J. Physiol. (1978), **275**, *pp.* 241–262
With 5 text-figures
Printed in Great Britain

CALCIUM AND STRONTIUM CONCENTRATION CHANGES WITHIN SKINNED MUSCLE PREPARATIONS FOLLOWING A CHANGE IN THE EXTERNAL BATHING SOLUTION

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(*Received 20 April 1977*)

SUMMARY

1. A method for producing rapid [Ca^{2+}] and [Sr^{2+}] changes in the frog skinned muscle fibre preparation while maintaining constant all other cationic concentrations (Moisescu, 1976*a*, *b*) is described and analysed in detail.

2. Different experiments, some of them involving the Ca^{2+}-sensitive photoprotein aequorin, as well as theoretical considerations, indicate that with this method one can produce a Ca^{2+} (or Sr^{2+}) concentration change within 0·1–0·15 sec in a *whole* preparation having a diameter of 50 μm.

3. The rate of force development was similar to that observed *in vivo*.

4. The radial diffusion coefficient of EGTA in relaxed myofibrillar preparations was measured and found to be $4{\cdot}6 \times 10^{-6}$ cm^2 sec^{-1} at 20 °C.

5. The sarcoplasmic reticulum in myofibrillar bundles was found to be active with respect to both Ca^{2+} and Sr^{2+} in the solutions used ([Mg^{2+}] 1 mM; [Na] 30 mM; [K] 140–170 mM; [Cl] $\leqslant$ 20 mM; pH 7·10).

6. The amount of Ca released by caffeine from internal stores (previously loaded with Ca) can raise the total Ca concentration in the muscle fibre preparation by at least 1·8 mM.

7. The presence of 10 mM-caffeine in *all* bathing solutions reduced drastically the ability of the sarcoplasmic reticulum to accumulate both Ca and Sr.

INTRODUCTION

Since Natori (1954) introduced the skinned frog muscle preparation, many investigators have used it to study both the properties of the contractile apparatus (e.g. Hellam & Podolsky, 1969; Ebashi & Endo, 1968; Podolsky & Teichholz, 1970; Julian, 1971; Godt, 1974; Donaldson & Kerrick, 1975, Endo, 1972*a*, *b*) and those of the sarcoplasmic reticulum (Endo, Tanaka & Ogawa, 1970; Ford & Podolsky, 1972*a*, *b*; Endo & Thorens, 1975; Thorens & Endo, 1975; Fabiato & Fabiato, 1977).

There are however a number of complex diffusion problems associated with the fact that the ionic species required for a physiological activation of the contractile apparatus are provided from outside the preparation.

A major source of errors for both transient and steady-state tension results using

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myofibrillar preparations arises from the ATP (adenosine triphosphate)-ase activity within the myofibrillar bundle. This ATP-ase activity raises not only the direct problem of maintaining the Mg-ATP concentration within the whole cross-section of the preparation between certain limits so that the process of tension production is not affected (Godt, 1974), but also that of maintaining a constant Ca^{2+} concentration throughout the myofibrillar bundle when using pH-sensitive Ca buffers. This second problem arises from the fact that the over-all process of ATP hydrolysis is associated with relatively large proton movements, which alter the pH and change the apparent binding constant of Ca to the Ca buffer (Portzehl, Caldwell & Rüegg, 1964). This in turn means that the Ca^{2+} concentration in the preparation differs from that in the bathing solution (see Results).

In addition, the inherent appearance of a gradient in the inorganic phosphate concentration in an activated myofibrillar preparation might also contribute to the formation of a Ca^{2+} – and/or Mg^{2+} – concentration gradient within the fibre, if the $[Ca^{2+}]$ – and/or $[Mg^{2+}]$ – buffering capacity of the activating solutions are not great enough (see also Results and Discussion).

Yet another aspect of the diffusion problem must be solved in order to obtain information about the Ca^{2+} kinetics in the process of force activation. This is to provide a much more rapid Ca^{2+}-concentration change throughout the whole skinned muscle preparation, as compared to the recorded force changes.

The main aim of this paper is to show that one can overcome the above mentioned problems generated by diffusion and that one can produce relatively rapid $[Ca^{2+}]$ and $[Sr^{2+}]$ changes in skinned muscle preparations by adequately controlling the constituents of the bathing solutions.

This work represents an extention of some previously published results (Moisescu, 1973, 1976*a*, *b*; Ashley & Moisescu, 1973, 1975).

METHODS

Dissection and mounting of the preparation

The myofibrillar bundles were prepared under a microscope from single twitch muscle fibres (iliofibularis) of healthy frogs (*Rana esculenta*). After being blotted dry, the muscle was placed in a Petri dish filled with paraffin oil (Merck). On the bottom of the dish was a layer of Sylgard 184 (Dow Chemicals) in which fine pins could be impaled to facilitate the dissection. One single fibre was subsequently isolated and skinned under oil with two pairs of no. 5 jewellers forceps and sharp needles. Alternatively, the fibres were skinned under oil in a droplet of 'relaxing solution' (Table 2, solution B). Thinner bundles of myofibrils were obtained from the skinned fibre by splitting the thicker bundles obtained initially. One end of the myofibrillar bundle chosen for an experiment was carefully tied with a very fine surgical silk or nylon thread (20 μm), and the dissecting chamber was then moved under the mounting device. Subsequently this end of the preparation was securely tied under oil to a stainless-steel rod attached directly to the anode peg of the RCA 5734 mechanoelectric transducer. The other end was clamped in a pair of fine stainless steel Barcroft forceps (J. Weiss & Sons, London) which when closed do not hold liquid between the points (see Ashley & Moisescu, 1977 for the diagram of the experimental set-up). The myofibrillar bundle was then cut behind the forceps, the dissecting chamber was removed, and the preparation was lowered into a standard relaxing solution B (Table 2). After at least 10 min the preparation was slightly stretched until the measured resting force was about 0·01 mN, and then the diameter and the sarcomere lengths were measured. This resting force corresponds to average sarcomere lengths in the range 2·2–2·4 μm if the preparations had a diameter roughly

between 40 and 70 μm (see also Endo, 1972*a*). For measuring the sarcomere length, a Leitz UMK 50/0·6 long working-distance objective was used. The fibres were kept cold throughout the preparation procedure and were used within 6 hr after the animals had been sacrificed.

Measuring and recording apparatus

The mechanical arrangement of the tension transducer is similar to that previously described (Ashley & Moisescu, 1977). The output from the bridge circuit went either to a Tektronix storage oscilloscope or to a pen recorder (Schwarzer, Varioscript V 242). The sensitivity of the measuring system was 300 V/N and the natural frequency 166 sec^{-1}. The overall extraneous compliance was under 2 mm/N.

Chamber and changing of solutions

The solutions were contained in a series of thirteen rectangular wells (vol. 4 ml.) drilled out of a Perspex block and were covered by a hollow Perspex lid containing an opening through which the myofibrillar preparation could be immersed into each particular solution. The chamber was constructed such that a mixture of water and glycerol could be circulated around each well and through the lid. By using a thermostat with pump (Haake, Typ E12) and a refrigerating unit (Haake K11) it was possible to control the temperature of the solutions and of the air above them within $\pm$ 1 °C. The Perspex block containing the solutions could slide in the space between the lid and a metallic frame which was attached to a micromanipulator. A photomultiplier tube (EMI 9635) could be placed under the chamber and this was used to measure the intensity of light emitted by the Ca-sensitive photoprotein aequorin (Ashley, Moisescu & Rose, 1974) in some experiments.

The method employed for changing solutions was similar to that of Hellam & Podolsky (1969) and provided a rapid and uniform way for applying a solution along the whole length of the preparation.

The procedure of changing solutions was started by lowering the whole chamber by 5–6 mm until the preparation (whose position did not change) was above the walls which separate the wells, but still under the lid; then the block with solutions was slid until the desired solution was under the preparation, and finally the whole chamber was raised, such that the preparation was about 3 mm under the level of the solution. This set of movements could be carried out in 1–2 sec. The preparation does not appear to be damaged by being moved through an air-solution interface (Ford & Podolsky, 1972*a*; Ashley & Moisescu, 1977).

