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# FACTORS INFLUENCING FREE INTRACELLULAR CALCIUM CONCENTRATION IN QUIESCENT FERRET VENTRICULAR MUSCLE

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SUMMARY

1. The photoprotein aequorin was injected into cells of ferret papillary muscles to monitor the resting intracellular free Ca concentration ($[Ca^{2+}]_i$).

2. Increasing the external Ca concentration ($[Ca^{2+}]_o$) increased both resting $[Ca^{2+}]_i$ and resting tension. The tension and $[Ca^{2+}]_i$ both rose to a peak and then declined to a steady-state level which was higher than the control. Qualitatively similar, but larger, effects were observed if $[Ca^{2+}]_i$ was first elevated with strophanthidin. The increase of $[Ca^{2+}]_i$ was accompanied by the development of spontaneous oscillations of $[Ca^{2+}]_i$.

3. When a steady level of $[Ca^{2+}]_i$ had been reached in high $[Ca^{2+}]_o$, $[Ca^{2+}]_o$ was reduced back to the control level for a brief period. A subsequent increase of $[Ca^{2+}]_o$ produced a rise of $[Ca^{2+}]_i$ to the same steady level as that previously found in the high $[Ca^{2+}]_o$ but the initial peak and subsequent decline were absent. It is suggested that the decline of $[Ca^{2+}]_i$ from the initial peak is mediated by a fall of intracellular Na concentration ($[Na^+]_i$) limiting Ca entry on a Na–Ca exchange.

4. Increasing external K concentration ($[K^+]_o$) from 5 to 30 mmol/l had no detectable effect on $[Ca^{2+}]_i$ under control conditions. However, if $[Ca^{2+}]_i$ was first increased either by applying strophanthidin or by increasing $[Ca^{2+}]_o$, increasing $[K^+]_o$ produced a transient rise of $[Ca^{2+}]_i$ and tension. This rise was unaffected by D600. It is suggested that the secondary decline of $[Ca^{2+}]_i$ after the initial rise may, again, be produced by a fall of $[Na^+]_i$ acting on an Na–Ca exchange.

5. Acidification produced by increasing $[CO_2]$ had no detectable effect on $[Ca^{2+}]_i$ under control conditions. However, if $[Ca^{2+}]_i$ was increased by strophanthidin, acidification produced a rise of $[Ca^{2+}]_i$. This rise of $[Ca^{2+}]_i$ was partly transient even when the intracellular acidification was presumably maintained (raising $CO_2$ at constant $[HCO_3^-]$). Acidification in Na-free solutions had qualitatively similar effects to those in Na-containing solutions.

6. In Na-free solutions (Na replaced by K) the $[Ca^{2+}]_i$ could be maintained at a low level for at least several hours. Increases of $[Ca^{2+}]_o$ in Na-free solutions led to a decrease of $[Ca^{2+}]_i$, and similarly decreasing $[Ca^{2+}]_o$ led to an increase in $[Ca^{2+}]_i$. These anomalous effects of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ could be abolished by Mn ions or D600.

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It is suggested that changes in $[Ca^{2+}]_o$ may have reciprocal effects on Ca permeability and hence on $[Ca^{2+}]_i$.

7. The application of the mitochondrial uncoupler FCCP in Na-free solutions led to an increase of resting tension followed, after a substantial delay, by an increase of $[Ca^{2+}]_i$. It is therefore suggested that in these conditions the maintenance of a low $[Ca^{2+}]_i$ involves a metabolism-dependent process which fails at an [ATP] lower than that at which rigor develops.

8. It is concluded that: (i) in Na-containing solutions changes of $[Na^+]_i$ may affect the response of $[Ca^{2+}]_i$ to changes in $[Ca^{2+}]_o$ or membrane potential and (ii) there is a metabolism-dependent mechanism which is capable of maintaining a low $[Ca^{2+}]_i$, even in the absence of Na–Ca exchange.

## INTRODUCTION

The resting intracellular free Ca concentration ($[Ca^{2+}]_i$) in cardiac muscle is of the order of $10^{-7}$ mol/l (Marban, Rink, Tsien & Tsien, 1980; Lee, Uhm & Dresdner, 1980), while the free Ca concentration in plasma is about $10^{-3}$ mol/l. The low $[Ca^{2+}]_i$ is therefore maintained in spite of a large gradient favouring the entry of Ca into the cell. Ca is thought to enter the cell by passive leaks, by voltage-dependent channels and by an Na–Ca exchange. Three systems have been proposed for removal of Ca from the cell cytoplasm: (i) a sarcolemmal Na–Ca exchange which uses the free energy released by Na ions entering the cell to remove Ca from the cell (Reuter & Seitz, 1968). (ii) A sarcolemmal ATPase which expels Ca, powered directly by the hydrolysis of ATP (Caroni & Carafoli, 1980). (iii) Intracellular organelles, in particular the mitochondria and sarcoplasmic reticulum (for review see Carafoli, 1982).

However, there is still uncertainty regarding the relative importance of these different mechanisms. This is partly due to the paucity of experimental information about the response of $[Ca^{2+}]_i$ even to such simple manoeuvres as changing the membrane potential, the external Ca concentration, or pH. Previous measurements of changes of resting tension in response to such manoeuvres (Eisner, Lederer & Vaughan-Jones, 1983; Vaughan-Jones, Lederer & Eisner, 1983) cannot give unambiguous information about $[Ca^{2+}]_i$ because tension is not a unique function of $[Ca^{2+}]_i$: consequently direct measurements of $[Ca^{2+}]_i$ are desirable.

In the present experiments we have used the photoprotein aequorin to measure $[Ca^{2+}]_i$ and have studied the effects of membrane potential, pH and extracellular Ca concentration on $[Ca^{2+}]_i$. These experiments have been carried out both in Na-contaning solutions in which all of the above regulatory mechanism should be active, and Na-free solutions, in which Na–Ca exchange should be eliminated. The results show that, although Na–Ca exchange has significant effects on $[Ca^{2+}]_i$, there is also a metabolism-dependent process which can maintain $[Ca^{2+}]_i$ at low levels, even in the absence of Na–Ca exchange.

## METHODS

The methods are essentially the same as described by Allen & Kurihara (1980) and Allen & Orchard (1983). Ferrets were killed by an intraperitoneal injection of pentobarbitone. The heart was removed rapidly, washed in Tyrode solution and a thin, uniform papillary muscle (mean

diameter 0·67 mm, range 0·4–1·0 mm) dissected from the right ventricle. The muscle was mounted horizontally in the experimental chamber between a fixed hook and a tension transducer based on an Akers strain gauge. The experimental chamber was maintained at 30 °C.

