final mixture was 30 mM calcium/200 mM-EGTA.

Procedure and assay of radioactivity

The efflux was followed by placing the fibre for given times into a series of vessels containing aliquots of normal or modified crab saline at a temperature of 16–25° C. In the experiments with *Maia*, the calcium was precipitated from the saline at the end of each experiment by the addition of sodium carbonate and absolute ethanol. The precipitate, after centrifugation, was dissolved in hydrochloric acid and the solution evaporated to dryness. The residue was dissolved in 1 ml. distilled water and transferred to a counting vial together with 11 ml. liquid scintillator (*p*-terphenyl, 2 g; 1,4-di-[2-(5-phenyloxazolyl)]-benzene, 0·1 g; naphthalene, 40 g; 1,4-dioxan to 1 l.). In the experiments with *Balanus*, 1 ml. aliquots of saline were used and were transferred directly to counting vials to which was added 1 ml. distilled water and 15 ml. modified Bray solution (Bray, 1960) (where ethylene glycol was replaced by 2-ethoxy ethanol to improve the solubility of the saline in the scintillator). At the end of the experiment the fibres were digested in concentrated nitric acid or 100 vol. (30%, w/v) hydrogen peroxide. The radioactivity in each vial was assayed by liquid scintillation counting.

The efflux was expressed as a rate constant based on the total fibre count:

$$\text{rate constant (time}^{-1}) = \frac{\text{counts lost/collection time}}{\text{mean counts in the fibre during the collecting period}}.$$

Electrical stimulation

Single *Maia* fibres were stimulated by passing current between a 200 μm platinum wire inserted into the cannula and an indifferent silver–silver chloride electrode in the external saline. The amplitude of the stimulus pulse was 6 V. Stimulus frequencies above 4/sec were not used as there was a noticeable degeneration of the fibre.

Salines

The crab saline described by Fatt & Katz (1953) was used. In experiments involving potassium contractions, the sodium in the saline was replaced by potassium, although occasionally potassium chloride solutions isotonic with crab saline were employed. The caffeine was B.P. grade. All salines were buffered with sodium bicarbonate or with 2 mM-TES (N-Tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid), the range of pH being 6·8–7·5.

Determination of the calcium content

Single muscle fibres were rinsed briefly in calcium-free saline and then digested in 100 vols. (30%, w/v) hydrogen peroxide at 90° C. The residues were assayed for calcium by atomic absorption spectrophotometry. Lanthanum was added (4 mM) to each sample to suppress interference from phosphate.

RESULTS

The calcium content of the fibres

The mean calcium content found for *Maia* fibres was 1·1 m-mole/kg S.E. ± 0·08 (eleven fibres) and that for *Balanus* fibres 2·2 m-mole/kg S.E. ± 0·11 (six fibres).

The efflux of ^{45}Ca after injection into Maia muscle fibres and the effect of ouabain

Fig. 1 shows an example of the time course of the calcium efflux from a single *Maia* muscle fibre after injection of a [^{45}Ca]calcium chloride solution containing 41 mM calcium. This induced a contraction in the fibre which lasted 1–2 min. Initially there is a rapid efflux of calcium which reaches its peak in 5–10 min and this is followed by a slow decline lasting some 40 min to a steady low residual level with a rate constant of about 7×10^{-4} min^{-1}. In a series of seventeen experiments the initial rapid efflux was on average 80 times higher than the eventual steady level. During this period of rapid loss of calcium following the contraction induced by the injection, about 40% of the injected ^{45}Ca is lost from the fibre. Subsequently, the remaining 60% of the injected calcium is lost at a steady low rate, characteristic of the resting fibre. These proportions seemed independent of the concentration of calcium chloride injected.

Details of the various stages of the calcium efflux from a series of *Maia* fibres are summarized in Table 1, the steady efflux in p-mole/cm^2.sec being calculated on the basis that at this stage the injected ^{45}Ca had come into isotopic equilibrium with the total fibre calcium. It was also assumed that the efflux takes place solely across the surface membrane and that the internal calcium was 1·1 m-mole/kg. If account is taken of the increase in the surface area due to the cleft system (Discussion) and the increase in the

internal calcium produced by the initial injection, the efflux could be some 10 times smaller. Treatment of the fibre at a later stage of the experiment with 5×10^{-4} M ouabain saline showed that this known inhibitor of the sodium pump in *Maia* (Bittar, Caldwell & Lowe, 1967) has no effect on the processes concerned with the steady calcium efflux.

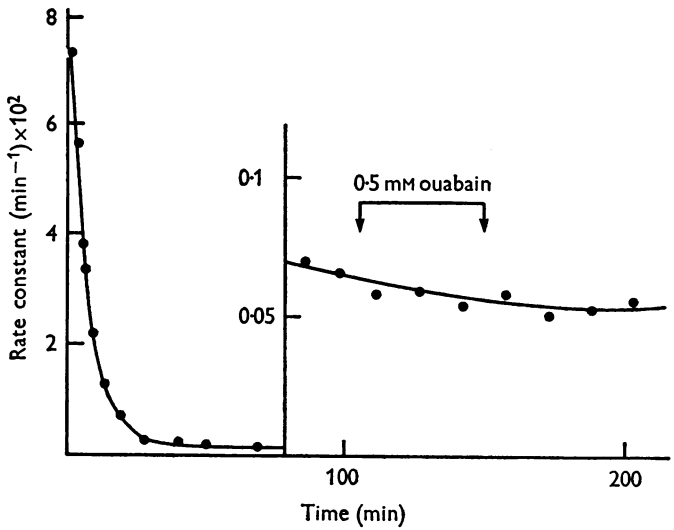

Fig. 1. The efflux of ^{45}Ca from a *Maia* muscle fibre after the injection of [^{45}Ca]calcium chloride. The effect of 0·5 mM ouabain saline on the residual calcium efflux is shown. Mean resting potential −48·5 mV. Wt./surface area: 24·8 mg/cm². Temp. 18° C.

