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CALCIUM INFLUXES AND TENSION DEVELOPMENT IN PERFUSED SINGLE BARNACLE MUSCLE FIBRES UNDER MEMBRANE POTENTIAL CONTROL

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SUMMARY

1. Single giant barnacle muscle fibres from *Megabalanus psittacus* (Darwin) were used to measure the Ca entry and the development of tension in the fibres under membrane potential control.

2. Fibres bathing in 60 mM-$MgCl_2$ sea water, free of Ca, did not develop tension with sudden displacements of the membrane potential towards more positive values. This failure to develop tension with depolarizations was observed with and without the internal application of Ca buffers.

3. Fibres bathing in artificial sea water with either 10, 20, 60 or 100 mM-$CaCl_2$ developed tension with depolarization even after 60 min of internal perfusion of the fibres with solution containing no Ca buffers. In this case the maximum tension recorded during a voltage clamp run decreased with time from nearly 2·5 to 0·2 kg/cm^2. However, addition of 10 mM-Tris-EGTA (ethyleneglycol-bis (β-aminoethyl ether) N, N' – tetra-acetic acid) to the perfusing solution rapidly eliminated the development of tension; after 10 min of internal perfusion with Ca buffers no tension could be elicited by electrical stimulation.

4. Ca-influx determinations were carried out only in the fibres in which the outward K^+ currents were blocked by internal application of TEA (tetra-ethylammonium). The ratio of 'measured extra Ca influx/computed ionic flux of divalent cations during the inward current' was $1{\cdot}06 \pm 0{\cdot}41$.

5. For fibres bathed in either natural sea water or in artificial sea water with various concentrations of Ca, the temporal course of development of isometric tension was similar to the temporal course of the integral of the inward current due to Ca^{2+}.

6. In a fibre from *M. psittacus* bathing in natural sea water the calcu-

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lated extra entry of Ca required to increase its internal concentration to about 50 μM was 500 p-mole per depolarization (60 mV); while the corresponding average influx calculated from the inward current record in natural sea water is 474 p-mole.

7. Evidence was obtained for the accumulation of Ca in an internal compartment.

INTRODUCTION

It is generally agreed that Ca ions play a key role in the generation and control of contraction in muscles (Huxley & Huxley, 1964; Ebashi & Endo, 1968; Ebashi, Endo & Ohtsuki, 1969). The experimental evidence suggests that during the development of tension there is a transient increase in the concentration of sarcoplasmic Ca^{2+} (Jöbsis & O'Connor, 1966; Ashley & Ridgway, 1970). This increase may be due to a release of Ca^{2+} from the sarcoplasmic reticulum (SR), as in the vertebrate twitch fibres, or possibly, at least in part, a direct movement of Ca across the sarcolemma and the transverse tubules in response to depolarization, as in heart muscle (Niedergerke, 1963*a*, *b*; Beeler & Reuter, 1970*a*, *b*, *c*), slow striated fibres of vertebrates and many crustacean muscles (see review by Ebashi *et al.* 1969).

Ashley & Ridgway used the Ca-induced luminescence of aequorin (Shimomura, Johnson & Saiga, 1962) to monitor the changes in concentration of free calcium during a single twitch in a barnacle muscle fibre (Ashley & Ridgway, 1970). Although this experiment provided clear evidence for the existence of a transient increase in the concentration of Ca^{2+} during a twitch, it cannot be used to determine to what extent this increase in calcium concentration was due to a direct inward movement of Ca^{2+} across the sarcolemma or tubules in response to depolarization. Hagiwara & Naka (1964) measured an extra influx of Ca of 35–85 p-mole/cm^2 per action potential in muscle fibres from barnacle bathed in a saline which contained 20 mM-$CaCl_2$. According to Ashley & Ridgway (1970) this influx of Ca would not be sufficient to account for the transient increase in internal Ca observed in their experiments with aequorin. It is possible, however, that the longer membrane depolarization which they used may have been more like a series of action potentials so that the Ca entry was in any case greater.

In a previous paper (Keynes, Rojas, Taylor & Vergara, 1973) we have shown that the total ionic current which flows across the membrane of a barnacle muscle fibre after a step change in potential in a voltage clamp experiment can be resolved into a sustained inward current independent of a later outward current, also sustained. The method which led to this separation of the currents was the replacement of the Ca of the sea water

by Mg^{2+}, the result of which is the disappearance of the inward currents from the current records. The aim of the present research has been to measure directly, with tracer Ca the net movement of Ca in a voltage clamp experiment and to compare this Ca flux with that calculated by integration of the ionic current.

Our results strongly suggest that the inward current is in fact carried by Ca^{2+}. For a 60 mV depolarizing voltage clamp pulse an average total extra-influx of Ca per pulse of 652 p-mole has been measured from a 60 mM-$CaCl_2$ saline free of Na and Mg.

We have also recorded and measured the development of tension caused by a sudden depolarization of the membrane under potential control. Our results show a linear relationship between $\int_0^t i_{Ca}\,dt$ and tension where i_{Ca} is the total inward Ca current. This result was obtained with fibres bathed in either natural sea water or artificial sea water with various concentrations of Ca.

METHODS

Large specimens of the barnacle *Megabalanus psittacus*, readily available on the coast of Chile, were used in this research. A detailed description of the combined perfusion technique and voltage clamp procedure used can be found in the preceding paper (Keynes *et al.* 1973).

Measurement of tension

As a general procedure, the fibres were bathed in a 60 mM-$MgCl_2$ sea water containing no Ca and were internally perfused with a K-aspartate solution for a few minutes (see Table 1 for the composition of the solutions used); then, the tendon was attached to the shaft of a mechano-electronic transducer (5734 RCA) which was mounted on a micro-manipulator. The resting length in this solution was taken into account to adjust the output of the transducer to zero. Then the external solution was replaced by a sea water with Ca (see Table 1). On several occasions the resting tension slowly increased during the experiment. The transducer was calibrated on several occasions and found to be fairly linear up to 10 g using the whole length of the shaft. It was usually necessary to connect the string at shorter lengths in order to get higher ranges.

Measurement of membrane current

Currents were radially applied from an internal spiral electrode to a pair of external platinum plates coated with black platinum. These were electrically connected to a current–voltage amplifier in a virtual-earth configuration. The output of the current amplifier was recorded on film. This system of external plates measures the total membrane current

$$I_m(t) = \int_0^L \frac{\delta I_m(x, t)}{\delta x}\,dx,$$

where L is the length of the fibre bathed in sea water.

The uniformity of the current density, $\delta I_m(x, t)/\delta x$, was controlled once with a pair of differential C-shaped electrodes which recorded the voltage drop in the resistance of the external sea water and which could be moved along the fibre axis.

Measurement of the Ca influx

In previous publications (Atwater, Bezanilla & Rojas, 1969; Bezanilla, Rojas & Taylor, 1970) we have described experiments in which sodium influxes were measured in a squid giant axon during rest and during voltage clamping. The same methods were employed during the present work. Tracer flux measurements were carried out with either TEA in the internal solution or Cs^+ in place of K^+. TEA blocks the outward currents and Cs^+ is impermeable; this makes possible a more accurate measurement of the inward currents (Keynes *et al.* 1973).

TABLE 1. Composition of the solutions used for internal and external perfusion

A. Internal solutions (mM)	K^+	Cs^+	Tris-Cl	Tris-EGTA	TEA-Cl	Sucrose
1*a* K aspartate	180	—	5	—	—	600
1*b* K aspartate plus EGTA	180	—	5	5, 10 or 20	—	600
1*c* K aspartate plus TEA	180	—	5	—	60	600
1*d* K aspartate plus TEA plus EGTA	180	—	5	10	60	600
1*e* Cs-aspartate plus EGTA	—	180	5	20	—	600
2*a* K acetate	200	—	—	—	—	800
2*b* K acetate plus TEA	200	—	—	—	60	600
2*c* K acetate plus TEA	100	—	—	—	100	600
2*d* Cs acetate	—	180	—	—	—	600
B. External solutions (mM)						
Sea waters	K^+	Na^+	Ca^{2+}	Mg^{2+}	$Tris^+$	pH
7 O-Ca ASW	10	430	—	60	5	7·5
7*a* ASW	10	430	10	50	5	7·3
7*b* K-free ASW	—	440	10	50	5	7·3
7*c* 20 Ca ASW	—	430	20	50	5	7·0
11*a* 60 Ca ASW	—	430	60	—	5	7·0
11*b* 100 Ca ASW	—	310	100	—	5	7·0
Salines						
8 Na and Ca-free, Mg saline	10	—	—	60	435	7·5
9*a* Na and Mg-free, Ca saline	10	—	60	—	435	7·3
9*b* Na and Mg-free, high Ca saline	10	—	100	—	310	7·0

Tonicity of internal solutions ranged from 910 to 1080 m-osmole. ^{45}Ca was added to solution 9*a* to give an average activity of 300,000 c.p.m./μl. The isotope was a carrier-free sample and the concentration of Ca was 60 mM.