The muscle was initially stimulated at 0·33 Hz and set to the length at which it produced maximum tension. Stimulation was stopped, so the present experiments were performed on quiescent preparations.

Membrane potentials could not be recorded during these experiments because the position of the photomultiplier tube precluded the insertion of micro-electrodes. The measurements of membrane potential made for the experiment of Fig. 3 were therefore obtained in a parallel experiment.

All the experiments presented are representative of at least three preparations.

*Aequorin.* Aequorin was injected into 50–100 superficial cells of the preparation. The light from the aequorin was detected with a photomultiplier tube. In order to minimize the current produced by the photomultiplier tube in the absence of light (the dark current), the photomultiplier tube was mounted in a cooled housing (Cannell & Allen, 1983). A shutter could be interposed between the preparation and the photomultiplier tube in order to distinguish the dark current from the current due to light from the experimental chamber. At the end of each experiment the muscle was removed from the experimental chamber and the light level in the chamber measured to ensure that there was no extraneous light source.

Aequorin light signals were smoothed with a low-pass filter to reduce the photon noise associated with the very low levels of light. The band width of the filter is stated in the legend to each Figure.

In some experiments the aequorin light was calibrated using the method described by Allen & Blinks (1979). This involves discharging all the aequorin remaining in the preparation at the end of the experiment so that the fractional luminescence (defined in Allen, Eisner, Lab & Orchard, 1983*a*) of light levels recorded during the experiment could be determined. In those experiments in which the calibration procedure was performed the conversion factor for aequorin light (as photomultiplier current) to fractional luminescence is given in the Figure legend. Fractional luminescences can be converted to the $[Ca^{2+}]_i$ with an appropriate *in vitro* calibration curve of aequorin light against $[Ca^{2+}]$. An *in vitro* calibration was performed in a solution containing 157 mmol/l $K^+$, 3 mmol/l $Mg^{2+}$, pH 7·0, 30 °C and the results empirically fitted by the following equation which relates $[Ca^{2+}]$ (in mol/l) to fractional luminescence ($FL$):

$$[Ca^{2+}] = \frac{7 \times 10^{-5} (FL)^{0.33} - 4 \times 10^{-7}}{1 - (FL)^{0.33}}.$$

If gradients of $[Ca^{2+}]_i$ are present, as will occur if $[Ca^{2+}]_i$ oscillates asynchronously across the preparation (see Discussion), then this calibration procedure tends to over-estimate the $[Ca^{2+}]_i$ (Blinks, Wier, Hess & Prendergast, 1982). For this reason the resulting estimates of $[Ca^{2+}]_i$ should be regarded as an upper limit.

The drugs used in these experiments (strophanthidin, D600, Triton-X and FCCP (carbonyl cyanide *p*-trifluomethoxyphenylhydrazone)) were each examined *in vitro* for effects on aequorin and found to have no effect on either the quantum efficiency or the light emission at a buffered $[Ca^{2+}]$ of $4 \times 10^{-7}$ mol/l. $Mn^{2+}$ was not tested in this way because it binds to EGTA more tightly than Ca and thus prevents EGTA being used as a Ca buffer (Blinks, Prendergast & Allen, 1976). However, the similarity of the effects of Mn and D600 (which we have shown does not affect aequorin) in Fig. 6 suggests that the mechanism of the two drugs is similar. Replacement of external Na by K will decrease $[Na^+]_i$ which will be replaced by K, and this is known to have negligibly small effects on aequorin luminescence (Prendergast, Allen & Blinks, 1977). pH changes do affect aequorin luminescence (Allen & Orchard, 1983), an intracellular acidification of 0·2 pH units leading to a reduction in aequorin light emission of 10% or less when $[Ca^{2+}]$ is in the intracellular range. Such effects are negligible for the pH changes produced in the present work.

*Solutions.* The standard solution contained (in mmol/l): $Na^+$, 135; $K^+$, 5; $Mg^{2+}$, 1; $Ca^{2+}$, 2; $Cl^-$, 104; $HCO_3^-$, 20; $HPO_4^{2-}$, 1; acetate, 20; glucose, 10; insulin, $4 \times 10^{-5}$. In experiments in which Na was to be replaced by K, the Na salts were replaced by equimolar amounts of the corresponding K salts. When using Na substitutes for which such salts are difficult to obtain (e.g. choline), and in order to avoid precipitation of Ca when using high Ca concentrations, we have also used a solution buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid). In this case the control solution contained (in mmol/l): $Na^+$, 135; $K^+$, 5; $Mg^{2+}$, 1; $Ca^{2+}$, 2; $Cl^-$, 120; HEPES, 5; glucose, 10; insulin, $4 \times 10^{-5}$.

## RESULTS

### $[Ca^{2+}]_i$ *in Na-containing solutions*

*$[Ca^{2+}]_i$ in control conditions.* Resting light emission was unmeasurably low in the majority of aequorin-injected preparations examined in control solution. Using an estimate of the minimum detectable light emission with our apparatus and the calibration procedure, the absence of detectable light suggests a $[Ca^{2+}]_i$ of $< 160$ nmol/l (Allen *et al.* 1983*a*). Even when resting light was detectable it was so close to the threshold for detection that it was not feasible to investigate the effects of interventions, except those which produced large increases.

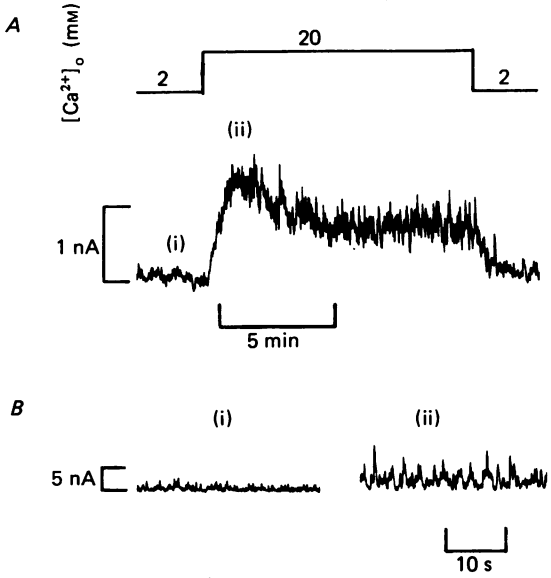


Fig. 1. The effect of increasing $[Ca^{2+}]_o$ on resting light. *A*. Time course of changes of aequorin light (band width 0–0·1 Hz). $[Ca^{2+}]_o$ was increased to 20 mmol/l for the period shown above. *B*. Specimen records of aequorin light obtained at the times indicated on *A* (band width 0–3 Hz). All solutions were buffered with HEPES. A current of 1 nA represents a fractional luminescence of $3 \times 10^{-6}$.