TABLE 1. Characteristics of the calcium efflux from a series of *Maia* muscle fibres

Eventual steady efflux (p-mole/cm².sec)	0·39 (S.E. ± 0·03) (seventeen observations)
Eventual steady rate constant (min⁻¹)	$7{\cdot}0 \times 10^{-4}$ (S.E. ± 0·4) (seventeen observations)
% ^{45}Ca lost in the first 45 min after injection	38·4 (S.E. ± 3·2) (eighteen observations)
Temperature	16–22° C.

The effect of electrical stimulation on the efflux of ^{45}Ca from Maia

Fig. 2 shows the results of an experiment in which the effect on the steady calcium efflux of electrical stimulation was studied at three different frequencies. It can be seen that the increase in efflux over the residual efflux is roughly proportional to the rate of stimulation. If the average value of 0·4 p-mole/cm².sec is taken for the residual efflux, then

the values obtained for the extra efflux per stimulus for the three rates of stimulation shown in Fig. 2 are 0·6, 0·5 and 0·5 p-mole/cm^2. Contraction was just visible at the lower frequencies, but was well marked at 3·7 stimuli/sec.

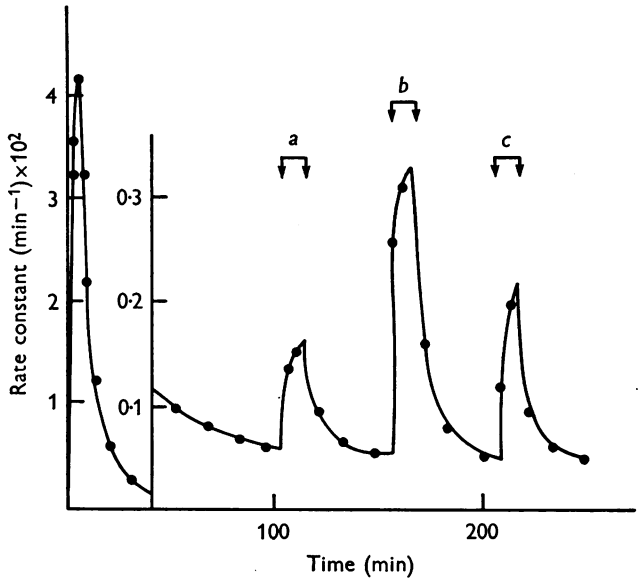

Fig. 2. The effect of electrical stimulation at (*a*) 1·2, (*b*) 3·7 and (*c*) 2·1 stimuli/sec on the residual calcium efflux from a *Maia* fibre. Mean resting potential −54·5 mV. Wt./surface area: 30·6 mg/cm^2. Temp. 16° C.

The effects of caffeine and injected calcium on the efflux of ^{45}Ca

The results in Fig. 2 suggest that the increase in calcium concentration in the sarcoplasm brought about by electrical stimulation and shown directly with aequorin (Ashley & Ridgway, 1970) leads to an increased loss of calcium from the fibre to the surroundings. Increases in sarcoplasmic calcium can be brought about in other ways, for example, by treatment of the fibre with caffeine. Fig. 3 shows an experiment in which the fibre was treated with 2 mM caffeine saline. The fibre contracted during the treatment and the efflux rose to a value about 15 times higher than the preceding residual efflux. When caffeine was removed, the fibre gradually relaxed and the efflux fell back to near the residual level. The sharp rise in calcium efflux brought about by caffeine treatment reflects the increase in sarcoplasmic calcium associated with contraction.

The injection of caffeine as well as its external application can also cause the contraction of *Maia* fibres (Caldwell & Walster, 1963). Fig. 4 shows how injection of a solution of 50 mM caffeine in distilled water affected the calcium efflux. The injection brought about a contraction of the fibre and

this was associated with an increase in the calcium efflux, reflecting the release of calcium from binding sites in the fibre by the caffeine. Injected unlabelled calcium can also cause a contraction, and this can also release ^{45}Ca from binding sites in the fibre. This is shown by the second injection

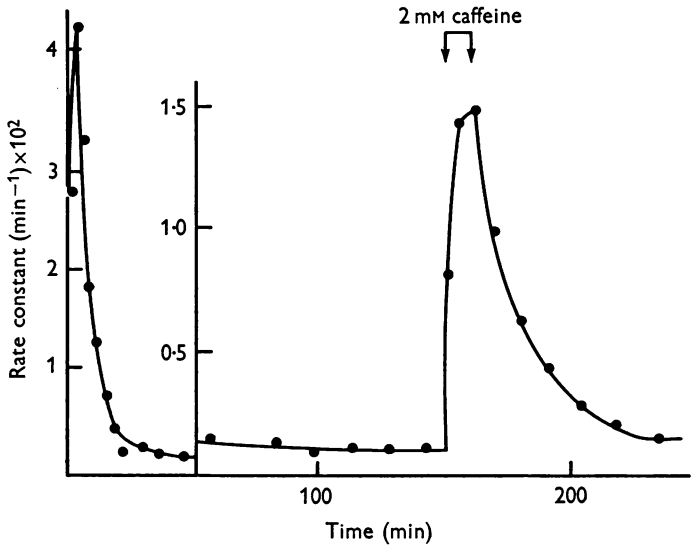

Fig. 3. The effect of 2 mM caffeine saline on the residual calcium efflux from a *Maia* fibre. Mean resting potential −53·5 mV. Wt./surface area: 28·8 mg/cm². Temp. 20° C.