There are some possible errors in the measurements of the currents: (1) the separation of the capacitative transient from the ionic currents at the beginning and at the end of the pulse; (2) the estimation of the leakage currents during the pulse and (3) the estimation of the remaining outward K current.

The ^{45}Ca influx as a function of time was measured in the following manner. About 5–10 min after the onset of perfusion, the external solution was replaced by a calcium saline containing ^{45}Ca (solution 9*a*, Table 1). Perfusate samples were

collected every 1 or 2 min. The volume of collected perfusate ranged from 0·08 to 0·4 cm^3 depending upon the rate of internal perfusion. The rate at which ^{45}Ca appeared in the perfusate reached steady value after no more than 20 min. Once this resting level of calcium inflow was reached, the measured resting potential was clamped, and then a few pulses of membrane depolarization were applied at a frequency of 0·1 pulses/sec.

Simultaneous recordings of membrane potential, membrane currents and development of tension

The intracellular perfusion method used in this work is adequate to study the electrical properties of the membrane system of the barnacle muscle fibres. By this procedure one can manipulate easily the internal ionic composition and measure the effects on potentials and currents during voltage clamp as the fibres remain in good condition and sustain electrical activity for several hours (Keynes *et al.* 1973). However, to study muscle contraction, perfused fibres are not ideal. We encountered several difficulties which need to be discussed.

During intracellular perfusion, drastic changes in the fibre take place. First, there is a rapid increase in volume (increasing the diameter 3 to 4 times), which reaches, however, a steady state in which no further changes in volume are noticeable. During this increase in fibre size, the gross organization (myofibrils) as examined with the polarization microscope appeared unmodified (Vergara & Rojas, 1971). However, this continuous intracellular perfusion removed several intracellular substrates. The temporal course of the removal of some substrates seems to be exponential and the time required to reduce the initial concentration inside the fibre to one half is a function of the intracellular perfusion rate. For example, in experiments in which the ATP, the N-phosphoryl arginine (PArg.) and P_i contents of the perfusate were measured in order to determine the time course of removal of the ATP, PArg. and P_i, it was found that, for an internal perfusion rate of 0·0206 cm^3/min, the initial ATP, PArg. and P_i content dropped to one half in about 60 min. When the internal perfusion rate was 0·051 cm^3/min this time was only 20 min. From these wash out curves and from the ATP, PArg. and P_i content of the fibre after it had been perfused, the initial ATP, PArg. and P_i content could be calculated and were found to be about 1·5 m-mole for ATP, 14·5 m-mole for PArg. and 25 m-mole for P_i per kg of wet tissue (Nassar & Rojas, 1973).

The enzymatic activity of the structural proteins is a function of the concentration of Mg^{2+} and Ca^{2+} and ATP (Davies, 1963; Ebashi & Endo, 1968; Dreizen, Gershman, Trotta & Stracher, 1967; Hellam & Podolsky, 1969). In the present research we have used either 180 mM-K aspartate or 200 mM-K acetate plus sucrose at pH 7·3 as internal solution (see Table 1). The contractile machinery of the fibres operates even after 60 min of internal perfusion with these solutions but the maximum tension developed by the fibre decreases with time under perfusion.

RESULTS

Resting Ca influxes in perfused fibres

Fig. 1 shows the results of two experiments in which the temporal course of the resting influx was followed during about 4000 sec. This experiment was designed to determine the influence of internal application of Ca buffers and TEA upon the resting influx of Ca.

The experiment was begun (Fig. 1*A*) with perfusion with the usual

180 mM-K aspartate plus sucrose (solution 1*a* of Table 1). At time 0, the external saline was replaced by another of the same ionic composition but containing ^{45}Ca (solution 9*a* of Table 1). Fig. 1*A* shows that the resting inflow of radioactivity slowly increased until a steady level was reached. After this, the internal solution was replaced by another containing

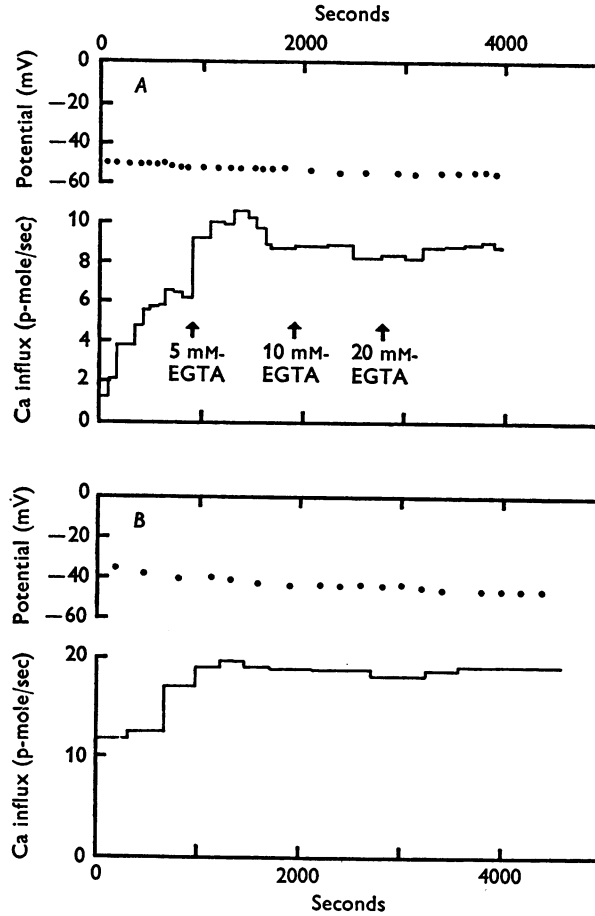

Fig. 1. Effects of EGTA and TEA upon the resting Ca influx. Part *A*: internal perfusion with K aspartate first, then with K aspartate plus various concentrations of EGTA (solutions 1*a* and 1*b* of Table 1) starting at the times indicated by the arrows. Note the transient increase with the first application of EGTA, then the steady state unchanged by further increases in the concentration of EGTA.

Part *B*: internal perfusion with K aspartate plus TEA plus EGTA (solution 1*d* of Table 1) continuously. Note that after about 700 sec, the steady-state influx is maintained and that it is higher than that without TEA.

TABLE 2. Resting Ca influxes in perfused barnacle muscle fibres

a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
Expt.	$[EGTA]_i$ (mM)	$[TEA]_i$ (mM)	Time to reach steady resting influx (sec)	Apparent surface (cm^2)	Resting potential (−mV)	Influx (p-mole/sec)	Influx per unit apparent area p-mole/(sec cm^2)	Influx per unit area p-mole/(sec cm^2)
AR 1	0	0	700	1·1	50·3 (5)	6·1 (5)	5·5	0·26
	5	0			51·0 (4)	9·3 (4)	8·5	0·39
	10	0			52·0 (7)	8·9 (7)	8·1	0·37
	20	0			52·6 (6)	8·9 (6)	8·1	0·37
				Ave. (22) ± S.D.		8·4 ± 1·4		
AMV 2	10	60	1000	1·8	41·6 (5)	18·8 (5)	10·4	0·48
AMV 1	10	60	700	1·4	27·1 (7)	28·6 (7)	20·4	0·94
				Ave. (12) ± S.D.		24·5 ± 5·1		
VR 2	10	25	1200	2·4	36·0*	15·9 (4)	6·5	0·31
VR 3	20	25	800	1·9	52·0*	42·8 (2)	21·9	1·00
				Ave. (6) ± S.D.		24·9 ± 14·0		
AR 4	0	20	900	0·4	49·0*	2·8 (3)	7·0	0·32
AR 6	0	20	1100	0·9	51·6(4)	15·2 (4)	16·2	0·75
				Ave. (7) ± S.D.		9·9 ± 6·7		
VR	0	0	600	1·6	20 (2)	32·5 (2)	20·3	0·94

( ): number of determinations used to calculate the average. *h*: influxes calculated with the data in column *g* divided by the apparent surface area of the fibre. *i*: Influxes calculated assuming that the calcium influx takes place across 21·6 cm^2 of membrane for every cm^2 of apparent surface. * indicates that the membrane potential was under control. ** In this experiment the internal solution was 180 mM-CsAsp. plus sucrose instead of the usual 180 mM-KAsp. plus sucrose. All experiments in this Table were performed at room temperature of about 19° C.