*The effects of elevating $[Ca^{2+}]_o$.* In the experiment shown in Fig. 1, $[Ca^{2+}]_o$ was increased from 2 to 20 mmol/l. This produced an increase of the filtered aequorin light signal (Fig. 1*A*). This increase then decayed to a steady state which was higher than control. It is known that in Ca-loaded preparations, spontaneous release of Ca from the sarcoplasmic reticulum can lead to cellular movements (Lakatta & Lappé, 1981) produced by oscillations of $[Ca^{2+}]_i$ (Allen, Eisner & Orchard, 1983*b*; Orchard, Eisner & Allen, 1983; Wier, Kort, Stern, Lakatta & Marban, 1983). In an attempt to identify the extent to which this phenomenon may be occurring, higher time-resolution sections from Fig. 1*A* with less filtering are shown in Fig. 1*B*. When $[Ca^{2+}]_o$ is elevated, a component of light with a frequency of $\sim 0{\cdot}25$ Hz is detectable.

In order to see appreciable increases of aequorin light on increasing $[Ca^{2+}]_o$, in most muscles it was necessary to increase $[Ca^{2+}]_o$ to about 20 mmol/l. However, much

smaller increases of $[Ca^{2+}]_o$ gave measurable increases of $[Ca^{2+}]_i$, if $[Ca^{2+}]_i$ was initially increased with strophanthidin. Exposure to strophanthidin (10 $\mu$mol/l) for 1 h increased $[Ca^{2+}]_i$ to a level of $540 \pm 120$ nmol/l (mean $\pm$ S.E., $n = 3$). Fig. 2 shows such an experiment in the presence of strophanthidin. The level of aequorin light is elevated detectably, as shown by the fall when the photomultiplier tube was closed. Subsequently $[Ca^{2+}]_o$ was increased from 2 to 8 mmol/l, resulting in an increase of aequorin light emission which then decayed to a steady level which was higher than the control. $[Ca^{2+}]_o$ was then reduced to 2 mmol/l for a brief period (2 min) before returning it to 8 mmol/l. This second increase of $[Ca^{2+}]_o$ produced a rise of aequorin

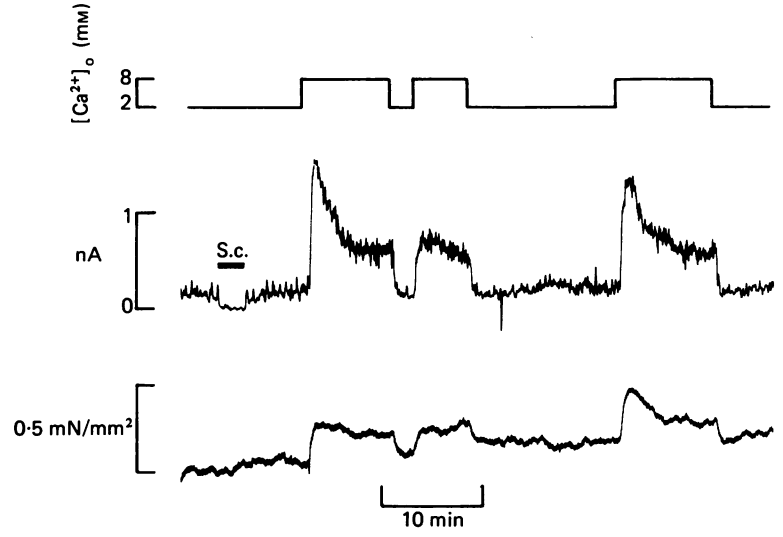


Fig. 2. The effect of increasing $[Ca^{2+}]_o$ in the presence of strophanthidin. Traces show: top, aequorin light, band width 0–0·02 Hz; bottom, tension. The solution protocol is shown above the record. All solutions contained $10^{-5}$ mol/l strophanthidin and were buffered with $CO_2/HCO_3^-$. S.c. indicates a period when the photomultiplier tube shutter was closed (see Methods). A current of 1 nA represents a fractional luminescence of $5 \times 10^{-6}$.

light which had a smaller transient component than that produced by the first exposure although the final steady level of $[Ca^{2+}]_i$ was similar in both cases. $[Ca^{2+}]_o$ was then reduced for a longer period (15 min) to 2 mmol/l. When $[Ca^{2+}]_o$ was subsequently increased to 8 mmol/l, the increase of aequorin light emission again showed a large transient component. A component of resting tension is detectable which shows the same characteristics as the light record. This experiment therefore shows that a significant recovery period in normal $[Ca^{2+}]_o$ is required before a subsequent exposure to high $[Ca^{2+}]_o$ will produce the transient component of the increase of $[Ca^{2+}]_i$.

*The effects of* $[K^+]_o$ *on* $[Ca^{2+}]_i$. In these experiments the membrane potential was increased from its initial value of $\sim -70$ mV to either (i) $\sim -35$ mV (by increasing $[K]_o$ to 30 mmol/l) or (ii) to $\sim 0$ mV (by increasing $[K]_o$ to 140 mmol/l). Control experiments showed that merely increasing the osmolarity of the solution (by adding NaCl or sucrose) had no significant effect on resting light.