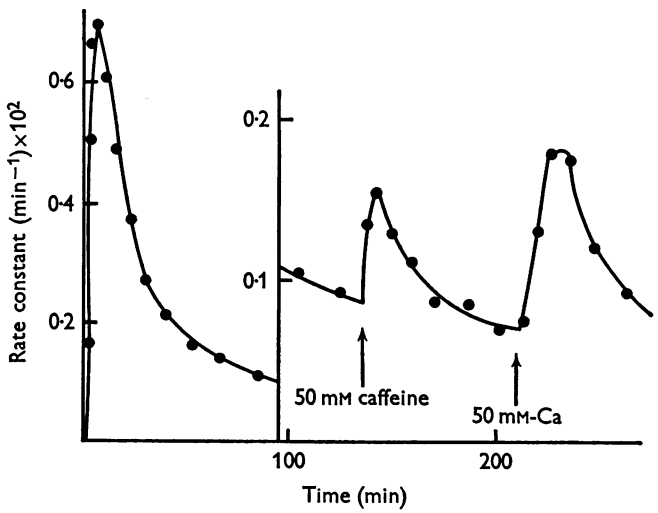

Fig. 4. The effect of the injection of 50 mM caffeine and 50 mM calcium chloride on the residual calcium efflux from a *Maia* fibre. Mean resting potential −46·5 mV. Wt./surface area: 31·0 mg/cm². Temp. 19° C.

in Fig. 4, where the increased efflux represents the mobilization inside the fibre of previously injected ^{45}Ca. Although this experiment was performed on the same fibre, the result was not connected with the preceding injection of caffeine, since a similar response was obtained in fibres in which caffeine had not been pre-injected. The increase in efflux found in these experiments was not an artifact produced by osmotic or ionic shock, since the injection of distilled water alone caused no increase in calcium efflux.

The effects of changes in external potassium concentration on the efflux of ^{45}Ca

Fig. 5 shows first the effect of removal of external potassium and then the effect of greatly increasing the external potassium on the residual calcium efflux. Immersion of the fibres for a period of about 40 min in a

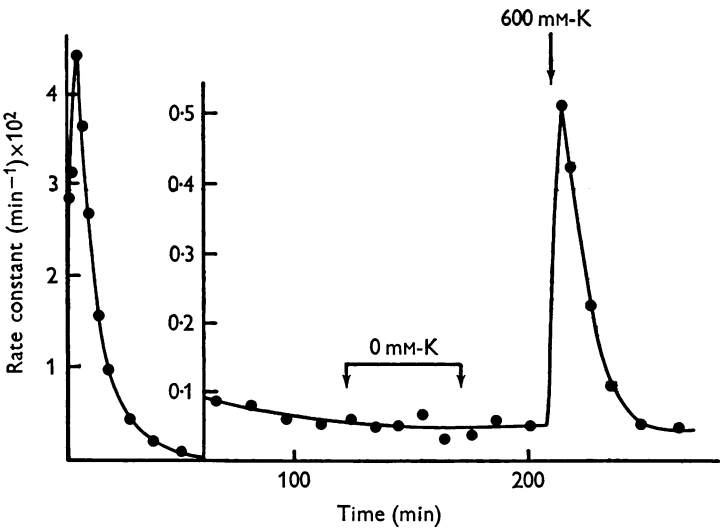

Fig. 5. The effect of 0 mM potassium saline and 600 mM potassium chloride on the residual calcium efflux from a *Maia* fibre. Mean resting potential −54 mV. Wt./surface area: 25·4 mg/cm². Temp. 19° C.

potassium-free medium has no effect on the calcium efflux and caused no obvious mechanical responses. On the other hand immersion of the fibre for 20 sec in a potassium chloride solution (600 mM) roughly isotonic with crab haemolymph caused an immediate contraction and a very sharp but temporary rise in calcium efflux, the increase being about 10 times over the residual level. The depolarization caused by high potassium concentrations leads to a release of calcium into the sarcoplasm (Ashley & Ridgway, 1970) which causes contraction and this is reflected in the increased calcium efflux.

Increases in external potassium insufficient to cause significant mechanical responses can, nevertheless, increase the heat production of muscle (Solandt, 1936). Fig. 6 shows the results of an experiment in which a fibre was exposed to raised external potassium concentrations of 20, 40 and 60 mM. It will be seen that appreciable rises in the calcium efflux took place even though there was no definite mechanical responses. These rises presumably reflect a rise in sarcoplasmic calcium insufficient to cause a distinct contraction, but sufficient to stimulate metabolism.