5 mM-Tris-EGTA, the remaining constituents of the perfusing solution being unmodified. As a result of this treatment there was a transient increase in the apparent influx from an average value of 6·2 p-mole/sec up to 10·3 p-mole/sec. After about 300 sec the influx decreased to another level of 8·6 p-mole/sec. Any further increase in EGTA content of the perfusing solution did not have any appreciable effect on the influx (see Fig. 1*A* for 10 and 20 mM-Tris-EGTA). One possible interpretation for the initial increase in the apparent influx is to attribute to EGTA a specific effect upon the Ca permeability. However, the initial increase could be due to the release of ^{45}Ca bound to intracellular binding sites other than the SR, caused by the presence of the chelating agent EGTA (Martell & Calvin, 1956). The transient nature of the increase argues in favour of the second alternative. Fig. 1 *B* depicts another typical experiment to illustrate the results when TEA is added to the perfusing solution. In general, fibres perfused with TEA have higher resting influxes (from Table 2, the average resting influx in fibres perfused with TEA is 16·5 p-mole/sec) and lower resting potentials than those fibres perfused without TEA (Lakshiminarayanaiah & Rojas, 1973).

The steady resting influxes (more than fifty determinations) were carried out in more than ten fibres, and the results are summarized in Table 2, which shows that fibres prefused with TEA (experiments AMV 2, AMV 1, VR 2, VR 3, AR 4, AR 6) have lower resting potentials and higher resting influxes than those fibres perfused without TEA (experiment AR 1). In an attempt to study the effects of the resting potential *per se* we measure the resting influx holding the membrane potential either at its initial value (before TEA had any depolarizing effect, as in experiment VR 3 and AR 4) or after the resting potential reached a steady value (as in experiment VR 2). It can be seen that in this last experiment the steady influx was lower than in fibre VR 3 in which the resting potential was kept at a relatively high value and in which the influx was rather low (2·8 p-mole/sec), indicating the influxes are quite variable and that the increase in the resting influx is not caused by the depolarizing effect of the TEA.

In order to obtain the influxes in Table 2 under column (*i*), we assumed that 1 cm^2 of apparent surface membrane (calculated considering the fibres as perfect cylinders of ellipsoidal cross-section) is equal to 21·6 cm^2 of external membrane. This includes areas of membrane in the clefts and in the tubules (Keynes *et al.* 1973).

Extra Ca influx during voltage clamp

Fig. 2 illustrates part of an experiment. It contains a plot of the Ca influx in p-mole/sec as a function of time in sec. Each column represents the average influx determined during an interval of time of approximately

200 sec. After the resting influx reached a steady level (about 16 p-mole/sec) the measured resting potential of -45 mV was clamped and 20 rectangular pulses of 50 mV (an absolute membrane potential, V_p, of $+5$ mV during the pulse) were applied at a frequency of 0·1 sec^{-1}. It can be seen that the net effect of these pulses is to increase the rate at which the Ca enters the fibre. The beginning and end of the voltage clamp period are indicated in the Figure with arrows. The flux data show a long delay in returning to the previous resting influx level. This could possibly be due partly to the time lag for the removal of the tracer from the dead space of the outlet cannula and the fibre and partly to the slow removal of

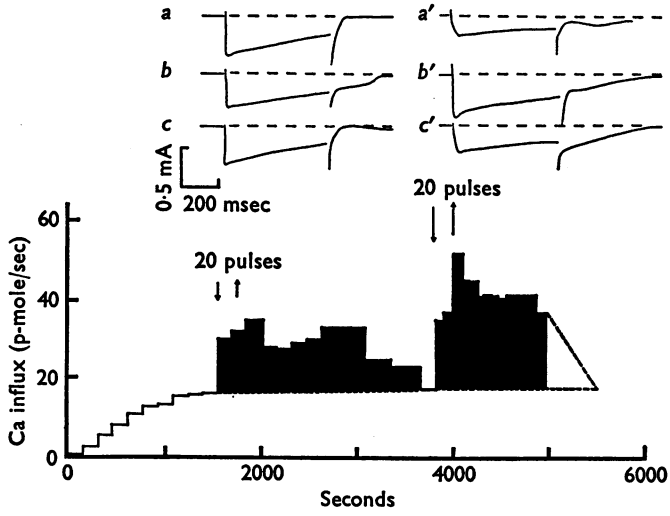

Fig. 2. Extra Ca influxes during voltage clamping. The upper part of the figure shows the current records for three of the twenty 50 mV voltage clamp pulses applied during the period indicated by the arrows. The area enclosed by each of the twenty current records was measured and the total compared to the currents represented by the extra influx (shown in black in the lower part of the Figure).

Ca bound to intracellular sites. The increase in Ca influx during the voltage clamp period is represented by the area enclosed by the columns above the resting influx level indicated. These areas are nearly 21,800 and 23,800 p-mole for the first and the second period of stimulation respectively. The dashed line in Fig. 2 has been drawn as a continuation of the resting influx before the clamp period. Three of the current records obtained during each of the two runs are shown above the corresponding extra Ca influx (*a*, *b*, and *c* for the first run and *a′*, *b′*, and *c′* for the second run), in the upper part of the Fig. 2. It may be noticed that the inward currents varied from pulse to pulse (compare for example record *a′* and *b′*).

TABLE 3. Determination of calcium influxes during voltage clamp

a	*b*	*c*	*d*	*e*	*f*	*g*	*h*
Fibre volume before perfusion (μl.)	Total extra entry (p-mole)	Extra influx per pulse (p-mole/pulse)	Average calcium influx calculated from $I_{Ca^{2+}}$ (p-mole/pulse)	Transport number (c/d)	Fibre volume after perfusion (μl.)	Estimated maximum changes in $[Ca^{2+}]_i$ (μ-mole/l.)	Estimated maximum changes in $[Ca^{2+}]_i$ corrected for volume increase $g \times \left(\frac{f}{a}\right)$ (μ-mole/l.)
32·2	23,500	1175	1090	1·10	177·0	6·6	36·3
	25,000	1250	1150	1·10		7·1	39·0
14·3	5,250	350	—	—	78·5	4·4	24·2
	3,812	423	—	—		5·4	29·6
5·3	6,580	263	481	0·55	9·5	27·5	49·3
25·1	8,675	578	645	0·90	143·0	5·1	29·1
	6,640	166	184	0·90		1·5	8·5
20·1	5,080	1016	575	1·80	28·0	36·0	50·1
Average				1·06		11·7	33·3
± S.D.				0·41		12·7	13·6

a: this volume was calculated assuming that the fibres were perfect cylinders of ellipsoidal section; the diameters used to calculate this volume were measured before internal perfusion; only the length of the fibre facing the current electrodes was considered.

b–d: extra influx calculated from the inward current records, $I_{Ca^{2+}}$ as follows:

$$\frac{1}{2Fn}\sum_{k=1}^{n}\int[i_1(t)-i_2(t)]_k\,dt,$$

where $i_1(t)$ is the current measured with $[Ca]_o$ = 60 mM and $i_2(t)$ is the current recorded with $[Ca]_o$ = 0 mM.

On some occasions a sustained inward current which never returned to the base line during the cycle was obtained and this introduced some uncertainty in the value of the extra Ca influx calculated from the current record.