Increasing $[K^+]_o$ to 30 mmol/l had no observable effect on aequorin light emission in normal muscles when the resting light was undetectable. In order to investigate the effects of depolarization we therefore increased $[Ca^{2+}]_i$, either by using strophanthidin and/or by elevating $[Ca^{2+}]_o$. When strophanthidin ($10^{-5}$ mol/l) was added, the same increase of $[K^+]_o$ produced a transient increase of resting light. In the experiment shown in Fig. 3 $[Ca^{2+}]_i$ was elevated by addition of $10^{-5}$ mol/l strophanthidin and by increasing $[Ca^{2+}]_o$ to 16 mmol/l. When $[K^+]_o$ was then increased from

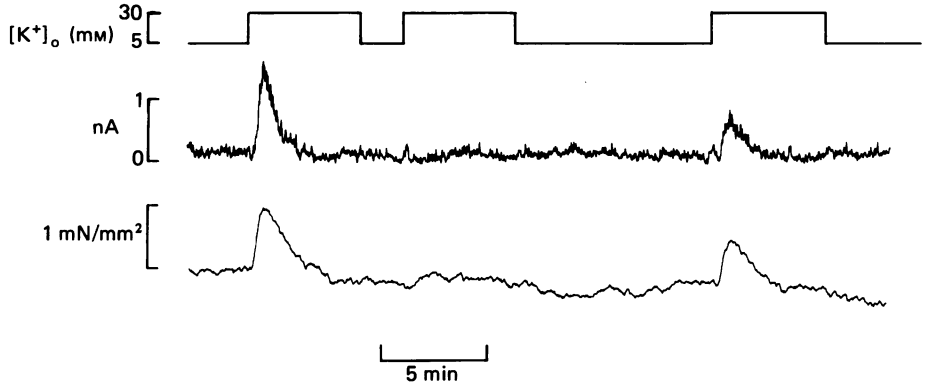


Fig. 3. The effect of depolarization produced by increasing $[K^+]_o$. Traces show: top, aequorin light, band width 0–0·1 Hz; bottom, tension. The solution protocol is shown above the record. All solutions contained $10^{-5}$ mol/l strophanthidin and 16 mmol/l $Ca^{2+}$, and were buffered with HEPES.

5 to 30 mmol/l, the resting light increased rapidly to a peak and then declined towards the control level. If $K^+$ was increased again, after only a short period in 5 mmol/l $K^+$, resting light increased very little; after a longer period in 5 mmol/l $K^+$ a large effect of 30 mmol/l $K^+$ was again apparent.

D600 (20 $\mu$mol/l) substantially reduced the twitch and light transient produced by stimulation (cf. Allen *et al.* 1983*a*), but had little effect (less than 20%) on the increase of light produced by increasing $[K^+]$ (not shown).

Exposure to solutions containing 150 mmol/l $[K^+]_o$ (at constant $[Na^+]_o$), produced similar but larger effects in all preparations without the need to first elevate resting light ($[Ca^{2+}]_i$) with either strophanthidin or elevated $[Ca^{2+}]_o$.

*The effects of pH on* $[Ca^{2+}]_i$. Allen & Orchard (1983) found that an acidification produced by elevating $[CO_2]$ from 5 to 15% did not produce a measurable increase of light in quiescent preparations. The present experiments confirmed this result (not shown). However, if resting light (and hence tension) was first increased with strophanthidin, increasing $[CO_2]$ to 15% (at constant $[HCO_3^-]$) produced a large increase of resting light which had a transient component. Resting tension was reduced throughout the exposure to elevated $[CO_2]$ (Fig. 4). This decrease of tension is presumably due to the decrease of apparent Ca sensitivity of the contractile proteins produced by acidification (Fabiato & Fabiato, 1978; Allen & Orchard, 1983). Light decreased on removing $CO_2$, although to a level higher than that before $CO_2$ application. This is probably because $[Ca^{2+}]_i$ continued to increase during exposure to strophanthidin.

*$[Ca^{2+}]_i$ in Na-free solutions*

In the conditions in which the previous series of experiments were carried out, Na–Ca exchange may be an important factor in the control of $[Ca^{2+}]_i$. Therefore the following experiments were carried out in Na-free solutions (Na replaced by K), in which Na–Ca exhange can no longer operate.

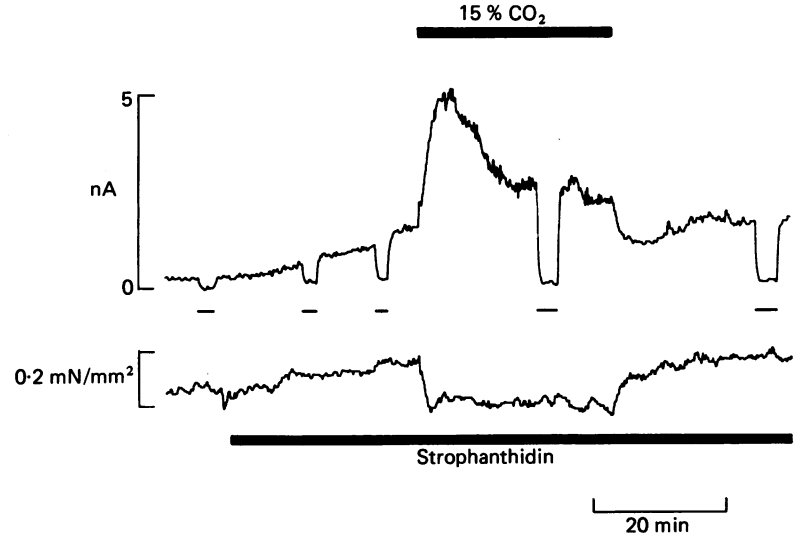


Fig. 4. The effect of increasing $[CO_2]$ in the presence of strophanthidin. Traces show: top, aequorin light, band width 0–0·02 Hz; bottom, tension. $10^{-5}$ mol/l strophanthidin was present in the solutions during the period shown. The solutions were buffered with $CO_2/HCO_3^-$. During the period indicated above the record the perfusate was changed from a solution equilibrated with 5% $CO_2$ (pH = 7·4) to one equilibrated with 15% $CO_2$ (pH = 6·9). The black bars below the aequorin light trace show periods when the photomultiplier tube shutter was closed (see Methods).

Immediately after application of Na-free solution, the $[Ca^{2+}]_i$ rises dramatically (Allen *et al.* 1983*a*), but on prolonged exposure the $[Ca^{2+}]_i$ falls to a low level. In three preparations in which a steady elevation of aequorin light was detectable in Na-free (K) solution, the calibration procedure gave a $[Ca^{2+}]_i$ of $240 \pm 60$ nmol/l (mean ± S.E., $n = 3$).