The apparent affinity constants of Ca^{2+}, Sr^{2+} and Mg^{2+} to different ligands

Significantly different values have been quoted in the literature particularly for the affinity constants of Ca^{2+}, Sr^{2+} and Mg^{2+} to EGTA and ATP (Sillén & Martell, 1964, 1970; Ogawa, 1968; Godt, 1974). Furthermore the values reported have been obtained for conditions (e.g. temperature, ionic strength) different from those used in many physiological experiments. We have developed some relatively simple potentiometric methods for determining the *apparent* affinity constants of Ca^{2+}, Sr^{2+} and Mg^{2+} for ATP, EGTA (ethanedioxy*bis* (ethylamine) tetraacetic acid), creatine phosphate, and other ligands in a medium having the desired cationic composition These methods have been used to determine the apparent affinity constants of the divalent cations for various ligands under a wide range of experimental conditions (see Table 1, Moisescu, 1976*a*, *b*; Miller & Moisescu, 1976; Ashley & Moisescu, 1977), and will be described in detail elsewhere.

Solutions

The bathing solutions were prepared using a similar procedure to that previously described (Moisescu, 1973; Ashley & Moisescu, 1977).

Table 2 lists the solutions of type A and B (Ashley & Moisescu, 1977) from which a set of solutions having different free Ca levels but an otherwise identical ionic composition were directly obtained. All precautions mentioned in this paper concerning the preparation of solutions and the calculation of $[Ca^{2+}]$ were also taken.

For the preparation of the set of solutions with a different free Sr concentration but with an otherwise identical ionic composition we also mixed only two solutions, B and A′. The solution of

TABLE 1. Measured values for the apparent binding constants of various ligands, K_L^{app}, for Ca^{2+}, Mg^{2+}, Sr^{2+}

Ligand	Cation	K_L^{app} (M^{-1})	Experimental conditions				Observations
			K^+ (M)	Na^+(M)	pH	t(°C)	
ATP	Mg^{2+}	4900 ± 400	160	35	7·10	20	See Moisescu, 1976*a*
		5800 ± 400	135	35	7·10	0	
		6500 ± 500	135	35	7·10	20	
	Ca^{2+}	3400 ± 300	135	35	7·10	20	
		2900 ± 300	135	35	7·10	0	
	Sr^{2+}	1400 ± 200	135	35	7·10	20	
EGTA (mol. wt. 380·4)*	Mg^{2+}	46 ± 6	100–200	35	7·10	20	See Moisescu, 1976*a*,
		25 ± 5	100–200	35	7·10	0	Ashley & Moisescu,
	Ca^{2+}	$(5 \pm 0{\cdot}5)10^6$	100–200	35	7·10	0–20	1977; Moisescu &
	Sr^{2+}	$(2 \pm 0{\cdot}2)10^4$	100–200	35	7·10	0–20	Ashley, 1977.
HDTA (mol. wt. 348·4)*	Mg^{2+}	$8 \pm 1{\cdot}5$	100–200	35	7·10	0–20	See Moisescu, 1976*a*
	Ca^{2+}	$6{\cdot}5 \pm 1$	100–200	35	7·10	0–20	
	Sr^{2+}	negl.	100–200	35	7·10	0–20	
CP	Mg^{2+}	$12 \pm 1{\cdot}5$	100–200	35	7·10	0–20	See Moisescu, 1976*a*
	Ca^{2+}	$\leqslant 20$	100–200	35	7·10	0–20	
	Sr^{2+}	$8 \pm 1{\cdot}5$	100–200	35	7·10	0–20	
TES	Mg^{2+}, Ca^{2+}, Sr^{2+}	negl.	100–200	35	7·10	0–20	See Moisescu, 1976*a*

* Note that the diffusion coefficient of EGTA should be very similar to that of HDTA.

Abbreviations used: TES = *N* − tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid; CP = creatine phosphate; HDTA = hexamethylenediamine *NNN'N'*-tetraacetic acid; EGTA = ethanedioxy*bis*(ethylamine)tetraacetic acid; ATP = adenosine triphosphate; ADP = adenosine diphosphate; P = inorganic phosphate; CK = creatine kinase.

TABLE 2. Composition of solutions

Solution	K (mM)	Na (mM)	TES (mM)	ATP total (mM)	Mg total (mM)	CP (mM)	CK (U/ml.)	HDTA (mM)	EGTA (mM)	CaEGTA (mM)	SrEGTA (mM)	Mg^{2+} (mM)	Cl (mM)	pH	temp. (°C)
A	137	36	60	8	8·05	10	15	—	—	50	—	1	20	7·10	25
A′	137	36	60	8	8·5	10	15	—	10	—	40	1	20	7·10	25
A″	137	36	60	8	8·3	10	15	—	5	—	45	1	20	7·10	25
B	137	36	60	8	10·25	10	15	—	50	—	—	1	20·5	7·10	25
H	137	36	60	8	8·45	10	15	50	—	—	—	1	20	7·10	25
AL	137	36	140	8	7·94	10	15	—	—	50	—	1	18	7·10	2
A′L	137	36	140	8	8·18	10	15	—	10	—	40	1	18	7·10	2
BL	137	36	140	8	9·15	10	15	—	50	—	—	1	18·3	7·10	2
HL	137	36	140	8	8·33	10	15	50	—	—	—	1	18	7·10	2
A′1	160	36	140	8	2·8	10	20	—	12·66	—	37·33	0·1	6	7·10	25
B1	160	36	140	8	2·95	10	20	—	50	—	—	0·1	6	7·10	25
H1	160	36	140	8	2·8	10	20	50	—	—	—	0·1	6	7·10	25

LR = solution of type H, HL, H1 mixed with a solution of type B, BL, B1 in a proportion higher than 300:1, respectively (see also the legends of the Figures).

type B was identical to the solution B used for the preparation of a set of solutions covering a large [Ca^{2+}] range. The solution of type A′ contained however only 80% Sr-EGTA (and 20% EGTA) and its total Mg concentration, Mg_A, was accordingly modified (see Ashley & Moisescu, 1977).

Under our conditions the pH and the free Mg concentration in any intermediate solutions obtained from A′ and B did not differ by more than 0·01 and 5% respectively. We did not use a generating solution of type A containing equimolar amounts of Sr and EGTA as had been the case for the Ca^{2+} solutions due to the considerably smaller apparent affinity constant of Sr^{2+} to EGTA in comparison with that of Ca^{2+} (see Table 1), which would have led to significantly different pH values and free Mg concentrations in the intermediate solutions. The free Sr concentration has been calculated from an equation similar to that used for calculating [Ca^{2+}] (see Ashley & Moisescu, 1977).

In addition to the solutions of type A, A′ and B we have used another solution of type H, which contained HDTA (hexamethylenediamine-*NNN′N′*-tetraacetic acid; see Results and Discussion) instead of EGTA. The composition of this solution is also shown in Table 2. The total Mg concentration in this solution was calculated in a manner similar to that for solutions of type B.

The stock solutions of type A, A′ and B which contained 50 mM-total EGTA, and of type H which contained 50 mM-HDTA were prepared 24 hr before the experiment, except for the presence of ATP, creatine phosphate (CP) and creatine kinase (CK) and were stored overnight in the refrigerator. Before the experiment the solutions were brought to the working temperature. ATP and CP were then added solid and pH was adjusted to $7{\cdot}10 \pm 0{\cdot}01$ with known amounts of KOH (usually less than 1 mM). Then CK was added solid and the solutions were mixed in the appropriate proportions. The pH value was then checked for the whole set and did not differ usually by more than 0·01 pH unit.

Sr-EGTA was prepared in a similar manner to Ca-EGTA (Ashley & Moisescu, 1977) from equimolar amounts of EGTA and $SrCO_3$ in water (final pH < 4·5) aided by gentle heating (10–15 min at 80 °C) to displace the equilibrium of H_2CO_3 towards CO_2 and H_2O. Thus we have been able to increase to a maximum the amount of total EGTA in the solution for a given total concentration of monovalent cations.

In order to increase the accuracy of the estimation of the *relative* Ca^{2+} and Sr^{2+} concentrations, the amount of *free* EGTA has been directly titrated with $CaCl_2$ (Moisescu & Pusch, 1975) at the end of an experiment in each well in the presence of excess $MgCl_2$ ([Mg^{2+}] 10 mM). The values thus obtained have been generally very similar to those expected from the mixing of the solutions.