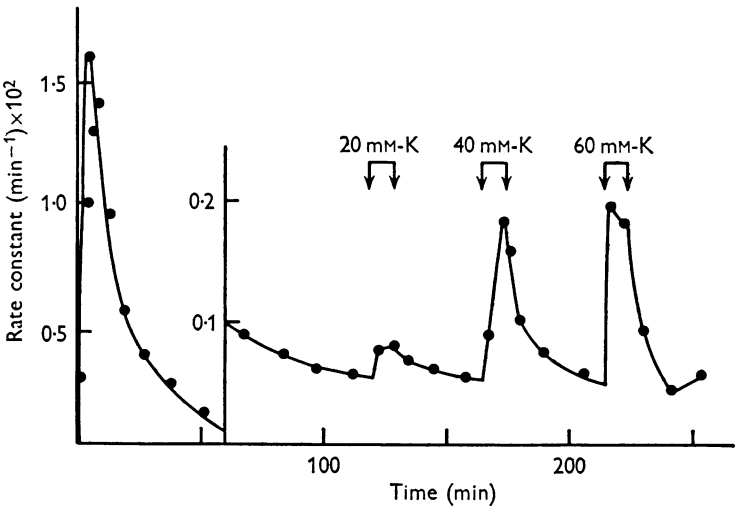

Fig. 6. The effect of 20, 40 and 60 mM potassium salines on the residual calcium efflux from a *Maia* muscle fibre. Mean resting potential −47 mV. Wt./surface area: 42·7 mg/cm². Temp. 17° C.

Treatment of the fibres with external potassium concentrations insufficient to cause contraction appears to reduce or abolish the ability of the fibre to contract when the potassium concentration is raised further to a concentration which normally causes contraction. Fig. 7 shows the calcium efflux in an experiment in which the fibre was exposed first to a raised external potassium concentration of 40 mM and then briefly (1 min) to a concentration of 600 mM. During the first treatment with 40 mM external potassium the efflux first rose some 2·5 times, but there was no detectable contraction. The efflux then fell away until it eventually reached a much lower value than the normal resting efflux. Treatment of the fibre some 10 min later with 600 mM external potassium failed to cause a contraction or a rise in calcium efflux. On transfer to normal crab saline, the efflux rose towards its normal resting value. An effect similar to that produced by an external potassium concentration of 40 mM could also be produced by treatment of the fibre with caffeine saline at a concentration

(0·5 mM) which is also insufficient to cause contraction (see Fig. 8). The 0·5 mM caffeine saline caused a small rise in efflux which dies away, but no detectable contraction could be observed. If the fibre was now immersed in 600 mM potassium chloride for 30 sec neither a contraction nor an increase in calcium efflux was seen. The effects of a higher concentration of caffeine (2 mM) on the calcium efflux from a fibre previously treated with

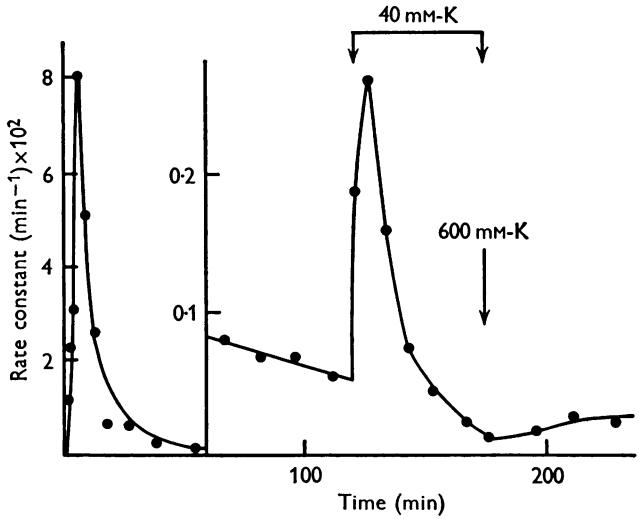

Fig. 7. The effect of 40 mM potassium saline and 600 mM potassium chloride on the residual calcium efflux from a *Maia* fibre. Mean resting potential −50·5 mV. Wt./surface area: 29·5 mg/cm². Temp. 20° C.

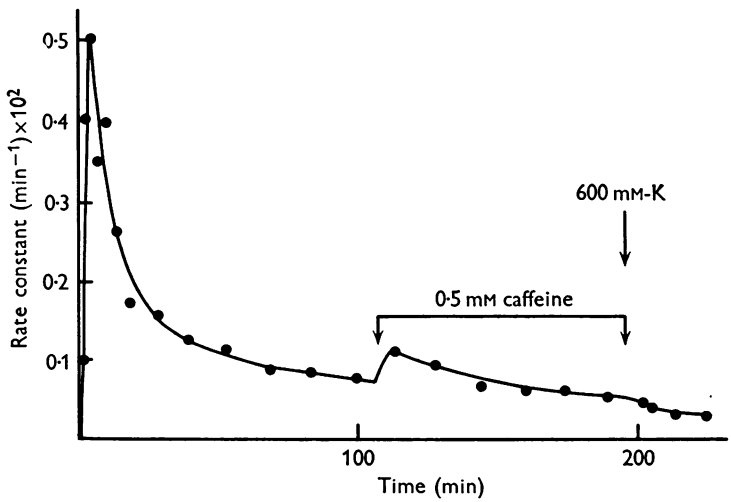

Fig. 8. The effect of 0·5 mM caffeine saline and 600 mM potassium chloride on the residual calcium efflux from a *Maia* muscle fibre. Mean resting potential −42·5 mV. Wt./surface area: 35·8 mg/cm². Temp. 18° C.

external potassium at a concentration of 40 mM were also investigated. These are shown in Fig. 9. It can be seen that the 2 mM caffeine caused some rise in calcium efflux, but much less than that observed in the experiment in Fig. 3. Contraction was observed but this took about 5 min rather than a few seconds to develop.