Table 3 summarizes the results obtained with five different fibres. The extra Ca influx is a function of time with dimensions of p-mole/sec

$$\Delta m^i_{\mathrm{Ca}} = \Delta m^i_{\mathrm{Ca}}(t).$$

Column *b* gives the values of the total Ca extra entry:

$$\int \Delta m^i_{\mathrm{Ca}}(t)\,\mathrm{d}t.$$

The numbers in column *c* represent the average of the integrated extra Ca influx over a few pulses, that is

$$\overline{\Delta m^i_{\mathrm{Ca}}} = \frac{1}{n}\int \Delta m^i_{\mathrm{Ca}}(t)\,\mathrm{d}t;$$

where n is the number of pulses during the stimulating period. Column *c* gives then the extra Ca entry per cycle. Column *d* gives the average of the ionic entry computed from the current records during and after the voltage clamp pulses. Column *e* shows the ratio of the values under columns *c* and *d*, i.e. *c*/*d*. These ratios represent the transport number of Ca ions during the inward current. Column *f* gives the calculated volume of the fibre in the chamber. These volumes can be used to calculate the maximum transient increase in the internal concentration of Ca during each step depolarization. The calculated increase in internal Ca concentration after inflation of the fibre due to the continuous intracellular perfusion is given under column *g* in μM. The values given under column *h* were calculated using the measured increase in volumes as a consequence of the perfusion and represent the change in concentration of Ca that would have occurred if the fibre had not been inflated.

The conclusion that follows from the data presented in Table 3 is that the inward currents measured during the voltage clamp are in fact carried by Ca ions and thus substantiates the suggestion made in an earlier publication (Keynes *et al.* 1973). Consequently, in the following description we treat the inward current records (and their integrals) as representing inward movements of Ca ions. Also included in this Table 3 are the calculated possible changes in the Ca level in the sarcoplasm of the fibres. We should recall here that the extra influxes have been measured in fibres bathed in a saline free of Na and that this could modify the passive movements of Ca as in the Na–Ca exchange studied in other preparations (Baker, 1972).

Effect of voltage clamp pulses and 5 mM caffeine on the release of ^{45}Ca from internal sites

Although the results on extra Ca influxes suggest that the inward currents are carried by Ca and therefore that by measuring

$$1/(2F)\int i_{Ca}\,dt$$

one can estimate the Ca entry, we have no way to determine in these experiments whether or not the Ca currents induce an additional liberation of Ca from intracellular reservoirs such as the sarcoplasmic reticulum (SR) at the terminal sacs. We have carried out two different experiments which possibly indicate the contrary but they are not conclusive.

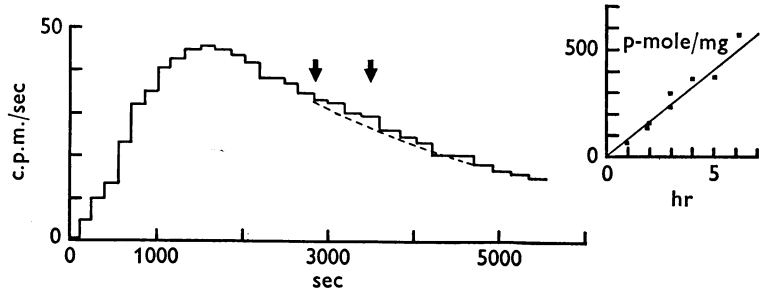

Fig. 3. Effect of voltage clamp pulses on the release of ^{45}Ca from internal sites. Fibre loaded with ^{45}Ca for 7·3 hr at 4° C. The external radioactivity was removed in part by soaking the fibre during 40 min in a 60 mM-$CaCl_2$ saline without ^{45}Ca. Fibre perfused with solution 1*c* of Table 1. Rate of perfusion was set at 20·6 μl./min. Resting potential decreased from −58 to −31 mV along the experiment. Holding potential set at measured resting potential of −43 mV during the application of the pulses. Temperature near 20° C. Insert: p-mole/mg wet weight of Ca taken up with time measured with ^{45}Ca.

Release of ^{45}Ca to the perfusate in fibres pre-loaded with ^{45}Ca

Single fibres were loaded with ^{45}Ca by immersion at various times in a 60 mM-$CaCl_2$ saline (solution 9*a* in Table 1) with ^{45}Ca (specific activity of this solution was 10·1 c.p.m./p-mole of Ca^{2+}). Next each fibre was rinsed in a large volume of the same solution but without ^{45}Ca for 30–45 min. Some fibres were used to estimate the Ca entry. In this case each fibre was weighed and following acid digestion, an aliquot was spread on a Teflon planchet, dried and counted. The results of these determinations were expressed in p-mole/mg wet wt. and have been plotted as a function of loading time in hours as shown in the insert of Fig. 3.

Other fibres were internally perfused as described in methods and the perfusate was collected in separate vials for counting.

Fig. 3 shows a wash out curve (internal ^{45}Ca being removed by the internal perfusing solution) obtained with a fibre which was pre-loaded with ^{45}Ca at 4° C for 7·3 hr. This fibre was perfused with 180 mM-K aspartate plus 600 mM sucrose plus 60 mM-TEA at a rate of 20·6 μl/min. The arrows indicate the period under stimulation with depolarizing voltage clamp pulses of 70 mV and 300 msec duration at a frequency of 1 pulse every 20 sec. Only visual inspection of the contraction was possible. The fibre was observed to contract every time a pulse was applied. 30 min before the period under stimulation, collection of samples of the external solution began. The external saline was replaced by 1 ml. of the same solution immediately after the collection of each sample of the perfusate. There was a slight increase in the radioactivity of the samples during the period under stimulation. Thus, before application of the pulses the fibre was losing radioactivity at a rate of 1·1 c.p.m./sec and during the period under stimulation the rate rose to 1·5 c.p.m./sec.

The ^{45}Ca wash out curve shows an initial rise, a peak value at 1600 sec and a monotonic decrease. A semilogarithmic graph of the radioactivity collected during the falling phase showed that the radioactivity removed from the fibre decreased exponentially. Both the time constant for the rising phase and for the falling phase varied from fibre to fibre. At the end of this experiment, that part of the fibre in the chamber was removed and used to measure the remaining radioactivity. For the experiment of Fig. 3 the remaining radioactivity was about 1060 c.p.m./mg. With this value and the wash out curve we can calculate the ^{45}Ca entry during the loading period as 8097 c.p.m./mg or 810 p-mole/mg.

The application of twenty-five depolarizing voltage clamp pulses induced a slight change in the temporal course of the wash out curve. To calculate the release of ^{45}Ca caused by the application of the pulses we assume that the temporal course of the wash out curve under resting conditions was exponential (dashed curve in Fig. 3). For the experiment plotted in Fig. 3 we obtain a total increase in the radioactivity of the samples of 2700 c.p.m.

The specific activity for the ^{45}Ca in the sarcoplasmic reticulum for the experiment in Fig. 3 is not known. Other experiments carried out to determine the loading time required to achieve equilibrium in the distribution of ^{45}Ca in the various compartments of the fibre showed that even after 7 hr of loading time equilibrium was not achieved. The small graph on the right side of Fig. 3 represents the Ca entry in p-mole/mg as a function of loading time for various fibres. It is apparent from this graph that the Ca entry is linear up to 7 hr of loading time at 4° C.

For the experiment in Fig. 3, after 7·3 hr of incubation in the ^{45}Ca saline, 218,619 c.p.m. entered the fibre (8097 c.p.m./mg × 27 mg). If we

assume that equilibrium of ^{45}Ca distribution had been reached after this time, it follows that the effective ionic concentration in the intracellular compartment should be

$$[Ca^{2+}]_i = ((\text{c.p.m.})_i/(\text{c.p.m.})_o) \times [Ca^{2+}]_o.$$

Since the volume of the fibre was about 27 μl. we calculate the effective $[Ca^{2+}]_i$ as 815 μM. This value is 8×10^3 times greater than the accepted level of internal ionized Ca and we conclude that a large fraction of the Ca entering the fibre is sequestrated by a system which prevents the ionized Ca from exceeding 0·1 μM.

A rough estimate of the Ca release can be obtained if we assume that the Ca available for exchange with extracellular Ca is distributed in several compartments and that one of these compartments, the Ca sequestering mechanism, is in isotopic equilibrium. Therefore, for every 10 c.p.m., 1 p-mole of Ca was liberated from the sequestering system (the external activity in the loading saline was 10 c.p.m./p-mole of Ca). During twenty-five twitches 270 p-mole of Ca had entered the perfusate from the entire fibre.