The standard solutions were aimed to give similar values to those found *in vivo* for: [Mg^{2+}] (1 mM; Ashley & Ellory, 1972; Endo & Thorens, 1975; [K^+] + [Na^+] (170 mM; Adrian, 1956), pH (7·1 Caldwell, 1958).

A solution in which no active steady-state force response is measured is generally called a *relaxing* solution as opposed to an *activating* solution. The free Ca concentration in what is called here a relaxing solution was lower than 3×10^{-8} M.

RESULTS

Changes in EGTA-buffered [Ca^{2+}] generated by a change in intramyofibrillar pH

One can calculate based on the stability constants of different cations to ATP, ADP (adenosine diphosphate) and inorganic phosphate (P_i) (Sillén & Martell, 1964, 1970) that the hydrolysis of 1 mole ATP to ADP and P_i releases about 0·5–0·7 mole H^+ at pH 6·8–7·1, [Mg^{2+}] = 0·1–1 mM, ionic strength 0·1–0·2 M and temperature 0–25 °C.

The use of an ATP – regenerative system such as creatine phosphate and creatine kinase to maintain constant the ATP concentration during activation does not reduce much the pH gradient in the preparation, it only changes its sign. This is because hydrolysis of 1 mole creatine phosphate into creatine and P_i would absorb 0·3–0·5

mole protons under similar conditions as above. This estimate is based on the observation that at pH 6·6–7·6, creatine phosphate is essentially a divalent anion, while creatine is neutrally charged.

Furthermore it can be calculated that even in the presence of 10 mM of an ideal pH buffer at pH 7·00, the measured ATP-ase activity of the frog fibres at 0 °C (about 1 mM sec^{-1}) (Infante & Davies, 1964) could lead to such a pH gradient within a 100 μm thick preparation that the apparent affinity constant of Ca^{2+} to EGTA in the preparation would differ up to a factor of two from that in the bathing solution. Accordingly, the free Ca concentration within the preparation will be quite different from that in the external solution, and then it is quite difficult to associate an observed steady-state level of force to a free Ca concentration in the bathing solution.

This discrepancy between the intrafibrillar and external [Ca^{2+}] is expected to be enhanced in activating solutions having a smaller pH buffering capacity, a higher temperature, or where the sarcolemmal diffusion barrier has not been completely removed.

The presence of an important pH effect due to the hydrolysis process of creatine phosphate or ATP associated with the mechanical response in skinned muscle fibres is suggested by the experiment shown in Fig. 1*A*.

Here the preparation has been initially activated in a Ca solution in which the relative tension response was about 10% of its maximum value, and then the preparation has been transferred into air with a high relative humidity at the same temperature. One can see that after the contact between the preparation and the activating solution has been interrupted, there is a drop in the force. This is most likely due to the hydrolysis of creatine phosphate which is associated with an alkalinization of the intrafibrillar medium (see above) and thus with a drop in free Ca concentration. From other experiments it is known that the Ca^{2+} activation curves of skinned muscle fibres are shifted by varying the pH in the bathing medium (Ashley & Moisescu, 1974; 1977; Ebashi, Nonomura, Kitazawa & Toyo-oka, 1975). The corresponding shift in the apparent affinity constant of Ca^{2+} to EGTA is however larger and consequently an increase in pH should lead to a lower force response whilst a drop in pH should result in a higher relative force level when the initial force response is submaximal. After about 30 sec, when presumably most of the creatine phosphate has been hydrolysed, the concentration of ATP starts to fall. The hydrolysis of ATP is associated with an acidification of the intrafibrillar medium and thus with an increase in [Ca^{2+}] buffered with EGTA. As a result the isometric force rises to much higher levels. Indeed, force is expected to reach higher values than the initial level since under our conditions the apparent hydrolysis of 10 mM-creatine phosphate should require fewer protons than would be produced during the hydrolysis of 8 mM-ATP. After another 20–30 sec it is possible that a rigor state is also developed in different regions of the preparation. When the myofibrillar bundle is reintroduced into the solution force falls to its initial level.

The immediate drop in force after the preparation has been lifted into the air cannot be explained by the activity of the sarcoplasmic reticulum since the activating solution contained 10 mM-caffeine and under this condition it can be shown that the sarcoplasmic reticulum cannot be loaded with Ca (see Fig. 4*D* and later). If the activating solution contains only ATP as in Fig. 1*B*, then force has immediately the

tendency to rise when the preparation is suspended in the air and not to fall as in Fig. 1*A*, since here the direct hydrolysis of ATP acidifies the intrafibrillar medium.

The pH-buffering capacity and the activity of creatine kinase in the solutions shown in Table 2 were adequate for experiments with myofibrillar bundles thinner than 90 μm in diameter.

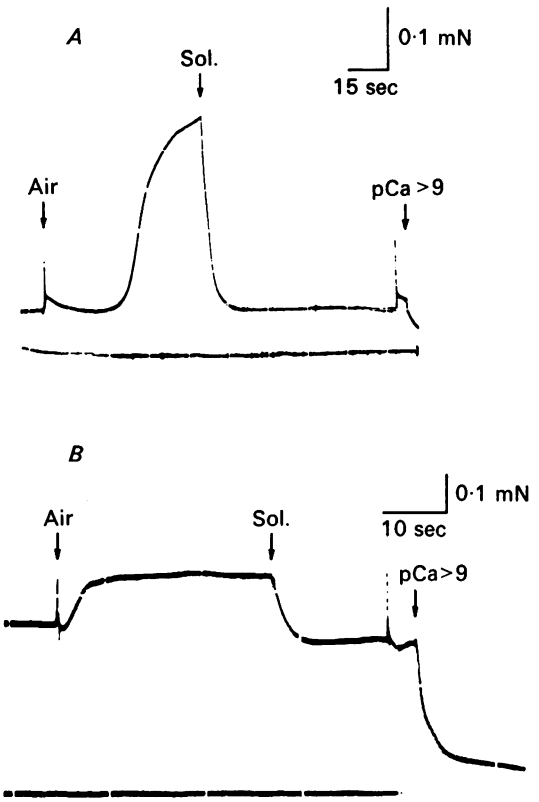

Fig. 1. Force responses in frog myofibrillar preparations when lifted into the air after being equilibrated in an activating solution at pCa 6. The activating solution for *A* was of type A/B (Table 2) and contained both CP and CK, while the activating solution for *B* was similar to that for *A* but did not contain CP and CK. The relative steady-state level for force in *B* is higher than in *A* due to a lower concentration of monovalent cations in the solution without creatine phosphate (Ashley & Moisescu, 1977). At the arrows marked with Sol, the preparations were reintroduced in the respective solutions. Both activating solutions in *A* and *B* contained 10 mM-caffeine. Characteristics of the preparations: diameter: 80 μm in *A*, 90 μm in *B*; length 1·1 mm in *A*, 1·2 mm in *B*; sarcomere length in *A* and *B* 2·2 μm. Temperature 22 °C.

An increase of both the TES concentration from 60 to 140 mM (4–22 °C) and that of creatine kinase from 15 to 50 U/ml. did not have any effect upon the rate of tension development or on the relative steady-state level. However, it appears from other experiments that the capacity of the preparation to develop force is gradually diminished in time when the concentration of the zwitterion form of TES is higher than about 200 mM, even if the myofibrillar bundle is kept only in a relaxing solution.

Like other workers in this field (Hellam & Podolsky, 1969; Julian, 1971; Endo,

1972*a*, *b*) we have also observed that force develops very slowly (over periods of minutes) in skinned muscle preparations from both barnacle and frog if the pH-buffering capacity of the bathing solution was low (TES 10 mM, pH 7·10, temp. 0–22 °C; Moisescu, 1973, 1975; Ashley & Moisescu, 1974) and if the relative steady-state force was around 10%.

It is very likely that this very slow process of force development in these experiments was due to the slow acidification of the intramyofibrillar medium which resulted in a slow increase in the internal free Ca concentration.