*The effects of changing $[Ca^{2+}]_o$ in Na -free solution*

Fig. 5 shows a record from a muscle which had been exposed to an Na-free solution (replaced by K) for 40 min. Fig. 5*A* shows the effects of increasing $[Ca^{2+}]_o$ from 2 to 4 mmol/l. There is a small but significant decrease of resting light. In Fig. 5*B* the muscle was exposed to a lowered $[Ca^{2+}]_o$ (0·1 mmol/l). This produced an increase of resting light which was reversed on returning $[Ca^{2+}]_o$ to 2 mmol/l. This anomalous effect of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ was examined in more detail in the experiment shown in Fig. 6 which was designed to investigate whether the apparent rise of $[Ca^{2+}]_i$ produced by decreasing $[Ca^{2+}]_o$ was sensitive to Ca channel inhibitors such as D600 (20 μmol/l) or Mn (2 mmol/l). The first exposure to 0·1 mmol/l $[Ca^{2+}]_o$ produced a

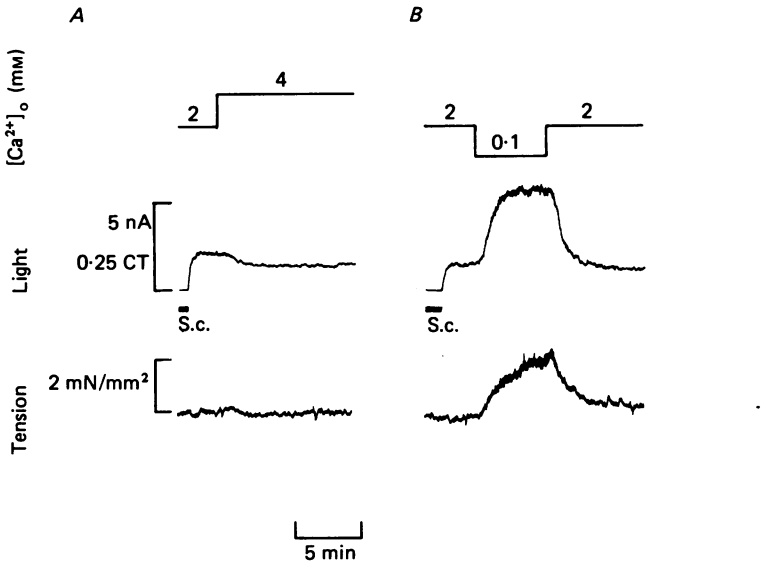


Fig. 5. The effect of changing $[Ca^{2+}]_o$ in an Na-free solution (replaced by K). Traces show: top, aequorin light, 0–0·01 Hz; bottom, tension. The solution protocols are shown above the records. S.c. indicates periods during which the photomultiplier tube shutter was closed. The muscle had been exposed to Na-free solution for 40 min before these records were obtained. *A*. $[Ca^{2+}]_o$ increased from 2 to 4 mmol/l. *B*. $[Ca^{2+}]_o$ decreased from 2 to 0·1 mmol/l. All solutions were buffered with $CO_2/HCO_3^-$. A current of 1 nA represents a fractional luminescence of $8 \times 10^{-6}$.

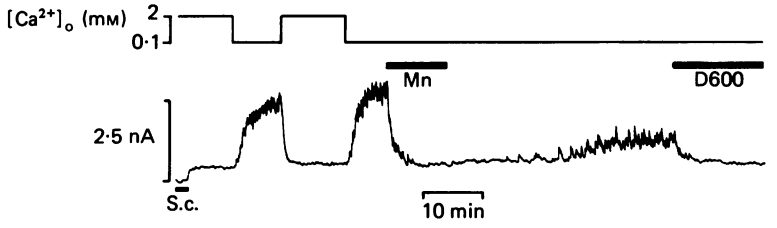


Fig. 6. The effect of D600 (20 $\mu$mol/l) and $Mn^{2+}$ (2 mmol/l) on the increase of resting light produced by decreasing $[Ca^{2+}]_o$ in an Na-free solution. The trace shows aequorin light (band width 0–0·01 Hz) in an Na-free (K substituted) solution when $[Ca^{2+}]_o$ was decreased from 2 to 0·1 mmol/l. The muscle had been exposed to Na-free solution for 30 min before this record was obtained. The solution protocol is shown above the record. All solutions were buffered with $CO_2/HCO_3^-$. S.c. indicates the period during which the photomultiplier tube shutter was closed.

clear increase of resting light which was reversed when the muscle was returned to 2 mmol/l $Ca^{2+}$. The second exposure to 0·1 mmol/l $Ca^{2+}$ produced a similar increase of $[Ca^{2+}]_i$ which was terminated by the addition of Mn to the perfusate. Mn was then removed, and when resting light had recovered partially the organic Ca channel blocker D600 was added. This also decreased resting light.

In two experiments $[Ca^{2+}]_o$ was lowered to 0·1 mmol/l and at the same time $[Mg^{2+}]_o$ was increased by 1·9 mmol/1, so that the total divalent cation concentration

remained constant. The rise in light was similar to that observed when $[Ca^{2+}]_o$ was simply lowered to 0·1 mmol/l.

Resting light levels were not detectable in choline-substituted solutions, so we could not determine whether the anomalous effects of Na-free (K) solutions are dependent on depolarization.

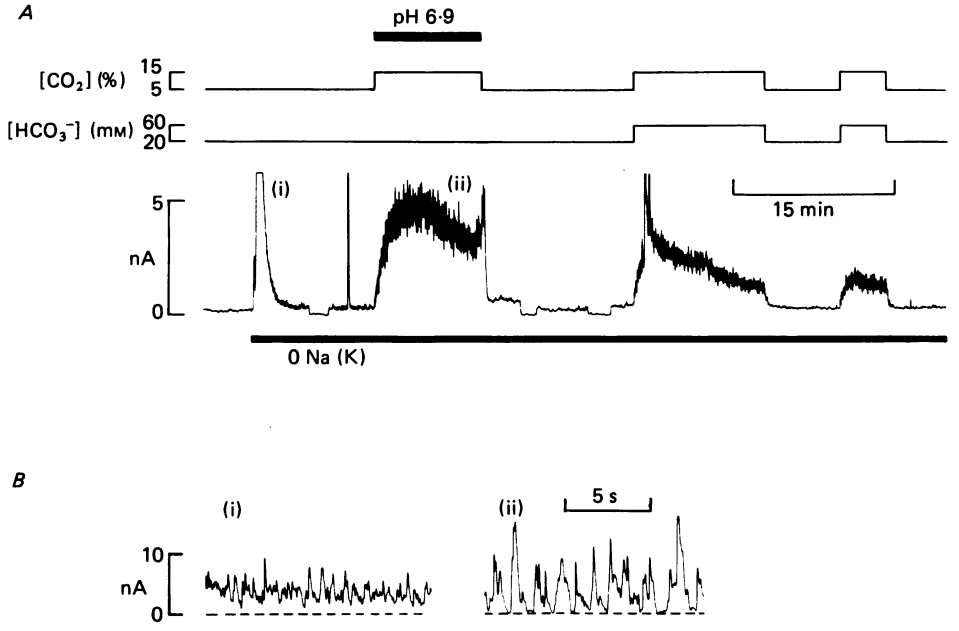


Fig. 7. The effect of increasing $[CO_2]$, with and without changes of extracellular pH, in an Na-free (substituted with K) solution. All solutions were buffered with $CO_2/HCO_3^-$. The trace in *A* shows aequorin light, band width 0–0·05 Hz. The solution protocol is shown above the record, the duration of exposure to Na-free solution is shown below the record. *B* shows light signals from the regions indicated on *A* with less filtering (band width 0–3 Hz).