We have no way to identify the sites from which the ^{45}Ca found in the perfusate originated. If we assume that only the SR participated in this Ca exchange and if we even further assume that this compartment was in equilibrium with the loading solution, the SR contribution to the internal changes in ionic Ca level would be 10·8 p-mole/twitch. For the same experiment the external contribution,

$$1/(2F)\int i_{Ca}\,dt,$$

was 1080 p-mole/cycle (ranged between 855 and 1380 p-mole/cycle). It could be argued, however, that as a consequence of our perfusion procedure (no ADP, ATP and Mg^{2+} in the perfusate and other abnormal conditions) the mechanism for internal Ca release from the SR was rendered inoperative. In an attempt to resolve this question we performed a different experiment with a technique similar to that used by Hodgkin & Keynes (1953) to detect longitudinal diffusion of radioactive K in single giant nerve fibres.

Longitudinal spread of a ^{45}Ca-patch and mechanical activation induced by 5 mM caffeine in single fibres

A muscle fibre was spot micro-injected with ^{45}Ca along 1 mm at the centre of a zone delimited by two air bubbles (about 0·3 mm in diameter) placed 20 mm apart. One of these air bubbles was near the tendon end. The separation of the air bubbles was adjusted by slight stretching of the fibre; to stretch the fibre tension was applied with a torsion wire balance

attached to the chamber in such a way that the entire arrangement could be displaced horizontally under a gas flow detector (D-47 gas flow detector with a micromil window, Nuclear Chicago). By means of the slit-window counting method (Hodgkin & Keynes, 1953) the longitudinal distribution of ^{45}Ca could be followed in time. For each set of determinations, the solution bathing the fibre was removed during 2 or 3 min. The chamber was displaced with its axis at right angles with the 2 mm slit in front of the window of the gas flow detector. Measurements were made every mm. After a set of c.p.m.: distance (x) values (c.p.m. (x)) was recorded the 60 mM-$CaCl_2$ saline was reintroduced in the chamber and the fibre was

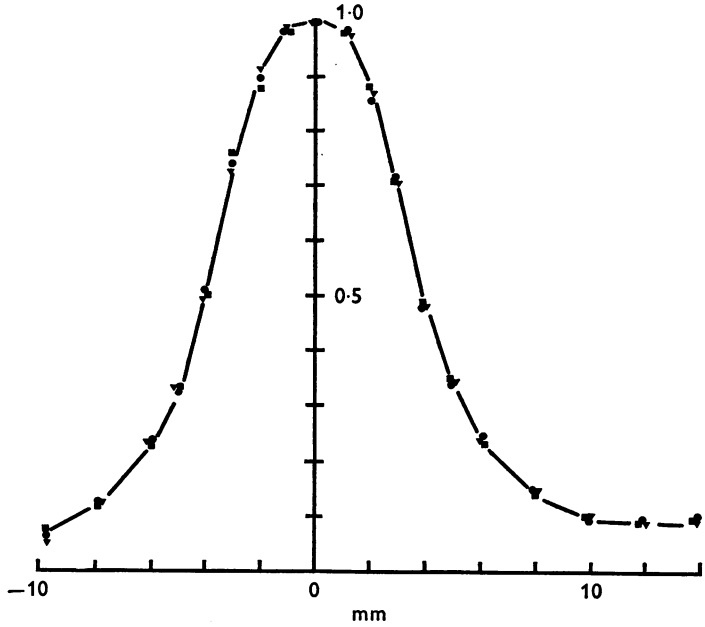

Fig. 4. Fibre cannulated and micro-injected with ^{45}Ca in a chamber similar to that described by Cohen *et al.* (1970) under dark field. The tip of the micro-injector was rinsed before insertion from the cannulated end. The external diameter of the micro-injector was 150 μm and was introduced up to 8 mm of the tendon end. The first air bubble was injected over 2 mm as the injector tip withdrew towards the cannulated end and then 1 μl. 180 mM-K acetate plus 600 mM sucrose plus 20×10^6 c.p.m. of ^{45}Ca (carrier free sample) were slowly delivered.

The experimental arrangement for counting radiation from the ^{45}Ca has been described (Rojas & Tobias, 1965) and was used here with only minor modifications.

Experiment carried out at room temperature. Diameter near the injected spot was 1·9 mm. ■ 10 min after injection; ● 35 min after injection; ▲ 65 min after injection.

kept in this solution during various times. Figure 4 shows the results of a typical series of determinations. The vertical axis represents the ratio

$$\text{c.p.m.}(x)/\text{c.p.m.}(x = 0)$$

where $x = 0$ was chosen as the horizontal position which gave the maximum c.p.m. The horizontal axis in Fig. 4 represents the lateral displacement, x, in mm. In nineteen of the twenty-seven experiments a single maximum was found; in twenty experiments the position of the spot where the ^{45}Ca was injected was almost at $x = 0$; in five experiments some tracer moved up the track left behind as the injector was withdrawn. In spite of these anomalies in the distribution curves, for each experiment the distribution of ^{45}Ca along the fibre did not change with time. For example, in Fig. 4 compare filled squares with filled triangles which were measured after 1·0 hr. Two contractures were induced with 5 mM caffeine dissolved in the same 60 mM-$CaCl_2$ saline. For the fibre of Fig. 4 the maximum tension developed during the contractures was 2·2 kg/cm^2 and the fibre shortened to about one third of its resting length and then returned to its original length when the caffeine was removed. Filled circles in Fig. 4 represent the distribution of ^{45}Ca after these contractures for an identical separation of the air bubbles. It can be seen that the spot did not spread.

The ^{45}Ca could have been bound to structures not involved in the contractile process, or the ^{45}Ca, once liberated and used in contraction, could have been rebound again so hastily as not to have been allowed to diffuse longitudinally (not a very probable alternative since the contractions were, in some experiments, many and during each the muscle fibre shortened to about one third its resting length), or there may not be any liberation of internally bound Ca associated with excitation contraction in these fibres.

Mechanical activation of a barnacle muscle fibre with the Ca system and with and without the K system

Fibres bathed in artificial sea water with 10 mM-$CaCl_2$ stimulated with depolarizing voltage clamp pulses showed the typical oscillatory currents reported in a previous work (Keynes *et al.* 1973) and the development of tension began together with the appearance of the oscillations in the current record.

The oscillatory nature of the membrane currents was interpreted as being due to interactions of the K and Ca systems present in the membrane of a barnacle muscle fibre. If this interpretation is correct, this experiment shows that the mechanical activity begins together with the appearance of inward current which in this preparation is carried by Ca ions. It would be desirable to calculate the necessary Ca influx for mechanical

activation. It is not obvious how to estimate the extra Ca influx from oscillatory current records. For this reason in the following experiments, the K^+ currents were eliminated by the use of TEA in the perfusate.

Fig. 5 illustrates a typical experiment in which the fibre was perfused with 100 mM-K acetate plus 100 mM-TEA-Cl plus 600 mM sucrose at pH 7·5. The external solution was 100 mM-$CaCl_2$ artificial sea water (solution 11*b* of Table 1). The Figure contains four sets of records; the lower trace represents the record of tension, *p*. When the absolute membrane

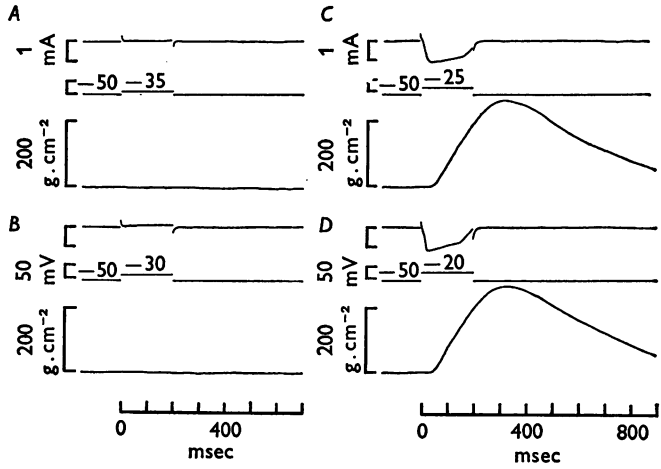

Fig. 5. Determination of the threshold for mechanical activation. Fibre bathed in Na and Mg free, high Ca saline (see Table 1) and internally perfused with K acetate plus TEA (solution 2*c* of Table 1).

potential during the pulse, V_p, was −35 mV or −30 mV no tension was recorded. For $V_p = -25$ mV a clear twitch was obtained. Any further displacement of the membrane potential towards more positive values (for V_p between −25 and +50 mV) gave a similar twitch, thus indicating the existence of a threshold for mechanical activation. Furthermore, it is clear from these records that the development of tension is associated with the flow of inward Ca current.