Evidence for this interpretation is provided by the experimental records obtained in the presence of a high concentration of pH buffer which are shown in Figs. 2 and 4 (also Fig. 2*a*, Moisescu 1976*a*, and Fig. 3 for low temperature). Here force rises quite rapidly with half times of about 1 sec or less although the relative steady-state force levels correspond to not more than 10%.

Rapid [Ca^{2+}] *and* [Sr^{2+}] *changes in myofibrillar preparations*

In recent years we have drawn attention to an activating procedure which can speed up the changes in the buffered concentration of an activator (Ca^{2+} or Sr^{2+}) throughout the skinned muscle preparation (Moisescu, 1973, 1975; Ashley & Moisescu, 1973). More recently this procedure has been further developed such as to enable a quantitative interpretation of the isometric force responses (Moisescu, 1976*a*).

This procedure of producing rapid concentration changes of an activator within a myofibrillar bundle is based on a simple principle. Let it be assumed that the preparation is represented by a cylinder, in which the free Ca concentration is maintained very low (10^{-9} M) by the presence of say 0·15 mM-$EGTA^{2-}$. At a given moment the cylinder is introduced in a solution containing for example 25 mM-$EGTA^{2-}$ and 25 mM-Ca-$EGTA^{2-}$ ([Ca^{2+}] ~ $2 \cdot 10^{-7}$ M, Table 1). From the formula describing the diffusion of a substance into a cylinder of radius a (see Crank, 1967, Fig. 5·3) one can calculate that in the middle of the cylinder the concentration of $EGTA^{2-}$ will reach a value of 2·82 mM at the moment $t = 0 \cdot 085\, a^2/D$ (where D is the diffusion coefficient for $EGTA^{2-}$).

If the diffusion coefficient of Ca-$EGTA^{2-}$ is considered to be identical to that of $EGTA^{2-}$, then the concentration of Ca-$EGTA^{2-}$ in the middle of the cylinder at the same moment $t = 0 \cdot 085\, a^2/D$ would be 2·69 mM. This makes a ratio of 0·95 between the concentrations of Ca-$EGTA^{2-}$ and $EGTA^{2-}$ in the middle of the cylinder as compared to 1 in the solution outside. This further implies that at time $t = 0 \cdot 085\, a^2/D$, the concentration of Ca^{2+} in *any* point in the cylinder would have been within 5% of that in the bathing solution. In fact the *average* value for [Ca^{2+}] in the cylinder would have been over 99% of that in the external solution at that moment.

If however the initial concentration of the *total* EGTA buffer in the cylinder ([Ca^{2+}] = 10^{-9} M) were the same as that in the 'activating' solution ([Ca^{2+}] = $2 \cdot 10^{-7}$ M), then the time elapsed (after introducing the cylinder in the 'activating' solution) to obtain the same ratio of 0·95 between Ca-$EGTA^{2-}$ and $EGTA^{2-}$ in the middle of the cylinder would be one order of magnitude higher.

The superiority of the first procedure to 'activate' the preparation in producing a more rapid [Ca^{2+}] change should be now quite obvious.

One could make an estimate for the time required for [Ca^{2+}] to reach in the middle of a preparation at least 95% of the value in the external solution (this being equivalent to an *average* value of over 99% for the whole preparation) if the diffusion coefficient of $EGTA^{2-}$ in the myofibrillar bundle was known.

We have actually measured the radial diffusion coefficient of $EGTA^{2-}$ in large myofibrillar preparations from barnacle by titrating with $CaCl_2$ (Moisescu & Pusch, 1975) the total amount of $EGTA^{2-}$ entering the preparation after different periods

of time. If the preparation was relaxed, the average value of the diffusion coefficient was $4{\cdot}6 \times 10^{-6}$ cm^2 sec^{-1} at room temperature, i.e. only about 20% smaller than that expected for free solution (Stein, 1967). However, if the solutions did not contain Mg-ATP and the preparation was in rigor, then the diffusion coefficient of $EGTA^{2-}$ was only about half the value mentioned above.

Using the value of $4{\cdot}6 \times 10^{-6}$ cm^2 sec^{-1} for D one could estimate that the time required to increase $[Ca^{2+}]$ to at least 95% of its value in the external solution throughout the preparation approximated to a cylinder of diameter $2a = 40$ μm (i.e. the *average* $[Ca^{2+}]$ in the preparation to reach 99% of its value outside) might be lower than 100 ms if the initial concentration in the cylinder was 0·15 mM and the preparation was activated in a solution with 50 mM total EGTA ($[Ca^{2+}] = 2 \times 10^{-7}$ M).

In more complicated calculations done on an IBM 470 Computer (Moisescu, 1973; Ashley & Moisescu, 1975) there have also been taken into consideration (*a*) additional Ca-binding sites (0·1–0·2 mM) distributed uniformly within the cylinder; (*b*) the rate constants of the Ca^{2+} binding to EGTA and release from Ca-EGTA (Podolsky, Berger & Czerlinski quoted by Hellam & Podolsky, 1969) and (*c*) different boundary conditions. These calculations also indicate that the free Ca concentration can be considered to be practically equilibrated (average $[Ca^{2+}]$ within preparation 95% of that outside) within 0·15 sec in the whole cross-section of a 50 μm thick bundle of myofibrils if (i) the activating solutions had 50 mM total EGTA (pCa 8–5·7); (ii) the initial EGTA concentration in the cylinder was between 0·05 and 0·15 mM and (iii) the solution around the cylinder was stirred during the first 0·15 sec after the change of solutions (see legend Fig. 2).

Similar calculations indicate that the time required for $[Ca^{2+}]$ to reach an *average* value of 95% of that in bulk solution within the 50 μm thick cylinder would be of the order of 15–20 sec if the initial EGTA concentration (pCa 9) was the same (50 mM) as in the 'activating' solution (pCa range 8–5·7), and if the solutions were not stirred (see Fig. 3*D*).

If the conditions imposed for the above theoretical treatment can be experimentally achieved, then one could design some experiments from which a direct estimate can be made about the length of time needed for actually changing $[Ca^{2+}]$ or $[Sr^{2+}]$ from one buffered level to another within the whole preparation.

The experimental procedure to achieve a rapid change in $[Ca^{2+}]$ or $[Sr^{2+}]$ in a myofibrillar bundle seems fairly straightforward at first sight. Thus the preparation should be initially equilibrated in a relaxing solution ($[Ca^{2+}] < 3 \times 10^{-8}$ M) with a low EGTA concentration (e.g. 0·15 mM) called thereafter LR (or 'low relaxing') see also Table 2. Then the preparation should be activated in a solution with a high Ca^{2+}- (or Sr^{2+}-) buffering capacity (e.g. EGTA 25 mM, Ca-EGTA 25 mM; $[Ca^{2+}]$ 2×10^{-7} M). In addition sarcoplasmic reticulum should be made ineffective in accumulating or releasing Ca^{2+} (or Sr^{2+}) and the solution around the preparation should be stirred immediately after the preparation was introduced into it.

The problem becomes quite obvious when one considers the composition of these solutions, since one has to find a substitute for the Ca^{2+} (or Sr^{2+})-buffer (in this case EGTA) to be used in the 'low relaxing' solution, such that no changes in the cationic composition, ionic strength, and osmolarity will take place within the whole preparation for the whole duration when the higher EGTA concentration in the

activating solution diffuses within the preparation. Changes in any of these parameters may severely alter the level and thus the time course of force response (Ashley & Moisescu, 1977).

In order to satisfy all these conditions, the substitute has to be identically charged at neutral pH as the Ca- (Sr-) buffering system, to have a very low apparent affinity constant for Ca^{2+} (or Sr^{2+}) but a diffusion coefficient and an apparent affinity constant for Mg^{2+} similar to those of the Ca- (Sr-) buffer.

There are two such substances which very closely approach these ideal conditions when using EGTA to buffer Ca^{2+} and Sr^{2+}. These substances are: HDTA (Anderegg, 1964; Moisescu, 1976*a*, commercially available from Fluka A.G., F.R. Germany) and ODTA (octamethylenediamine-*NNN'N'*-tetraacetate; Anderegg, 1964).

In all the experiments reported here we have used only HDTA to replace EGTA (see Table 2).