*The effects of pH on $[Ca^{2+}]_i$ in Na-free solution.* Fig. 7 *A* shows the effect of an increase in external $[CO_2]$ from 5 to 15 % (at constant $[HCO_3^-]$) in an Na-free solution. This manoeuvre should produce a maintained intracellular acidification (Ellis & Thomas, 1976). Increasing $[CO_2]$ produced an increase of light which decayed slowly and which reversed on returning $[CO_2]$ to 5 %. To confirm that the change of light was due to changes of intracellular rather than extracellular pH, $CO_2$ was again increased to 15 % but $[HCO_3^-]$ was simultaneously raised from 20 to 60 mmol/l to maintain external pH constant. This also produced a rise of light. The increase was, however, smaller and decayed faster than that produced by raising $CO_2$ alone. The smaller increase and faster decay of light presumably reflect the fact that the intracellular pH change produced by simultaneously increasing $CO_2$ and $[HCO_3^-]$ has an initial transient component which then decays to a steady state which is still lower than control (Ellis & Thomas, 1976).

Fig. 7 *B* shows records of aequorin light on a faster time base and with less filtering: (i) during exposure to an Na-free (K substituted) solution, (ii) during exposure to 15 %

$CO_2$ in Na-free (K) solution. These records were obtained at identical mean aequorin lights. During exposure to Na-free (K) solution, oscillations of light are present (cf. Orchard *et al.* 1983). These oscillations are much more pronounced during the exposure to 15 % $CO_2$ in Na-free (K) solution, suggesting that pH may have an effect on the mechanism of these oscillations, and may thus alter the filtered aequorin light level (see Discussion).

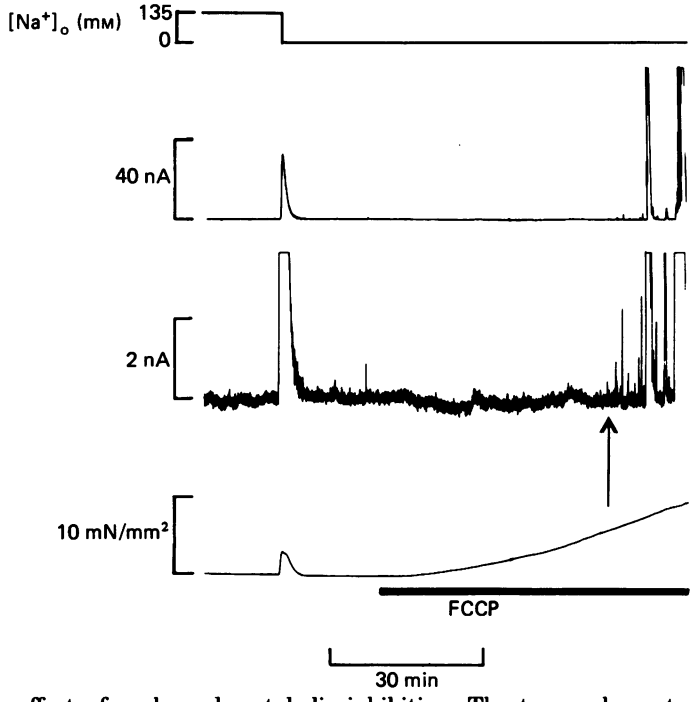


Fig. 8. The effect of prolonged metabolic inhibition. The traces show: top, low-gain aequorin light, band width 0–0·1 Hz; middle, high-gain aequorin light, band width 0–0·1 Hz; bottom, tension. Na was removed completely and replaced by K for the period shown above the record. FCCP (1 $\mu$mol/l) was added as indicated. All solutions were buffered with $CO_2/HCO_3^-$. A current of 1 nA represents a fractional luminescence of $4 \times 10^{-6}$. At the time indicated by the arrow there was no detectable light emission and we estimate light to be less than 0·25 nA. This corresponds to a $[Ca^{2+}]_i$ of less than 300 nmol/l.

*Effect of mitochondrial uncouplers on $[Ca^{2+}]_i$ in Na-free solution.* The previous experiments have shown that $[Ca^{2+}]_i$ can be maintained at a comparatively low level even in the absence of Na–Ca exchange. One possible explanation of this is that the Ca is taken up into intracellular organelles. In the experiment shown in Fig. 8 we investigated whether the regulation of $[Ca^{2+}]_i$ in Na-free solutions depends on Ca being taken up into mitochondria, as has been suggested previously (Jundt, Porzig, Reuter & Stucki, 1975; Chapman, Coray & McGuigan, 1983). Exposure of the muscle to an Na-free solution produced the usual transient increase of both $[Ca^{2+}]_i$ and tension. The mitochondrial uncoupler FCCP, which has been shown to produce a rapid release of Ca from loaded mitochondria (Brinley, Tiffert, Scarpa & Mullins, 1977), was

then added. However, as shown in Fig. 8, there is no detectable increase of resting light and, at the point indicated by the arrow, $[Ca^{2+}]_i$ was less than 300 nmol/l. FCCP produced a gradual increase of tension which, since it was unaccompanied by a rise of $[Ca^{2+}]_i$, is presumably due to the formation of rigor bridges produced by a fall of [ATP]. $[Ca^{2+}]_i$ does eventually rise in a series of bursts and, at the time the record was terminated, the level of $[Ca^{2+}]_i$ was sufficiently high that consumption of aequorin became significant.