Fig. 6 illustrates the effects of graded depolarizations on the contractile response of another fibre in 20 mM-$CaCl_2$ artificial sea water (solution 7*c* of Table 1) and internally perfused with solution 2*c* of Table 1.

In Fig. 6*A* several records of tension were superimposed; the number given next to each record represents the magnitude of the pulse in mV. Fig. 6*B* was constructed from the data shown in Fig. 6*A*. The vertical axis represents the fractional tension defined as the ratio between the peak tension for each depolarization, p_o,/the maximum peak tension for the run, p_{max}. The horizontal axis represents the absolute membrane

potential during the pulses, V_p. The threshold for mechanical activation in this fibre was near -20 mV. Comparison of the mechanical activation in this fibre in 20 mM-$CaCl_2$ artificial sea water with that of the fibre in 100 mM-$CaCl_2$ artificial sea water (Fig. 5) indicates that the steepness of the fractional tension curve is dependent on the concentration of Ca

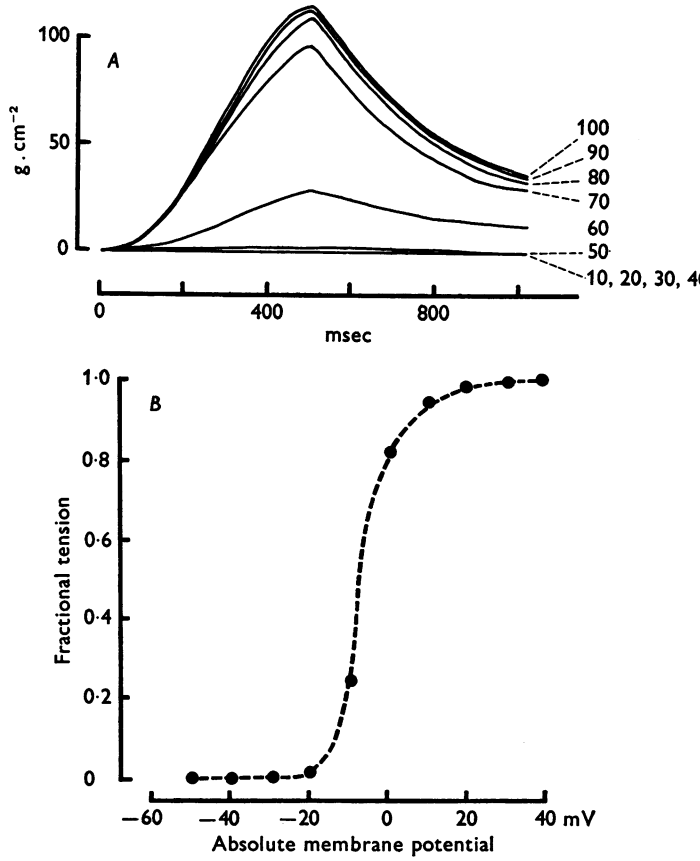

Fig. 6. Determination of the fractional tension, p_o/p_{max}, diagram, stimulating with voltage clamp pulses. Fibre internally perfused with solution 2*c* of Table 1 during 30 min before the records shown in Part *A* were made. The external sea water contained 20 mM-Ca^{2+}. Pulse duration was 500 msec for all voltages. Clamp voltages indicated on right.

in the external solution. The fractional tension curve for the fibre in 100 mM-$CaCl_2$ is much steeper and therefore the threshold more abrupt than it is for the fibre in 20 mM-$CaCl_2$ artificial sea water. For $V_p > +50$ mV, p_o/p_{max} began to decrease. This effect of V_p on p_o/p_{max} was examined in various fibres. In general, for $V_p > +50$ mV, p_o/p_{max} decreases, exponen-

tially with V_p from 1·0 to 0·15. For $V_p > +150$ mV, p_o/p_{max} decreases very little towards 0·15. This result might be expected if the Ca required for mechanical activation was provided by the inward currents. Thus, for V_p exceeding the reversal potential of the inward currents (Keynes *et al.* 1973), although the net extra influx of Ca is reduced, the inward Ca movement is still considerable (Rojas & Luxoro, 1974).

TABLE 4. Some electrical and mechanical parameters measured in fibres bathed in natural sea water and internally perfused during 10 min or less

a	*b*	*c*	*d*	*e*	*f*
Average apparent surface (cm^2)	Average fibre volume (μl.)	Maximum calcium current (μA)	Maximum calcium influx calculated from $I_{Ca^{2+}}$ $\left(\frac{\text{p-mole}}{\text{pulse}}\right)$	Maximum tension recorded (kg/cm^2)	Over-all increase in $[Ca]_i$ (μM)
1·25	65	120	309	0·27	4·6
1·18	42	150	375	0·32	8·9
1·20	45	130	325	0·92	7·2
1·30	32	200	503	0·86	15·6
1·22	36	250	625	> 1·16	17·4
1·26	48	190	475	> 1·50	9·9
1·36	53	213	706	0·89	13·3
Average		179	474		11·0
± S.D.		50	130		5·0

Tensions were recorded using a light-photodiode transducer and records were made on paper using a fast recorder (Physiograph, type DMP-4A, E. and M. Instrument Co., Texas, U.S.A.). In two experiments the fibre broke; the last record of tension was used to obtain the value given in the Table. The duration of the depolarizing pulse was kept constant at 500 msec. To estimate the volume of the fibre three measurements of diameter were considered, near the tendon end, near the cannulated end and at the centre. Fibres perfused with 200 mM-K acetate plus 60 mM-TEA plus 600 mM sucrose during 10 min or less. Experiments carried out at room temperature of about 19° C.

One important question which deserves some attention is whether there might be a stimulation of the extra Ca influx induced by the absence of Na in the external salines used in most of the tracer experiments. In Table 4 we present some of the most relevant results obtained with seven fibres bathed in filtered natural sea water and internally perfused for no more than 10 min. The kinetics of i_{Ca} measured in natural sea water compares rather well with that described in a previous paper (Keynes *et al.* 1973). It can be seen in Table 4 that the tensions recorded are greater than the tensions developed by fibres perfused during longer times. In two cases the fibres suffered disrupture at the cannulated end during contrac-

tion (see Table 4). These results suggest that the development of tension in these barnacle muscle fibres is associated with the appearance of inward currents regardless of the presence or absence of Na ions in the external solution. Since some of the tensions recorded in fibres perfused less than 10 min exceeded 1 kg/cm^2 (see Table 4), it is likely that the elapsed time under perfusion determined the decrease in p_{max} observed in the other experiments.

Effects of sub-threshold pulses applied sequentially

Further support for the association between inward currents and mechanical activation was obtained from the following experiment. Two pulses, which individually produced neither inward current nor tension, were applied sequentially. The experiments began by determining a fractional tension curve similar to Fig. 5*B*. For example in one experiment, for an absolute membrane potential of -28 mV during a 100 msec pulse, no mechanical activity could be detected while for a potential of -25 mV a twitch was recorded. Two subsequent sub-threshold pulses were not sufficient to activate the contractile machinery. Other experiments changing these pulses in different ways did not give any indication of 'Summation' of the two stimuli. Mechanical activation in these fibres was always associated with an inward current. The data on mechanical threshold collected with nine fibres showed that the threshold varies from fibre to fibre and is between an absolute membrane potential of -36 mV and -10 mV with a mean of $-24{\cdot}4 \pm 4{\cdot}7$ mV. This range of membrane potential which constitutes the threshold for mechanical activation is similar to the range of membrane potentials at which the Ca system is abruptly turned on (Keynes *et al.* 1973).

Relation between development of tension and the Ca influx indicated by the membrane current

One point which deserved attention in particular was the temporal relation between the development of tension and the extra Ca influx during

Fig. 7. Relationship between tension and the integral of the inward Ca current. For each set of records, upper trace represents the tension and the lower traces represent the membrane currents. Current records in Ca-free saline (solution 8 of Table 1), $i_2(t)$ in the text, are shown above the base line in a Na and Mg-free, Ca saline are clearly inward during the pulses. The area between these two currents was measured with a planimeter. The points drawn on top of the tension records are proportional to these areas.