It seems that in the experiments shown in Figs. 6–9, a 4–6 times increase in the external potassium or treatment with 0·5 mM caffeine causes a stimulation of the calcium-release mechanism in the fibre, but that the

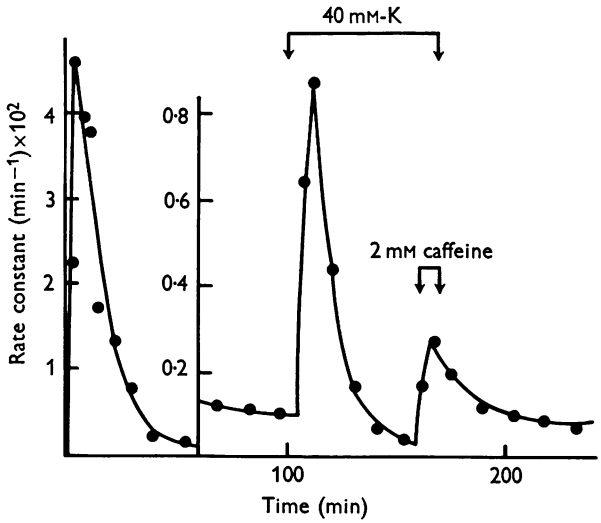

Fig. 9. The effect of 40 mM potassium saline and 2 mM caffeine saline in 40 mM potassium saline on the residual calcium efflux from a *Maia* fibre. Mean resting potential −51 mV. Wt./surface area: 31·2 mg/cm². Temp. 17° C.

calcium released is insufficient to cause a significant contraction. Some part of the release mechanism becomes exhausted and the calcium efflux falls. As a result of this exhaustion subsequent treatment of the fibre with 600 mM potassium chloride or 2 mM caffeine causes either no calcium release, no increase in efflux and no contraction (Figs. 7, 8) or else a greatly impaired operation of these processes (Fig. 9).

Effects of a calcium chelating agent (EGTA) on the calcium efflux and contraction of Maia muscle fibres

Ethylene glycol-*bis*-(β-amino-ethyl ether)-N, N′-tetra-acetic acid (EGTA) chelates with calcium fairly selectively in the presence of magnesium, potassium and sodium. When EGTA is injected internally it chelates much of the fibre calcium and partially reduces the concentration of free ionized calcium (Ashley & Ridgway, 1970; Ashley, 1971). This reduction is

reflected in a decrease in the calcium efflux from the fibre (Fig. 12). Fig. 10 shows the effects of EGTA when it is injected into the fibre with the ^{45}Ca. In this case the ionized calcium concentration was about 2×10^{-8} M. No contraction was observed when this solution was injected, and the usual large transitory calcium efflux was absent, the efflux starting at the residual rate normally obtained at a later stage. Immersion of the fibre in saline containing 5 mM caffeine caused no visible contraction. Enough internal calcium was, however, released by the caffeine to cause an increase in the ionized calcium in the sarcoplasm, and this is reflected in a threefold rise in the calcium efflux, which is appreciably smaller than that normally caused by caffeine (Fig. 3). After removal of the caffeine the efflux declined and exposure of the fibre to 600 mM potassium chloride for 50 sec failed both to increase the efflux and to cause contraction.

Effect of high K and caffeine salines on the efflux of ^{45}Ca from Balanus muscle fibres

The time course for the loss of radioactive calcium from a barnacle muscle fibre is similar to that already described for *Maia*. The steady rate of efflux, characteristic of the resting state, is not achieved until 100–150 min after the injection (Fig. 11). This residual efflux was characterized by a rate constant of 1·7 (S.E. $\pm$ 0·14) $\times 10^{-3}$ min^{-1} (thirty-four fibres) at 20–25° C. If the free and bound calcium within the fibres are assumed to be in isotopic equilibrium, then the resting calcium efflux based upon the total fibre calcium concentration is in the range of 1–2 p-mole/cm^2.sec.

If a saline containing 200 mM potassium is applied to the fibre there is a contraction and generally an increased efflux of the isotope above the residual level (Ashley, 1967). In 11 fibres studied, five fibres showed no appreciable increase in efflux despite a vigorous contraction. In the remaining fibres the efflux increased and then decayed whilst the fibre remained depolarized. The highest values for the increased efflux (Fig. 11) were only some 2–3 times greater than the residual efflux (C. C. Ashley & J. C. Ellory, unpublished experiments). In *Maia* the values for the stimulated efflux were much higher, in the range of 10–15 times greater than the residual level.

Salines containing 5 mM caffeine also initiated a contraction and an increased efflux above the residual level. The peak values for this increased efflux were again smaller than seen in the *Maia* preparation. Injections of 30 mM strontium chloride or 30 mM caffeine in distilled water also produced contractions and a stimulation of the calcium efflux.