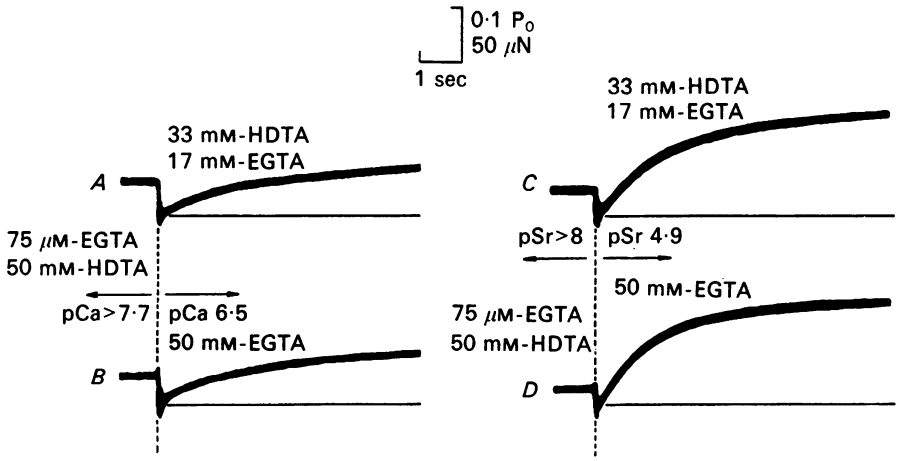

Fig. 2. Force development in a 1·4 mm long bundle of frog myofibrils when activated in $[Ca^{2+}]$- and $[Sr^{2+}]$-buffered solutions containing different concentrations of EGTA. The preparation (diameter 60 μm, sarcomere length 2·55 μm) has been equilibrated before each contraction in a 'low relaxing' solution (ratio sol. *B*/sol. *H* = 1/667). The vertical dashed lines indicate the moment when the preparation has been introduced in the activating solutions. Note that the solution round the preparation was always 'stirred' for about 0·2 sec while moving the preparation from the air-solution interface to its final position, about 3 mm under the surface. The activating solutions for *B* and *D* were of type A/B and A'/B respectively, and those for *A* and *C* were obtained by mixing one volume of solution for *B* or *D* respectively with two volumes of solution *H*. The thin horizontal lines represent the 'zero' level for active force. Temp. 25 °C.

A first type of experiment from which one can gain direct information about the time required to produce a 'rapid' Ca^{2+}- (or Sr^{2+}-) concentration change within a myofibrillar preparation is illustrated in Fig. 2. In this experiment the Ca^{2+}- (or Sr^{2+}-) buffering capacity of the activating solution is varied by replacing some of the EGTA with HDTA.

In Fig. 2*A*, *B* the preparation has been initially equilibrated in a relaxing solution containing only 75 μM total EGTA and then was activated in two solutions in which the free Ca concentration and the other cationic conditions were identical, but in which the total EGTA concentration was 16·7 mM for Fig. 2*A* and 50 mM for Fig. 2*B*

respectively. The activating solution for Fig. 2*A* contained 33·3 mM-HDTA to replace the same amount of EGTA. In Fig. 2*C*, *D* the preparation has been activated in two [Sr^{2+}]-buffered solutions, both having a pSr (= -log [Sr^{2+}]) value of 4·9 and the same free cationic concentrations but different total EGTA concentrations, similar to the solutions in Fig. 2*A*, *B*.

[Ca^{2+}] and [Sr^{2+}] are expected to equilibrate significantly faster (by a factor of at least 2) for the traces in Fig. 2*B* and *D* than for those in Fig. 2*A* and *C* respectively, since the difference in the Ca- and Sr-buffering capacity between the activating and the relaxing solutions is much higher in the former case than in the latter. However, the difference between the time course of the force responses in Fig. 2*A* and *B* and also in Fig. 2*C* and *D* is only minor, of the order of 0·1–0·2 sec.

This directly suggests that when using the activating procedure in Fig. 2*B*, *D*, the time of [Ca^{2+}] or [Sr^{2+}] equilibration within the whole preparation (diameter about 60 μm) is of the order of 0·1–0·2 sec. In addition, the result indicates that the rate limiting step in the process of force activation in this experiment was not that of [Ca^{2+}] and [Sr^{2+}] equilibration.

An important conclusion regarding the composition of the solutions can also be drawn from the experiment in Fig. 2. Thus one should notice that the absolute steady-state levels in each pair of traces in Fig. 2*A* and *B* and Fig. 2*C* and *D* are essentially identical, although the activating solutions contained quite different amounts of total EGTA. This strongly suggests that each pair of solutions were very well balanced: otherwise small differences in the cationic concentrations would have resulted in relatively large differences in the steady-state tension responses in this range. This shows that HDTA is indeed a good substitute for EGTA. In addition, other experiments have shown that the maximum Ca activated tension response in the presence of 50 mM-K_2-HDTA ([Ca^{2+}] = 20 μM) or 50 mM-K_2 Ca-EGTA is precisely the same (Moisescu, 1976*a*; Moisescu & Thieleczek, 1978) and is significantly larger than in the presence of 100 mM-KCl ([Ca^{2+}], 20 μM) (see also Ashley & Moisescu, 1977).

In another type of experiment we have used the Ca-sensitive photoprotein aequorin (see review by Blinks, Prendergast & Allen, 1976) to determine the time interval for [Ca^{2+}] equilibration in the myofibrillar preparation over a wide range of concentrations.

If one loads a myofibrillar bundle (diameter $\geqslant$ 50 μm) with aequorin and introduces it into an activating solution, then most of the aequorin will stay within the preparation for the first few seconds due to a very small value of its diffusion coefficient. Therefore one can draw conclusions about the free Ca changes which occur in the preparation immediately after activation from the changes in the intensity of the light emitted by aequorin.

The *radial* diffusion coefficient of aequorin was measured as about 10^{-7} cm^2 sec^{-1} at 20 °C (Ashley *et al.* 1974; Ashley & Moisescu, 1975) in myofibrillar preparations and is similar to the value of $0{\cdot}5–0{\cdot}7 \times 10^{-7}$ cm^2 sec^{-1} found by Rüdel, Taylor & Blinks (1976) for the *longitudinal* diffusion coefficient of aequorin in single frog fibres.

The feasibility of this kind of experiment has been demonstrated recently (Ashley *et al.* 1974; Ashley & Moisescu, 1975) and in Fig. 3 we present a set of records showing the simultaneous tension and light response in several preparations when activated in different Ca-buffered solutions.

The preparations were initially equilibrated in a relaxing solution for at least 3 min; then 30–60 nl. of the same relaxing solution but including 0·1–0·2 mM-aequorin was added to the

middle segment of the preparations while suspended in the air. Then the bundles of myofibrils were introduced into paraffin oil at the same temperature, for at least 5 minutes to allow aequorin to distribute uniformly within the cross section of the myofibrillar bundles. Subsequently the preparations were rinsed briefly (1–2 sec) in the relaxing solution in which they had initially been equilibrated, and finally they were introduced into the appropriate activating solution (see also Ashley *et al.* 1974; Ashley & Moisescu, 1975).

When the preparation was initially equilibrated in a relaxing solution with a relatively low Ca^{2+}-buffering capacity (total EGTA = 0·15 mM) and then activated in a solution with 50 mM total EGTA (pCa range 6·00–5·74), light intensity reaches a steady-state value much faster than the isometric force response (Fig. 3*A*, *B*, *D*).