## DISCUSSION

### *Aequorin*

The properties of aequorin and the interpretation of aequorin light signals from contracting cardiac muscle have been discussed elsewhere (Allen & Blinks, 1979; Allen & Kurihara, 1980; Blinks *et al.* 1982). In this paper we are concerned with the interpretation of light signals from resting preparations, and this involves some new considerations. (i) The relation between aequorin light and $[Ca^{2+}]_o$ is complicated over the range of $[Ca^{2+}]_i$ likely to be encountered in resting preparations (0·1–1·0 $\mu$mol/l) (Allen & Blinks, 1979). At the bottom of this range aequorin light emission is approximately linearly related to $[Ca^{2+}]$. However, at the upper end of the range and above, the relation between light ($L$) and $[Ca^{2+}]$ is approximately $L \propto [Ca^{2+}]^{2.5}$. Consequently quantitative interpretation of the light records is particularly difficult. (ii) Various lines of evidence suggest that when $[Ca^{2+}]_i$ is elevated in resting preparations, there are spontaneous oscillations of $[Ca^{2+}]_i$ and tension which occur asynchronously in cells across the preparation (Lakatta & Lappé, 1981; Kass & Tsien, 1982; Orchard *et al.* 1983; Wier *et al.* 1983). The high levels of $[Ca^{2+}]_i$ found transiently in a few cells would probably be in the range where $L \propto [Ca^{2+}]^{2.5}$. Because of this non-linearity, an oscillating $[Ca^{2+}]_i$ leads to a greater (time-averaged) light signal than a steady $[Ca^{2+}]_i$ with the same mean $[Ca^{2+}]_i$. In a single cell this situation should be easily recognizable because of the associated oscillations in the light emission, but in a multiply injected preparation, where oscillations in different cells may be unsynchronized, this information may be lost. Thus in the worst case, changes in aequorin light do not necessarily indicate changes in the mean $[Ca^{2+}]_i$ across the preparation but could indicate changes in the amplitude of oscillations of $[Ca^{2+}]_i$.

In the experiments presented in this paper, oscillations are detectable in the unfiltered aequorin light signal, so that some component of the increase in filtered light signal may be due to changes in oscillations. Nevertheless, for various reasons it seems likely that a major component of the filtered light signal is due to changes in mean $[Ca^{2+}]_i$. (i) There are changes in resting tension which mimic the changes in light. If, in a multicellular preparation, asynchronous oscillations of $[Ca^{2+}]_i$ developed randomly throughout the preparation (but with no change in the time and spatially averaged $[Ca^{2+}]_i$), then it would be predicted that tension would fall. This is because the cells which had a low $[Ca^{2+}]_i$ would be less activated, and therefore would have an increased compliance, and this would tend to prevent the force from cells with a high $[Ca^{2+}]_i$ from being transmitted through the preparation. Thus the fact that tension shows parallel changes with the light suggests that there must be changes in mean $[Ca^{2+}]_i$ occurring in the same direction. (ii) Since the magnitude of Ca

oscillations seems to depend on the Ca load in the cell it is difficult to see how, without interfering with sarcoplasmic reticulum function, the amplitude of the oscillations could change in the absence of some change of mean $[Ca^{2+}]_i$. (iii) The maintained component of increase in $[Ca^{2+}]_i$ after a change in $[Ca^{2+}]_o$ has also been observed in studies with Ca-sensitive micro-electrodes (Marban *et al.* 1980; Sheu & Fozzard, 1982). The response of electrodes is also non-linear, but of the type which leads to an oscillating $[Ca^{2+}]_i$ producing a smaller signal than a steady $[Ca^{2+}]_i$. However, it remains possible that some of the interventions used in these experiments, notably acidosis, have a direct effect on the mechanism of these fluctuations.

*$[Ca^{2+}]_i$ in Na-containing solutions*

*The effects of $[Ca^{2+}]_o$ and membrane potential on $[Ca^{2+}]_i$.* The results in the present paper have shown that, in Na-containing solutions, either increasing external Ca concentration or depolarization can increase $[Ca^{2+}]_i$. The fact that these increases of $[Ca^{2+}]_i$ can be seen even when Ca channels are blocked supports the idea that they result, at least partly, from a $[Ca^{2+}]_o$- and membrane potential-dependent Na–Ca exchange (cf. Chapman & Tunstall, 1980; Eisner *et al.* 1983). A striking feature of these increases of $[Ca^{2+}]_i$ is that a large component is transient. Previous studies with Ca-selective micro-electrodes have not shown a transient increase of $[Ca^{2+}]_i$ (Marban *et al.* 1980; Sheu & Fozzard, 1982). However, an elevation of $[Ca^{2+}]_o$ (Vaughan-Jones *et al.* 1983) or prolonged depolarization (Eisner *et al.* 1983) can produce a rapid rise of resting tension followed by a slow fall which is consistent with a rise and subsequent fall of $[Ca^{2+}]_i$.

The fall in tension following depolarization has been shown to be associated with a fall of intracellular Na concentration, $[Na^+]_i$ (Eisner *et al.* 1983). This fall of $[Na^+]_i$ will decrease the entry of Ca ions through Na–Ca exchange and can therefore account for the observed secondary fall of both tension and $[Ca^{2+}]_i$. A similar explanation could also account for the rapid rise and subsequent fall of $[Ca^{2+}]_i$ produced by elevating $[Ca^{2+}]_o$, since this manoeuvre also decreases $[Na^+]_i$ (Deitmer & Ellis, 1978). Both the initial rise and the subsequent fall of $[Ca^{2+}]_i$ and the fall of $[Na^+]_i$ produced by either depolarization or elevating $[Ca^{2+}]_o$ can be qualitatively reproduced, if it is assumed that these interventions affect Na–Ca exchange and that there is another mechanism capable of removing $Ca^{2+}$ from the cytoplasm. On this model either depolarization or elevating $[Ca^{2+}]_o$ increases the rate of Ca entry into the cell and therefore the rate of Na efflux. The resulting fall of $[Na^+]_i$ will then decrease the rate of Ca entry, resulting in the observed changes of $[Ca^{2+}]_i$.