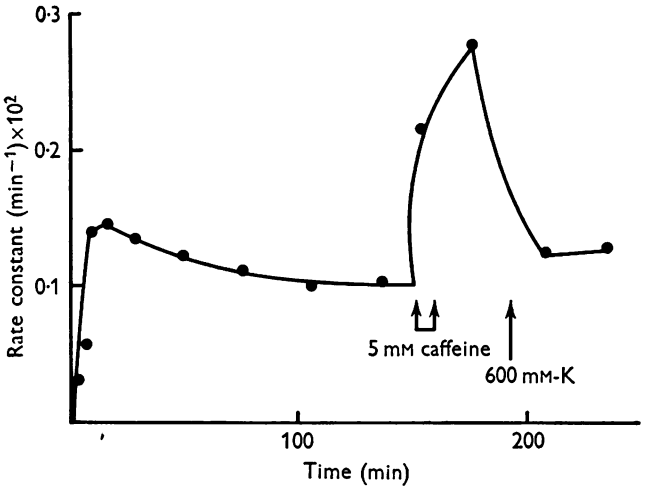

Fig. 10. The effect on the efflux of ^{45}Ca from a *Maia* fibre after the injection of a ^{45}Ca/EGTA buffer (*ca.* 2×10^{-8} M-Ca^{2+}) of 5 mM caffeine and 600 mM potassium chloride salines. Mean resting potential not recorded. Wt./surface area: 43·5 mg/cm². Temp. 16–22° C.

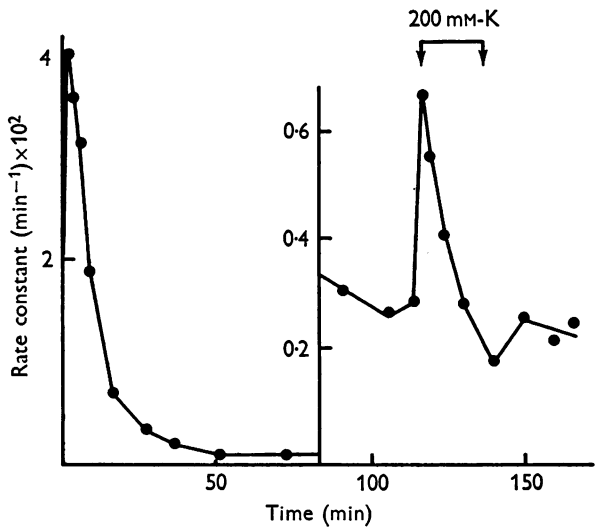

Fig. 11. The effect of 200 mM potassium saline on the residual calcium efflux from a *Balanus* fibre. The fibre gave a brisk contraction. Mean resting potential −41 mV. Fibre wt. 21·1 mg. Temp. 23–24° C.

Effect of the injection of EGTA on the residual ^{45}Ca efflux from Balanus muscle fibres

In Fig. 12 the time course of the efflux of radioactive calcium from a barnacle muscle fibre injected with 200 mM-EGTA (potassium salt) (pH 7·1) is shown. There is an immediate fall in the efflux following the injection, followed by a slow increase during the subsequent 20–30 min until the efflux approaches its initial value. In the six fibres investigated, the efflux was not reduced more than twofold after injection of the calcium-binding agent.

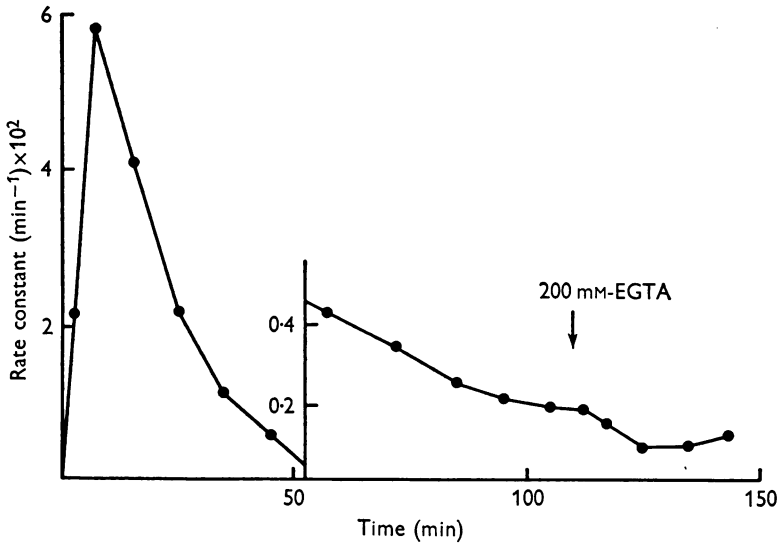

Fig. 12. The effect of the injection of 200 mM-EGTA on the residual calcium efflux from a *Balanus* muscle fibre. Mean resting potential −50 mV. Wt./surface area: 22·4 mg/cm^2. Temp. 16–22° C. Estimated EGTA concentration after fibre dilution *ca.* 3–4 mM.

The final concentration of EGTA assuming that it was distributed uniformly throughout the fibre was 3–4 mM. The finding that the rate constant for the efflux after some 20–30 min was similar to the initial value is in agreement with the findings for *Maia*, shown in Fig. 10, where an initial injection of a calcium-EGTA buffer solution containing ^{45}Ca produced an initial rate constant which was virtually identical with the residual efflux.

DISCUSSION

The loss of ^{45}Ca injected as calcium chloride into single *Maia* and *Balanus* muscle fibres does not follow first order kinetics in the period immediately following the injection, although it does so eventually when a slow residual rate of loss is reached. This is similar to the loss of injected radioactive strontium (Ashley, 1967), but is in contrast to the loss of injected radioactive sodium (Bittar *et al.* 1967), potassium (Lowe, 1964; Brinley, 1968) and magnesium (Ashley & Ellory, 1971*a*, *b*) which tend to follow first order kinetics more closely. On the other hand the loss of ^{45}Ca does follow first-order kinetics if it is injected as a complex with EGTA so that the concentration of ionized calcium introduced into the sarcoplasm is very small. A further point is that first order kinetics are found for the loss of ^{45}Ca injected as calcium chloride into squid giant axons (Blaustein & Hodgkin, 1969) which lack many of the structures found in muscle fibres.