Fibre internally perfused with K acetate (solution 1*d* in Table 1) and externally bathed in a 60 mM-$CaCl_2$ sea water (solution 11*a* of Table 1). Fibre was perfused during 5 min only at a rate of 10·3 μl./min. Experiment from a later series carried out in collaboration with Professor M. Luxoro.

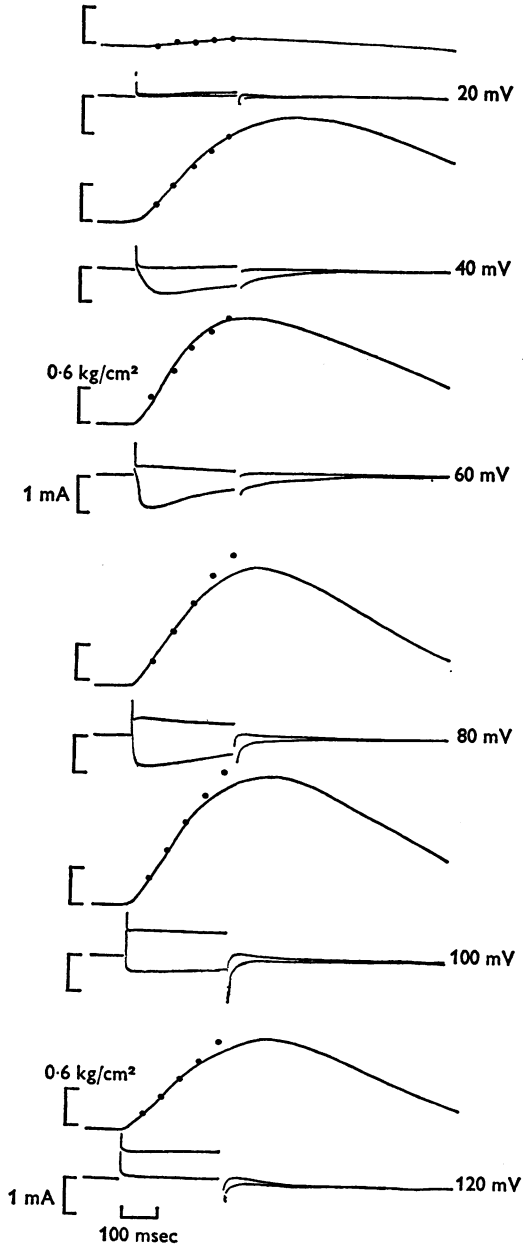

Fig. 7. For legend see facing page.

the voltage clamp pulse. Fig. 7 depicts one example. In the lower part three sets of records obtained near the threshold for mechanical activation are presented. It can be seen that the appearance of the mechanical response is, again, associated with the appearance of an inward current in the current trace. Filled circles drawn on top of the recording of the tension represent

$$K\int_0^t (i_1(t)-i_2(t))\,\mathrm{d}t,$$

where K is equal to 0·012 g/p-mole Ca, $i_1(t)$ is the membrane current recorded when the fibre was bathed in a 60 mM-$CaCl_2$ saline and $i_2(t)$ is the membrane current recorded when the fibre was bathed in a Ca-free saline. The records of tension when the fibre was bathing in a Ca-free saline have been omitted in Fig. 7, as no tensions were recorded. The maximum tension recorded during this voltage clamp run was about 2 kg/cm^2 but intact fibres from *Megabalanus psittacus* can produce tensions as high as 2·5 kg/cm^2 when activated with caffeine and when bathed in a 60 mM-$CaCl_2$ artificial sea water. Therefore, during the voltage clamp run illustrated in part in Fig. 7, p_{max} was not as severely reduced as in the other experiments where the fibres were perfused for longer times. The extra Ca entry varied from 600 to 6500 p-mole for the various depolarizations above threshold.

In twenty-nine fibres examined in a series of experiments the following were general observations. (*a*) A sudden decrease in membrane potential by itself is not sufficient to trigger a twitch of the barnacle muscle fibre; (*b*) development of tension was always associated with an inward Ca current and, (*c*) K currents are not necessary to turn on the contractile machinery.

DISCUSSION

Hagiwara and collaborators have shown that fibres micro-injected with calcium buffers are capable of generating 'calcium action potentials' (Hagiwara & Naka, 1964; Hagiwara *et al.* 1969). One of the possible explanations for these findings is that the internal Ca level controls the resting Ca permeability as well as the voltage dependent calcium permeability change of the membranes of barnacle muscle fibres. The effects of various internal levels of ionic Ca on the Ca influx were examined in one fibre. The influx increased from an average value of 5·6 p-mole/cm^2 sec (influx per unit of apparent surface) to a steady value of 7·8 p-mole/cm^2 sec after the addition of 5 mM-Tris-EGTA to the perfusing solution. This small difference falls however within the range of the experimental errors.

For crab muscle fibres it has been shown that reduction in external Na

increases the resting Ca influx (Baker, 1972). ^{45}Ca influxes were measured in the present work using a Na-free saline. Therefore, this condition would tend to increase the resting Ca influx. Furthermore, the external saline used contained 60 mM-$CaCl_2$, a concentration nearly 6 times that of natural sea water. Considering just the concentration effect we can estimate the resting influx in fibres bathed in sea water with 10 mM-$CaCl_2$ as 0·9 p-mole/cm^2 sec or one sixth of the value measured in the 60 mM-$CaCl_2$ saline. Here we assume a linear relationship between concentration and influx.

The Ca efflux from fibres of *Balanus nubilus* is near 1·5 p-mole/cm^2 sec (Ashley, Caldwell & Lowe, 1972). Supposing the same efflux for fibres from *Megabalanus psittacus* we can calculate the balance of fluxes in sea water as

$$0{\cdot}9 - 1{\cdot}5 = -0{\cdot}6 \text{ p-mole/cm}^2 \text{ sec.}$$

This result indicates that a fibre in sea water under resting conditions is either in equilibrium or is losing Ca at a rate of 0·6 p-mole/cm^2 sec.

In crab muscle fibres the sarcoplasmic reticulum maintains the concentration of free Ca near 0·1 μM (Portzehl, Caldwell & Rüegg, 1964; van der Kloot & Grovsky, 1965) and in barnacle muscle fibres the concentration of free Ca in the resting fibre is near 0·1 μM (Caldwell, 1970). Also it has been shown that a sudden injection of $CaCl_2$ into a barnacle muscle fibre produces a transient increase in the fibre-free Ca level (Ashley *et al.* 1972). It was suggested that the SR takes up approximately 60 % of the injected Ca in a few minutes. Baskin (1971) has measured an uptake of 16·3 μmole Ca per mg protein, working with SR of crustacean muscles. These results support the suggestion that an increase in Ca concentration in the sarcoplasm during depolarization can be quickly reduced by the SR bringing the internal concentration of free Ca back to its resting level (Caldwell, 1970). However, if during electrical activity Ca enters the fibre, unless there is a mechanism to unload the SR, the total calcium content of the SR would gradually increase. It has been suggested that the transverse tubular system unloads an important fraction of the Ca from the sarcoplasm (Caldwell, 1970; Bezanilla, Rojas & Vergara, 1974). The SR could pump Ca into the tubules at the level of the diads (Bezanilla *et al.* 1974). Further studies of the Ca balance in the SR of these barnacle muscle fibres are necessary to answer these questions.

The average Ca influx in fibres perfused with various concentrations of TEA is higher than the corresponding influx measured without TEA. For example, with 60 mM-TEA the average resting influx was 16·2 ± 4·8 p-mole/cm^2 sec (see Table 2). The resting potential was also affected by internal application of TEA. In two experiments (VR 2 and VR 3 of

Table 2) where the resting influx was measured at two different controlled membrane potentials, it was found that the increase in resting Ca influx could not be prevented by repolarizing the membrane.