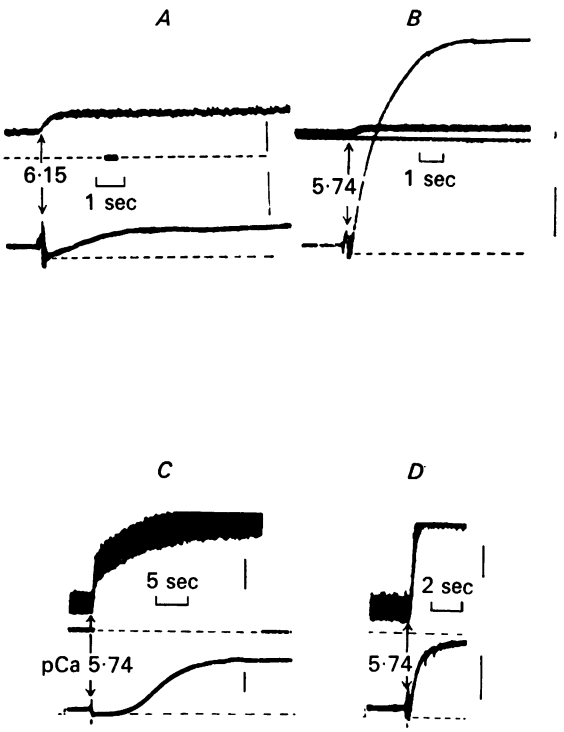

Fig. 3. Simultaneous force (the lower traces in each part) and light intensity (top traces) recordings from frog myofibrillar preparations loaded with aequorin (see text) after being activated in solutions of type AL/BL (Table 2) at 2–3 °C. The pCa values of the activating solutions are indicated by arrows. All solutions contained 10 mM-caffeine. In *A*, *B*, *D* the preparations have been initially equilibrated in a 'low relaxing' solution (BL/HL = 1/333, pCa about 8) and only then they have been activated. In *C* the preparation has been activated from a relaxing solution of type BL/AL (pCa 8) containing the same total concentration of EGTA as the activating solution. Calibration bars: 20 nA for the current from the photomultiplier tube which has been filtered with a time constant of 25 msec, and 0·1 mN for the force response. The 'zero' current and the 'zero active force are indicated by dashed lines. Diameter of the preparations: 65 μm for *A*; 85 μm for *B*; 70 μm for *C* and *D*. Sarcomere length around 2·2 μm in all three preparations. The 'dark' current was 15 nA in all experiments.

In Fig. 3*C* the same preparation as in Fig. 3*D* has been initially equilibrated in the relaxing solution of type B, which had the same total EGTA concentration as the activating solution (50 mM). Then it was loaded with aequorin and subsequently it

was activated in the same solution as for Fig. 3*D*. One can observe that in Fig. 3*C* both tension and intensity of light emitted by aequorin reach very slowly a steady-state level. This clearly demonstrates that the delayed tension responses in Fig. 3*C* as compared with Fig. 3*D* is due to the slow free Ca equilibration within the myofibrillar preparation. The aequorin light intensity was still increasing after about 15 sec, indicating that a substantial amount of aequorin was still within the preparation even after this long time. This observation is in agreement with the very low apparent diffusion coefficient of the photoprotein in the preparation (see above).

All solutions in Fig. 3 contained 10 mM-caffeine in order to avoid unwanted release of Ca from the sarcoplasmic reticulum and to inactivate the capacity of the preparation to accumulate Ca during the tension rise (see Discussion). These experiments have been done at 2 °C in the same solutions as those used for determining the kinetics of tension activation (Moisescu, 1976*a*, *b*) in order to obtain an experimental estimation of the $[Ca^{2+}]$ equilibration times in the preparation when using HDTA to replace most of the EGTA in the relaxing solution (see above).

From the aequorin experiments it can be estimated that the time required for $[Ca^{2+}]$ in the whole preparation to reach about 90–95% of the value in the activating solution is of the order of 0·1–0·15 sec for a myofibrillar bundle of *ca.* 50 μm in diameter, when using the activation method in Fig. 3*A*, *B*, *D*. One should also take into consideration that this value might be an overestimate since (i) at this temperature (2 °C) the aequorin light response follows the changes in free Ca concentration with considerable delay (rate constant 30 sec^{-1}; van Leuwen & Blinks, 1969), (ii) in this pCa range the intensity of light emitted by aequorin is proportional to $[Ca^{2+}]^n$, where n is at least 2 (Moisescu & Ashley, 1977), and (iii) the Ca^{2+}-binding properties of the relaxing solution containing aequorin might have considerably increased the Ca^{2+}-buffering capacity of the preparation immediately before activation (see above).

In conclusion there are several independent types of approaches which all indicate that when using the procedure described here, one can produce a nearly complete Ca^{2+}- and Sr^{2+} concentration change within 0·1–0·15 sec in a preparation of about 50 μm in diameter.

The part played by the sarcoplasmic reticulum

If one wishes to decrease as much as possible the time required for changing Ca^{2+} or Sr^{2+} concentrations within the preparation by using the method described above, one should try to stop the movements of these divalent ions associated with the sarcoplasmic reticulum.

Bearing this in mind we had to investigate some of the properties of the sarcoplasmic reticulum in our solutions and to find an efficient way of inactivating it without interfering with the contractile machinery. Thus we have found that the ability of the reticulum to accumulate and release Ca^{2+} and Sr^{2+} remained high in all present solutions unless additional substances such as detergents, caffeine, or Ca ionophores were included in the bathing solutions.

We were also satisfied that the presence of 10 mM-caffeine in all our bathing solutions did render the sarcoplasmic reticulum ineffective in accumulating or releasing large amounts of either Ca^{2+} or Sr^{2+}. These observations are summarized in Fig. 4.

Thus if the Ca-accumulating stores were initially depleted and the preparation was

activated in a caffeine containing solution, then force developed quite rapidly reaching a steady-state level which was maintained constant over long time intervals (the steady-state force in Fig. 4*A* corresponds to about 10% maximum force, P_0). If the preparation was however incubated for about 4 min in the same activating solution as for trace *A* but without caffeine and then was transferred into the same solution but with caffeine, then a large force transient was observed (trace *B*). Force rose relatively rapidly to a peak representing about 85% of maximum force P_0 under these conditions and then fell steadily to a value similar to that in the solution without caffeine.

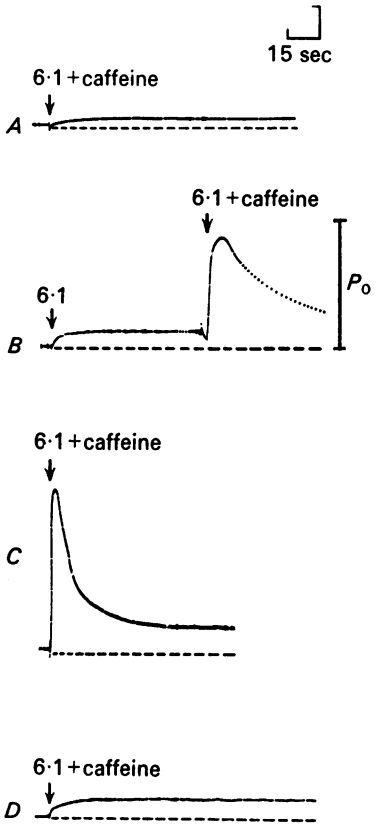

Fig. 4. Force responses from frog myofibrillar preparations when activated in a solution of type A/B, pCa 6·1 (Table 2) after equilibration for at least 3 min in a 'low relaxing' solution (pCa 8) with 0·1 mM-EGTA (B/H = 1/500). In *A*, the Ca accumulating stores have been initially depleted and only the activating solution contained 10 mM-caffeine. In *B*, the preparation (with depleted Ca stores) has been first activated in the solution without caffeine for 4 min and then it has been transferred in an otherwise identical solution which contained in addition 10 mM-caffeine. The transient force response reached about 85% of P_0. In *C*, the preparation has been loaded with Ca before being equilibrated in the 'low relaxing' solution, and here the activating solution contained 10 mM-caffeine. The peak reached by tension corresponds here to maximal tension P_0. In *D*, the preparation has been activated in an identical solution as for *C*, but before being equilibrated in the 'low relaxing' solution, the myofibrillar bundle has been maintained for 10 min in the activating solution with 10 mM-caffeine. Dimensions of the preparations: length for *A*, *B*, *D* 1 mm, for *C* 1·1 mm; diameter for *A*, *B*, *D* 50 μm, for *C* 70 μm; sarcomere length 2·2 μm for all traces. Calibration bars for force: 0·25 mM for *A*, and 0·1 mM for *B–D*. Temp. 24 °C.

By loading the preparation with Ca and then applying the same activation procedure as for trace 4*A*, then maximum force P_0 was very rapidly but transiently reached and then force fell to a steady value representing about 15% P_0 (trace 4 *C*).