In resting muscle fibres the sarcoplasmic calcium concentration is maintained at a value of $< 0{\cdot}3\ \mu M$ (Portzehl, Caldwell & Rüegg, 1964) mainly as a result of the calcium binding activity of the sarcoplasmic reticulum (Ebashi & Lipmann, 1962; Hasselbach & Makinose, 1961, 1963). The concentrations of the calcium chloride in the injected ^{45}Ca solutions were sufficient to raise the average concentration in the sarcoplasm of *Maia* and *Balanus* fibres by 0·3–0·5 mM. The fibres contracted for a period of 2–3 min as a result of this high sarcoplasmic concentration and then relaxed. Since the sarcoplasmic calcium must be reduced below about $0{\cdot}3\ \mu M$ for relaxation to take place (Portzehl *et al.* 1964) most of the injected calcium must have been removed from the sarcoplasm during the 2–3 min period following the injection. In support of this, work with the calcium-sensitive photoprotein, aequorin, has shown that calcium liberated by a tetanus is apparently removed from the sarcoplasm within 1 min of stopping stimulation (Ashley & Ridgway, 1968, 1970).

The behaviour of the efflux of ^{45}Ca following its injection suggests that it is removed from the sarcoplasm into two distinct compartments of the fibre during the initial contraction. About 60% of this ^{45}Ca is probably taken up by that part of the sarcoplasmic reticulum which has no direct connexion with the external medium, in particular the longitudinal sarcoplasmic reticulum. It is the later loss of this ^{45}Ca via the sarcoplasm which would give rise to the eventual slow residual efflux. The remaining 40% is removed from the sarcoplasm into regions of the fibre from which it can pass to the external medium, though with some delay. Although some of this loss will occur relatively rapidly across the surface membrane, most of it must be via other routes. It is likely that the structures involved in the delay are those associated with the outer membrane which have

already been suggested as being involved in certain effects found with the sodium efflux (Bittar *et al.* 1967). The structures most likely to be involved are the system of large invaginations of the surface membrane, the cleft system, which is estimated in *Balanus* to have an area 14–15 times greater than the surface membrane itself (Selverston, 1967). Also likely to be involved is the transverse tubular system which in frog muscle has an area 5–7 times the surface (Peachey, 1965). The main ^{45}Ca efflux is most probably through the membranes of the cleft system. The lumina of the clefts are extremely tortuous and filled with electron dense material and both factors could well delay the loss of ^{45}Ca to the external medium. This system is more extensive in *Balanus* fibres and this may account for the fact that the diffusion delays observed are often larger than those in *Maia*.

Various stimuli which cause contraction increase the efflux of ^{45}Ca above the residual level. In each case the increased efflux takes some time to decay after the stimulus and contraction have ceased. Here too the effects of a delayed diffusion through the cleft system seem to be apparent. The magnitude of the calcium efflux depends on the intensity of the stimulus and must bear some relationship to the internal calcium changes. There appear, however, to be differences between the two muscle fibre preparations. In *Maia* the maximum increase in the efflux as a result of stimulation by caffeine or high potassium is some 10–15 times greater than the residual efflux, while in *Balanus* it is only 2–3 times greater. Studies with aequorin have shown that in both *Maia* and *Balanus* fibres intense stimulation increases the concentration of calcium in the sarcoplasm from about 0·1 to 2 μM (Ashley, 1970*a*, 1971). When the stimulus is less intense the increase is smaller. It appears therefore that the calcium efflux from *Maia* fibres is proportional to the changes in internal calcium, at least in magnitude if not in time course (Caldwell, 1972). The smaller than expected increases in calcium efflux from *Balanus* fibres during intense stimulation may be in part due to a greater delaying effect of the more highly developed cleft system.

In experiments where either caffeine or calcium were injected into *Maia* fibres the resulting contraction was associated with an increased efflux of the isotope. In the case of the caffeine injection this result indicates that caffeine can release calcium from the sarcoplasmic reticulum into the sarcoplasm and this calcium then initiates a contraction. This has support from the finding that the contraction induced by a caffeine injection can be blocked by a simultaneous injection of approximately equimolecular proportions of the chelating agent EGTA (Ashley & Caldwell, 1964; Ashley, 1970*b*). The fact that injection of calcium also stimulates the increased efflux of ^{45}Ca as well as causing contraction can be interpreted most simply as follows. The injected calcium acts by stimu-

lating the additional release of radioactive calcium from the sarcoplasmic reticulum. This idea has some support from recent work on single skinned frog muscle fibres (Endo, Tanaka & Ogawa, 1970; Ford & Podolsky, 1970). Such an explanation could also be applied to the experiments where injections of strontium ions into *Balanus* fibres caused contraction and an increased efflux of ^{45}Ca.