$[Na^+]_i$ has not been measured in the present experiments and it is therefore necessary to compare the rate of fall of $[Ca^{2+}]_i$ with measurements of $[Na^+]_i$ from other tissues. Most of the data on the effects of $[Ca^{2+}]_o$ on $[Na^+]_i$ have been obtained in Purkinje fibres, where the decrease of $[Na^+]_i$ which parallels the secondary fall of tension (Lederer, Sheu, Vaughan-Jones & Eisner, 1984) has a half-time of about 5 min (cf. Deitmer & Ellis, 1978). This is comparable to the time taken for $[Ca^{2+}]_i$ to decay in the present experiments. In sheep ventricular muscle an elevation of $[Ca^{2+}]_o$ produces a decay of $[Na^+]_i$, which also has a half-time of 5 min (Sheu & Fozzard, 1982; Fig. 6). No precise agreement would be expected between the half-times for fall of $[Na^+]_i$ and $[Ca^{2+}]_i$, since Ca influx may well depend on a higher power of $[Na^+]_i$

(Glitsch, Reuter & Scholz, 1970; Mullins, Tiffert, Vassort & Whittenbury, 1983). Nevertheless the similarity of time courses suggests that the secondary fall of $[Ca^{2+}]_i$ on elevating $[Ca^{2+}]_o$ may be due to the simultaneous fall of $[Na^+]_i$. However, in the Purkinje fibre an intracellular acidification also accompanies the secondary fall of tension (Vaughan-Jones *et al.* 1983), which might be responsible for the relaxation. Since the present work shows that $[Ca^{2+}]_i$ falls, at least part of the relaxation must be accounted for by a fall of $[Ca^{2+}]_i$ rather than changes of pH. The fall of $[Na^+]_i$ produced by depolarization has a similar time course to the relaxation of tension (Eisner *et al.* 1983) and it would therefore seem likely that it can account for the fall of $[Ca^{2+}]_i$ observed in the present work.

*$[Ca^{2+}]_i$ in Na-free solutions*

*The effects of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ in Na-free solutions.* When $[Ca^{2+}]_o$ was elevated in an Na-free solution, $[Ca^{2+}]_i$ fell and, conversely, when $[Ca^{2+}]_o$ was decreased, $[Ca^{2+}]_i$ rose. It is difficult to see how decreasing $[Ca^{2+}]_o$ could release Ca from an intracellular store, and it therefore seems likely that the rise of $[Ca^{2+}]_i$ represents an increased Ca influx across the sarcolemma. In this case the decrease of $[Ca^{2+}]_o$ must have increased the Ca permeability (Linden & Brooker, 1982) sufficiently to offset the decreased driving force for Ca entry produced by decreasing $[Ca^{2+}]_o$. Presumably this fall of $[Ca^{2+}]_i$ is not seen in Na-containing solutions because the effects due to the actions of $[Ca^{2+}]_o$ on Na–Ca exchange dominate. The effects of Mn and D600 suggest that the increased permeability is an effect on Ca channels.

*The effects of pH on $[Ca^{2+}]_i$ in Na-free solutions.* The present experiments show that conditions which produce an intracellular acidification increase $[Ca^{2+}]_i$. The effects of pH on $[Ca^{2+}]_i$ in cardiac muscle have previously only been studied in the Purkinje fibre, where acidification has been reported to produce a rise (Bers & Ellis, 1982) or a fall (Hess & Weingart, 1980) of $[Ca^{2+}]_i$. Our results using $CO_2$ to change pH are in agreement with those of Bers & Ellis (1982), who used $NH_4Cl$.

Oscillations of $[Ca^{2+}]_i$ were particularly prominent in acidotic conditions even when compared with a similar elevation of light produced by Na removal (Fig. 7). This suggests that, in addition to effects on mean $[Ca^{2+}]_i$, acidosis may allow the sarcoplasmic reticulum to accumulate larger amounts of Ca before it is spontaneously released or have some direct effect on the release mechanism. Fabiato & Fabiato (1978), using skinned fragments of rat ventricular cells, found that acidosis at a constant $[Ca^{2+}]$ reduced the rate and amount of Ca uptake by the sarcoplasmic reticulum and reduced the frequency of the spontaneous release of Ca.

*Control of $[Ca^{2+}]_i$: sarcolemma versus intracellular organelles*

In many of the experiments in the present study Na–Ca exchange was presumably not contributing to active Ca extrusion from the cell. This is definitely so for the Na-free experiments and presumably so for the experiments in strophanthidin because, under these conditions, Na–Ca exchange is thought to produce net Na extrusion/Ca influx (Deitmer & Ellis, 1978). Nevertheless, steady levels of $[Ca^{2+}]_i$ can be reached in these conditions and, more important, various manoeuvres can decrease $[Ca^{2+}]_i$ (e.g. hyperpolarization and decrease in $[Ca^{2+}]_o$ in the presence of strophanthidin). There are two possible explanations for this control of $[Ca^{2+}]_i$: it could represent (i) a net

extrusion of Ca from the cell, perhaps by a Ca-ATPase or (ii) sequestration of Ca by intracellular buffers. Although sequestration cannot regulate Ca in the steady state it may be sufficient for the duration of the present experiments. Intracellular sequestration appears to be the dominant mechanism in the squid axon, where a steady $[Ca^{2+}]_i$ can be maintained in the presence of a large net entry of Ca and increase of total cell Ca produced by depolarization (Mullins *et al.* 1983). However, because the surface area-to-volume ratio is much greater in cardiac cells it seems likely that surface membrane fluxes will have a more important role in cardiac tissues. Measurements of free $[Ca^{2+}]$ such as those performed in the present study do not distinguish between the above two alternatives: analytical measurements of total Ca are required. Nevertheless, the experiments in the next section do shed some light on the relative contributions of sarcolemmal mechanisms *versus* intracellular organelles.

*The effect of metabolic inhibition on* $[Ca^{2+}]_i$. The increase of $[Ca^{2+}]_i$ after prolonged exposure to FCCP in Na-free solutions suggests that there is a metabolism-dependent process which can maintain $[Ca^{2+}]_i$ at low levels. FCCP both releases $Ca^{2+}$ from mitochondria and inhibits oxidative ATP production. The slow rise of $[Ca^{2+}]_i$ is inconsistent with a liberation of Ca from mitochondrial stores unless the release by FCCP is sufficiently slow that the Ca can be either re-sequestered elsewhere or pumped out of the cells. In either case it appears that there must be some non-mitochondrial Ca removal system which can regulate $[Ca^{2+}]_i$ in Na-free solutions. This could be either a Ca-ATPase extruding Ca from the cell (Caroni & Carafoli, 1980) or else sequestration by some other intracellular organelle. This system only failed after tension had begun to develop. As this tension developed in the absence of detectable increases of $[Ca^{2+}]_i$, it is presumably due to the onset of rigor.

Previous workers (Jundt *et al.* 1976) have shown that metabolic inhibition of cardiac muscle exposed to Na-free solutions produced an increase of resting tension which was attributed to an increase of $[Ca^{2+}]_i$ produced by Ca released from mitochondria. The present results show that the increase of tension is not accompanied by an increase of $[Ca^{2+}]_i$. Thus it is possible that the rise of tension seen by Jundt *et al.* (1976) was also due to formation of rigor bridges and cannot be used to indicate a release of Ca from intracellular organelles.

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