Evidence that the sarcoplasmic reticulum cannot be loaded with Ca^{2+} in the presence of caffeine is shown in Fig. 4*D*. Here the preparation was incubated for 10 min in a 'Ca-loading' solution as for traces *B* and *C*, which contained 10 mM-caffeine. Then the same procedure as for trace *C* was applied. However, the response is very similar to that in trace *A* rather than in trace *C* suggesting that the sarcoplasmic reticulum did not accumulate Ca^{2+} in the presence of caffeine. We have also increased both the ionized Ca level in the 'loading' solution with 10 mM-caffeine up to about 0·1 mM and the 'loading' time up to 15 min, but the response was consistently identical to that in Fig. 4*D*.

Similar results to those presented in Fig. 4 have also been obtained with Sr^{2+}. The ionized Sr concentrations were however about 25 times higher than those for Ca^{2+} to obtain equivalent values for the force responses.

It is noteworthy that if the preparations were first loaded with Ca (at pCa 6–6·1) or Sr (at pSr 4·5) as for the traces in Fig. 4*B* and *C* then one could obtain intermediate responses between traces *A* and *C* in Fig. 4 by simply 'reactivating' the preparations in the same loading solutions, after being initially equilibrated in a 'low relaxing' solution. This suggests that even in the presence of 1 mM-Mg^{2+} (Thorens & Endo, 1975; Endo & Thorens, 1975) one could still produce a kind of 'Ca-induced Ca-release' (Ford & Podolsky, 1972*a*, *b*; Endo *et al.* 1969; see also Fabiato & Fabiato, 1977) by *rapidly* increasing the level of Ca^{2+} or Sr^{2+} within the whole preparation to 10^{-6} and 3×10^{-5} M respectively.

We have also tried to inactivate the sarcoplasmic reticulum by using the non-ionic detergent Brij 36 (Orentlicher, Reuben, Grundfest & Brandt, 1974). This substance irreversibly inactivates the capacity of the myofibrillar preparation to accumulate Ca^{2+} or Sr^{2+}. However we have observed that the rate of tension development is very much decreased after the preparation has been maintained for several minutes in a relaxing solution with 0·5% Brij 36 (see also Endo, 1972*b*; Table 2). These and additional light microscope observations suggest that the homogeneity of the frog myofibrillar preparation is also partially destroyed by this detergent.

Fast force activation of skinned muscle fibres in Ca^{2+}- and Sr^{2+}-buffered solutions

It has been predicted (Ashley & Moisescu, 1972) and it has been recently demonstrated (Moisescu, 1976*a*) that the rate of tension development is strongly dependent upon the free Ca concentration in the activating solutions, under otherwise constant conditions. In this context we were interested to see if with the method described in this paper is possible to activate the frog myofibrillar preparation with rate constants approaching the values observed *in vivo* for tetanic stimulation. For this purpose the skinned muscle preparation was initially equilibrated in a relaxing solution containing 0·125 mM-EGTA (pCa ~ 8) and then maximally activated in solutions containing relatively high Ca^{2+} or Sr^{2+} concentrations buffered with 50 mM-EGTA.

Fig. 5*A–D* shows four such traces obtained at room temperature and at 2–4 °C with preparations of about 50 μm in diameter. The response in Fig. 5*A* was obtained with an activating solution containing equimolar amounts of Ca and EGTA (pCa ~ 4·6) at room temperature, while the other traces, *B–D*, represent force responses in different Sr^{2+}-buffered solutions. First one can observe that the apparent rate of tension development is about 4 times slower at 2 °C than at room temperature for both Ca^{2+}- and Sr^{2+}-activating conditions (see also Moisescu, 1976*a*, Fig. 2*h*), although the time course of the free Sr or free Ca concentration change within the

preparations due to the diffusion of the Sr^{2+} or Ca^{2+} buffer should be similar when the temperature difference is some 20 °C. As the relative tension–pCa (or pSr) relationship is also not greatly affected by these changes in the temperature (Ashley & Moisescu, 1977; D. G. Moisescu & R. Thieleczek, unpublished), the pronounced temperature effect upon the rate of force development can be only explained if the reaction steps in which Ca or Sr ions are apparently involved in producing force are 4 times faster at room temperature than at 2 °C under otherwise constant conditions.

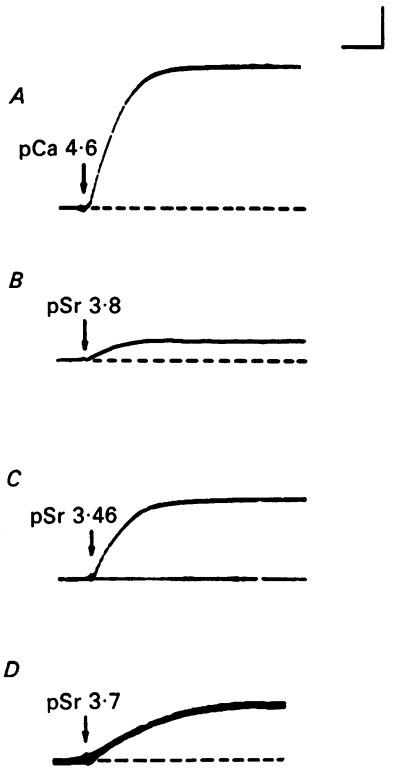

Fig. 5. Time course of force development in frog myofibrillar preparations activated in solutions with relatively high $[Ca^{2+}]$ or $[Sr^{2+}]$. *A*, the preparation (diameter 80 μm, length 1·4 mm, sarcomere length 2·3 μm) has been activated in solution A after being equilibrated in a 'low relaxing' solution (B/H = 1/400). Temp. 25 °C. *B*, the myofibrillar bundle (diameter 70 μm, length 2 mm, sarcomere length 2·25 μm) has been activated in solution A'1 (Table 2) after being equilibrated in a 'low relaxing' solution (B1/H1 = 1/400). Temp. 25 °C. *C*, the activation of the skinned fibre preparation (diameter 50 μm length, 1·5 mm, sarcomere length 2·2 μm) has been done in solution A'' after it has been equilibrated in a balanced 'low relaxing' solution (B/H = 1/400). Temp. 25 °C. *D*, after being equilibrated for several minutes in a 'low relaxing' solution (BL/HL = 1/400) the preparation (diameter 35 μm, length 1·2 mm, sarcomere length 2·2 μm) has been activated in a solution A'L. Temp. 2–3 °C. Calibration bars: vertical, 0·25 mN for *A* and *C*, 1 mN for *B*, and 0·1 mN for *D*; horizontal, 0·2 sec for *A*, *C* and *D*, and 0·14 sec for *B*.

Secondly, we have consistently obtained higher *maximal* rates of force development in Sr-EGTA solutions than in Ca-EGTA solutions, for otherwise similar conditions. This would be expected if the diffusion of the activator is still important in

determining the apparent rate of force activation for the experiments in Fig. 4*A–D*, since the *relative* [Sr^{2+}] should increase faster in the preparations over the range in which [Sr^{2+}] is higher than the apparent threshold for activation (Moisescu & Thieleczek, 1978) than the *relative* [Ca^{2+}] over the corresponding range (Moisescu, 1976*a*), due to a much lower affinity constant of EGTA for Sr^{2+} than that for Ca^{2+} under identical cationic conditions (see Table 2). In addition, the maximal rate of force development was always slightly faster for 0·1 mM-Mg^{2+} than for 1 mM-Mg^{2+} (Fig. 5, traces *B* and *C*). This observation can also be explained as above, by a diffusion limited process, since the relative force–pSr curve in the presence of 1 mM-Mg^{2+} is shifted towards lower [Sr^{2+}] when compared with the curve for 0·1 mM-Mg^{2+} (Donaldson & Kerrick, 1975).

In thinner preparations (between 30 and 40 μm) the maximal rate of tension development is consistently higher than in thicker preparations (above 60 μm). This also suggests that diffusion is still a limiting factor for the rate of tension development in Fig. 5*A–D*. However, the observed maximal rates for tension development are not inversely proportional to the square of the apparent diameter of the preparations. These results indicate that apart from diffusion there are also some other steps of reaction which limit the process of force activation and do not depend upon the Ca^{2+} or Sr^{2+} concentration. The apparent rate constants associated with these limiting steps of reaction should be of the order of 40–100 sec^{-1} at 20 °C for frog muscle fibres under our standard conditions.