Assuming that the influx is passive and using the data on resting influxes and membrane potentials, one can estimate the resting Ca permeability as follows,

$$P_{Ca} = m^i_{Ca} \frac{RT(1-\exp(2FV_M/RT))}{2F(-V_M)[Ca]_o},$$

where m^i_{Ca} is the resting Ca influx in p-mole/cm^2 sec, V_M is the membrane potential in mV and $[Ca]_o$ is the external Ca concentration in m-mole/cm^3 (Hodgkin & Katz, 1949). P_{Ca} for fibres internally perfused with 25–60 mM-TEA is found to lie in the range $5{\cdot}0$–$13{\cdot}9 \times 10^{-8}$ cm/sec which is higher than the value of 3×10^{-8} cm/sec found for fibres perfused without TEA.

To obtain direct proof that Ca ions are the carriers of the inward currents associated with a sudden decrease of the membrane potential in barnacle muscle fibre, we have compared the extra Ca influx measured with radioactive Ca and that calculated from the inward current record. On the assumption that for each depolarization the difference between the current obtained in the presence of external Ca, $i_1(t)$, and in its absence, $i_2(t)$, represents inward current carried by Ca ions, then during each pulse the equation

$$\Delta m^i_{Ca} = \frac{1}{2F}\int_0^t (i_1(t) - i_2(t))\,dt$$

should represent the extra Ca entry per pulse. The average extra Ca entry calculated in this way is 687·6 p-mole/500 msec pulse. The extra Ca entry measured with ^{45}Ca is 652·6 p-mole/500 msec pulse. The transport number for Ca ions is $1{\cdot}06 \pm 0{\cdot}41$ as given in Table 3, column *e*.

Even though the structures which undergo a transient change in Ca permeability during a depolarization are presumably distributed in the muscle fibre in such a way that, of the Ca ions which enter the fibre, a minimum necessary amount could reach and activate the contractile machinery, the internal perfusion of the fibres with high concentrations of Ca buffers (5–10 mM-Tris-EGTA) prevents the mechanical activation of the fibres by electrical stimulation even though the structures involved in Ca entry and release are presumably activated. For muscle fibres from *Maia squinado* it has been shown that an internal concentration of 3 mM-EGTA prevents contractures induced by KCl (Ashley, 1967). The Ca in the barnacle muscle fibre from *Megabalanus psittacus* is $0{\cdot}9 \pm 0{\cdot}1$ mM (Keynes *et al.* 1973). The results suggest that if the amount of EGTA inside the fibre is in excess of the total Ca then contraction cannot take

place (Caldwell, 1970). Furthermore, in the absence of ATP most of the intracellular Ca transporting systems would accumulate Ca at a minimum rate (van der Kloot & Grovsky, 1965; Hasselbach, 1964). Prolonged intracellular perfusion could render this physiological mechanism inoperative making it possible to recover the extra Ca influx in the perfusate. The large extra Ca influxes measured in the present work indicate that this is indeed the case for barnacle muscle fibres perfused with EGTA.

From the average extra Ca entry per pulse it is possible to calculate the maximum change in Ca concentration which would be associated with a pulse.

In inflated fibres the maximum Ca concentration which could be reached during a pulse is 11·5 μM (Table 3, column *g*) if the external solution contains no Na and 60 mM-$CaCl_2$ and is 11·0 μM for fibres bathing in natural sea water (Table 4, column *f*). As a consequence of the internal perfusion there is a nearly fourfold increase in the volume of the fibres. If the same change in Ca permeability also occurs in the intact fibres, with the data from Table 3 the maximum calculated increase in Ca concentration during a pulse would be near 33 μM. Hellam & Podolsky (1969) have determined the relationship between tension and ionic Ca concentration for skinned frog muscle fibres and found that for a level of ionized Ca of 1 μM the mechanical activation is maximal. If the same relationship holds for barnacle fibres (Caldwell, 1970) during each pulse the maximum possible concentration of free Ca in the sarcoplasm in barnacle muscle fibres would be about 33 times the level required to turn on the contractile machinery. Due to the long duration of the voltage clamp pulses utilized in most of the experiments presented in this report and to the high external concentration of Ca, the extra influx of Ca may be much greater than that of a fibre under physiological conditions. Also the use of internal TEA could activate the Ca exchange by holding the Ca channel open (Stinnarkre & Tauc, 1973). So if the amount of Ca which enters the fibre during the twitch is much less than the amount we measured during the voltage clamp pulse, then the system may require both the Ca released from the sarcoplasmic reticulum and the extra Ca influx at the tubular membrane. The possibility that this increase in sarcoplasmic Ca level due to the extra influx of Ca could trigger a change in Ca permeability of the membrane of the SR as has been shown *in vitro* (Endo, Tanaka & Ogawa, 1970) has been experimentally examined in the present work with inconclusive results.

The relation between the temporal course of the development of tension and the potential across the sarcolemma has been studied in skeletal muscle fibres (Hodgkin & Horowicz, 1960), in cardiac muscle (Nieder-

gerke, 1956), and in barnacle muscle fibres (Hoyle & Smyth, 1963) Hagiwara, Takashi & Junge, 1968).

Our experiments on mechanical activation on these perfused fibres have shown lack of summation of subthreshold pulses applied sequentially and appearance of a mechanical response always associated with the appearance of an inward current carried by Ca ions. The first result provides some evidence against the possibility of an internal liberation of Ca induced by changes in membrane potential and the second result demonstrates the association between extra Ca influx and development of tension.

A comparison of the temporal relation between the development of tension, $p(t)$, and the extra Ca influx during a depolarizing voltage clamp pulse, $\Delta m^i_{Ca}(t)$, showed that $\Delta m^i_{Ca}(t)$ follows the time course of the increase in tension. In most of the fibres examined both curves, $p(t)$ and $\Delta m^i_{Ca}(t)$, could be superimposed up to $2/3\,p(t)$ maximum and in no case did $\Delta m^i_{Ca}(t)$ preceed $p(t)$. For $p(t)$ greater than $2/3\,p(t)$ maximum, $\Delta m_{Ca}(t)$ was always greater than $p(t)$.

The experiments where the ratio $p_o(V_p)/p_{max}$, where $p_o(V_p)$ is the peak tension for each V_p was measured as a function of V_p showed that for large pulses this ratio decreases from 1·0, measured at about +50 mV, to 0·15 measured at +150 mV. One interpretation of this result is that for V_p approaching the reversal potential of the Ca currents (Keynes *et al.* 1973) the net transfer of Ca decreases and therefore that fraction of mechanical activation which depends on external Ca is smaller. From Boltzmann's principle the ratio 'extra calcium influx, Δm^i_{Ca}/extra calcium efflux, Δm^o_{Ca}' should be equal to

$$\frac{\Delta m^i_{Ca}}{\Delta m^o_{Ca}} = \frac{[Ca]_o}{[Ca]_i} \exp\left(\frac{-2FV}{RT}\right).$$

Combining this equation with the equation given on page 546, the extra influx becomes

$$\Delta m^i_{Ca} = \frac{2P_{Ca}F^2V[Ca]_o \exp(-2FV/RT)}{RT(1-\exp(-2FV/RT))}.$$

The close temporal relationship between $\Delta m^i_{Ca}(t)$ and mechanical activation suggest that the Ca influx due to i_{Ca} takes place presumably over the entire volume of the fibre and that there is an insignificant space between the structure with the Ca permeability change, possibly the tubules, and the proteins involved in mechanical activation.

Excitation-coupling schemes for the regulation of contraction and relaxation in muscle fibre with well developed SR have been proposed by various authors (Ebashi & Endo, 1968; Reuben, Brandt, Garcia & Grundfest, 1967). Even though the anatomical description of the SR of barnacle muscle fibres is available (Hoyle, McNeil & Selverston, 1973) it is not

complete enough to allow us to propose a logical scheme of excitation-contraction coupling in these fibres. For the striated muscle fibre of vertebrates the exact mechanisms of the coupling of the electrical activity in the tubular membrane (Schneider & Chandler, 1973) and the release of Ca by the SR terminal sac is not known (Adrian, Costantin & Peachey, 1969; Costantin, 1970; Bezanilla, Caputo, Gonzalez-Serratos & Venosa, 1972). Anyhow, this step could be avoided in barnacle muscle fibres, where it is possible that the change in Ca permeability takes place at the tubular membrane itself; the deep invaginations of the sarcolemma constitute the means of carrying electrical information from the surface towards the centre of the fibre. The electrical signal would then be conducted to the centre of each bundle of myofibrils by the tubules.

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