The results obtained with increased concentrations of potassium insufficient to cause contraction are relevant to the observation of Solandt (1936) that an increase of external potassium to 3–10 times above that in Ringer solution increased the resting heat production of frog muscle by up to 20 times. The increased calcium efflux observed in the present work when the external potassium is increased slightly suggests that the increased heat production observed by Solandt may be associated with a release and subsequent turnover of calcium which is insufficient to cause a detectable contraction. Some comment is necessary on the fact that in both the high and raised potassium salines the increased efflux was transitory. One explanation would be that the calcium release process from the sarcoplasmic reticulum was subject to inactivation or fatigue. A relevant observation is that the process of calcium entry across the outer membrane of the giant axon of the squid in response to high potassium salines followed a phasic time course (Baker, Meves & Ridgway, 1971). This process may well be related to the mechanisms which govern the release of calcium from the sarcoplasmic reticulum in muscle.

It is possible that part of the calcium released into the sarcoplasm in response to the raised potassium salines may well be taken up into some fibre compartment where it is no longer available for release on further stimulation, although caffeine may be able to bring about a slow release. This idea could explain the observation that no mechanical response or increase in calcium efflux was observed when fibres immersed for a time in 40 mM potassium were later immersed in 600 mM potassium. The idea that there is a compartment in muscle which can take up calcium but which does not release it on stimulation has been put forward by Weber, Herz & Reiss (1964) to account for fatigue. This compartment might be the mitochondria, but there are comparatively few of these in *Maia* and *Balanus* muscle, and it seems likely that some other part of the sarcoplasmic reticulum is involved (Winegrad, 1968; Connolly, Gough & Winegrad, 1971).

In experiments in which EGTA was injected into single fibres from both *Balanus* and *Maia* the residual calcium efflux was lowered by up to about half its usual value. It then gradually recovered over a period of about 30 min. The injection of EGTA should immediately lower the concentration of calcium ions in the sarcoplasm by roughly 100 times to about 1 nM. Since the efflux is cut by no more than a factor of 2, it seems likely

that the sarcoplasmic reticulum must release calcium ions into the sarcoplasm extremely rapidly after the EGTA injection to restore the calcium ion concentration there to near its initial value. It would, for example, be necessary to release 1·5 mM calcium, to bring the sarcoplasmic calcium ion concentration to about 0·1 μM if the sarcoplasmic EGTA concentration were 3 mM. Evidence that such a release takes place has been obtained from work with the photoprotein aequorin (C. Ashley, unpublished experiments) which has shown that the sarcoplasmic calcium ion concentration only falls by a factor of 2 or 3 after injections of EGTA, and not by a factor of 100. Aequorin has also shown that in the 30 min following the EGTA injection the sarcoplasmic calcium ion concentration, like the efflux, gradually increases towards its initial value.

The behaviour of the calcium efflux from *Balanus* and *Maia* muscle fibres injected with EGTA is in marked contrast to that from squid giant axons. Here the injection of similar concentrations of EGTA lowers the rate constant some hundred times from its resting value. This suggests that there is little readily releasable calcium in nerve (see Blaustein & Hodgkin, 1969).

When ^{45}Ca is injected with EGTA, the initial rapid loss of isotope is not observed, and the efflux rises to a value close to the residual calcium efflux. If the injected ^{45}Ca equilibrates with the calcium in the sarcoplasmic reticulum, stimulation would be expected to cause an increased loss of ^{45}Ca from the fibre. This was observed in response to a 2 mM caffeine saline, but there was no contraction. This is reflected in a rise of only twofold in the calcium efflux, compared with ten- to fifteenfold in the absence of EGTA. This experiment confirms the idea that the sarcoplasmic reticulum can release or remove sufficient calcium so that the final free sarcoplasmic concentration is similar to that before the injection. In the presence of concentrations of EGTA in the millimolar range an appreciable fraction of the fibre calcium will be as a calcium EGTA complex in the sarcoplasm (Ashley, 1971). When a subsequent stimulus activates calcium release from the sarcoplasmic reticulum, there is sufficient to increase the free calcium by only some 2 times. These efflux experiments interpreted simply suggest a discrete intracellular sarcoplasmic reticulum calcium store available for contraction. Similar results have been obtained in experiments where the contraction responses of these fibres are suppressed by progressive increases in EGTA internally (Ashley *et al.* 1965; Ashley, 1965, 1967).

In conclusion, one or two general observations can be made about the residual efflux from *Maia* and *Balanus* muscle fibres. Its lack of sensitivity to ouabain and to the removal of external potassium suggests that it is not directly linked to a coupled sodium-potassium pump. Like the calcium

efflux from squid giant axon, however (Baker, Blaustein, Hodgkin & Steinhardt, 1967; Blaustein & Hodgkin, 1969) it is sensitive to the removal of external sodium ions and appears to involve an appreciable sodium: calcium exchange (C. C. Ashley & J. C. Ellory, unpublished experiments).

It is perhaps instructive to compare the calcium fluxes in *Maia* and *Balanus* with those in frog muscle and squid axon (all at 17–25° C), bearing in mind that the fluxes for muscle, although calculated for the surface membrane, include considerable components across the internal structures. For *Maia* the efflux is 0·4 p-mole/cm^2.sec at rest; for *Balanus* it is 1–2 p-mole/cm^2.sec; for squid giant axon it is 0·2 p-mole/cm^2.sec (Blaustein & Hodgkin, 1969); while for frog muscle the resting calcium influx is 0·07 p-mole/cm^2.sec (Bianchi & Shanes, 1959). The calcium efflux found during a short contraction of a *Maia* muscle has been found to be in the region of 0·5–0·6 p-mole/cm^2, while Shanes & Bianchi (1960) found a minimum loss of 0·2 p-mole/cm^2 per twitch for frog muscle.

Part of this work was performed during the tenure of a grant from the Medical Research Council.

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