DISCUSSION

In this paper an analysis has been made of the requirements which must be met by the bathing solutions in order to be able to distinguish between the effect of the activating divalent cations Ca and Sr upon the isometric tension response and other effects which are mainly due to pH changes and variations in the MgATP concentration within the preparation during activation. Thus, when using bundles of frog myofibrils of 50–100 μm in diameter all solutions must contain an ATP-regenerating system (Godt, 1974), a very high concentration of an efficient pH-buffer, a high concentration of Ca^{2+}- (or Sr^{2+}-) buffer (particularly when the ratio between the *total* Ca (or Sr) in the solution and that of the Ca (or Sr) buffer approaches unity), as well as a relatively strong Mg^{2+}-buffer. In addition, one should try to maintain a constant concentration for each ionic species in the whole set of solutions. When replacing an ionic species for another one, it is important to make sure that the effect of both ions upon the absolute tension response is identical (see Ashley & Moisescu, 1977) as is the the case for HDTA and EGTA (Fig. 2*A–D*; Moisescu, 1976*a*).

The most important aim of this paper was, however, that of describing and analysing in more detail a method for rapidly equilibrating the free Ca^{2+} and Sr^{2+} concentrations within the skinned muscle fibre preparations without disturbing the equilibrium of the other cationic species (Moisescu, 1973, 1976*a*, *b*; Ashley & Moisescu, 1973). This rapid equilibration of [Ca^{2+}] or [Sr^{2+}] is brought about by introducing the preparation into a solution with a much higher buffering capacity for the relevant ion than that of the preparation as a whole (solution in the interfilament space, sarcoplasmic reticulum, myofibrillar proteins), and appears to be important for maintaining a homogeneous preparation during an experiment. One can then use the same

myofibrillar bundle for many activation-relaxation cycles without greatly modifying the properties of the contractile apparatus or the homogeneity of the preparation (Moisescu, 1976*a*; Moisescu & Thieleczek, 1978).

It should be noted that a decrease in the EGTA concentration in a 'low relaxing' solution below 0·1 mM would not significantly contribute to a faster $[Ca^{2+}]$ or $[Sr^{2+}]$ equilibration in the myofibrillar preparation since the Ca-binding sites on the regulator proteins alone should have a contribution similar to that of about 0·1 mM-EGTA (Moisescu, 1975; Ashley & Moisescu, 1975).

We wish to emphasize that basically the same procedure can be used for producing both a sudden *increase* or a sudden *decrease* in $[Ca^{2+}]$ or $[Sr^{2+}]$ within the myofibrillar preparation. For producing a sudden decrease in $[Ca^{2+}]$ for example, one should initially equilibrate the preparation in an activating solution containing a low concentration of Ca-buffer and then introduce it into a solution having a very high buffering capacity around a higher pCa value. We have used this procedure for producing a very fast relaxation of the preparation (Moisescu, 1976*a*, Fig. 2*f*). Using a similar type of approach as pointed out in the Results one can calculate that in that particular experiment (Moisescu, 1976*a*, Fig. 2*f*), the free Ca concentration should have dropped under 3×10^{-8} M within few tens of milliseconds in the whole preparation.

It is important that both the contractile apparatus and the capacity of the sarcoplasmic reticulum to accumulate Ca (or Sr) are well preserved in the presence of 50 mM-$EGTA^{2-}$ or 50 mM-$HDTA^{2-}$. The addition of 10 mM-caffeine to the solutions induces the release of Ca (and Sr) from myofibrillar stores, and diminishes to a minimum the capacity of the sarcoplasmic reticulum to accumulate Ca and Sr. Based on these effects of caffeine it is possible to estimate the minimum amount of Ca which can be stored by the sarcoplasmic reticulum.

In the experiment illustrated in Fig. 4*B*, the preparation had been equilibrated for 4 min in a solution in which the relative steady-state force response was about 15 % P_0. During this time Ca was also accumulated by the reticulum. Then the preparation was placed in an otherwise identical solution including 10 mM-caffeine which released Ca from the stores and caused a transient tension rise reaching 85 % P_0. The increase in the free Ca concentration within the preparation was produced by both the release of Ca from the sarcoplasmic reticulum and by the acidification effect due to the release of protons as a result of Ca binding to EGTA. The acidification in turn decreased the apparent affinity constant of Ca^{2+} to EGTA. The free Ca concentration must have increased by a factor of at least 2·4 within the whole preparation to account for an increase in relative tension from 15 to 85 % P_0 (see Moisescu, 1976*a*; Moisescu & Thieleczek, 1978) particularly when the pH has dropped (Ashley & Moisescu, 1977). If X is the concentration *in* the preparation which has been reached by Ca released from the sarcoplasmic reticulum, then the *free* Ca concentration within the preparation would have been modified by the factor F,

$$F = C \times H \tag{1}$$

where

$$C = ([\text{CaEGTA}] + X) \times ([\text{EGTA}]) \times ([\text{EGTA}] - X)^{-1} \times ([\text{CaEGTA}])^{-1} \tag{2}$$

represents the contribution of the released Ca to enhance the ratio [CaEGTA]/[EGTA], and

$$H = ([\text{TES}] + 2X)^2 \times ([\text{TES}^-] - 2X)^{-2} \times ([\text{TES}^-])^2 \, ([\text{TES}])^{-2} \tag{3}$$

represents the contribution of the pH change (due to the release of two protons for each Ca bound to EGTA) to the decrease of the apparent affinity constant of Ca^{2+} to EGTA.

In eqn. (3) $[TES^-]$ is the concentration of the negatively charged form of the pH buffer and [TES] is the concentration of the neutral form. For the conditions under which the tension response in Fig. 4*B* has been obtained ([CaEGTA] = 40 mM, [EGTA] = 10 mM, $[TES^-]$ = 17 mM, [TES] = 43 mM) and considering that $F \geqslant 2{\cdot}4$, then from eqns. (1)–(3) the result is that $X \geqslant 1{\cdot}8$ mM. This value is very similar to that obtained by Ford & Podolsky (1972*a*), when using radioactive Ca, and is much higher than those obtained with vesicles of the sarcoplasmic reticulum (e.g. Hasselbach, 1964; Ogawa, 1970) suggesting that the accumulating capacity of the *intact* reticulum is much higher than that of the vesicles. One can also observe that the increase in the EGTA-buffered $[Ca^{2+}]$ is mainly due to the changes in pH even in the presence of 60 mM-total TES. This again emphasizes the necessity of using very high concentrations of pH buffers when performing Ca release experiments in the presence of pH-sensitive Ca (or Sr-) buffers such as EGTA.

The rate of Ca^{2+} accumulation by the sarcoplasmic reticulum appears however to be low. Thus in order to load the reticulum effectively in the solutions used in Fig. 4 (pCa 6·1) one has to maintain the preparation for about 2 min after the rapid equilibration of $[Ca^{2+}]$. This corresponds to uptake rates of the order of about 20 n-mole (g muscle)$^{-1}$s^{-1}. This rate is expected to rise by at least 5 times if the free Ca concentration in the medium is higher, as some experiments of loading the sarcoplasmic reticulum at pCa = 4·6 suggest. Under these conditions the rate of Ca uptake by the intact reticulum appears to be similar to that obtained for the maximal rate of Ca accumulation by its vesicles (Inesi & Scarpa, 1972). The leakage of Ca from the reticulum is only of the order of 1–2 n-mole (g muscle)$^{-1}$s^{-1} if the free Ca concentration in the medium is lower than 10^{-9} M, since a preparation loaded with Ca must be kept for at least 30 min in a solution containing 50 mM-free EGTA in order that 10 mM-caffeine does not release substantial amounts of Ca for conditions like those in Fig. 4*B*, *C*.

We are grateful to Professor H. Ch. Lüttgau for continuous interest and support, Dr P. C. Caldwell, F.R.S. for his constant encouragement and Dr C. C. Ashley for providing the aequorin. This work was supported by the Deutsche Forschungsgemeinschaft.

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