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EFFECT OF EXTRACELLULAR CALCIUM ON CONTRACTILE ACTIVATION IN GUINEA-PIG VENTRICULAR MUSCLE

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SUMMARY

1. The problem of whether or not the alterations in twitch tension of mammalian cardiac muscle induced by changes in extracellular Ca concentration ($[Ca]_o$) are due to the Na–Ca exchange process across the surface membrane and/or the changes in the amount of Ca in the sarcoplasmic reticulum has been re-examined by using thin bundles (70–120 μm diameter) dissected from guinea-pig papillary muscle.

2. The observed time course of the change in the twitch tension due to a step change in $[Ca]_o$ was compared with that computed on a basis of the diffusion process of Ca ions in a circular cylinder and of the steady-state relation between $[Ca]_o$ and twitch tension.

3. After a sudden decrease in $[Ca]_o$ from 2 mM to various lower concentrations, the isometric twitch tension of the thin bundles first fell rapidly and monotonically and then showed a much smaller and slower secondary fall. The correspondence of the observed time course of the rapid phase with the predicted time course and the observed half-time of the rapid phase ranging from 1·0 to 2·5 s indicate that the rapidity of the twitch response may be dominated by simple diffusion of Ca ions through the extracellular space. If so, the effective diffusion constant of Ca ions inside the bundles was $1{\cdot}4 \pm 0{\cdot}2 \times 10^{-6}$ cm^2/s (mean ± S.E., $n = 9$).

4. The magnitude and direction of the step change in $[Ca]_o$ or the change at different stimulus frequencies gave rise to dissimilar time courses of the contractile change; the difference in the rapid time courses due to these factors could be explained by the simple diffusion models, but not in the much slower phase.

5. The half-time for the Ca effect was the same as that for the rapid effect of Na ions in the external solutions.

6. The time course of twitch decline due to $[Ca]_o$ decrease in the Na-free (Li) solution was identical to that predicted from the time course measured in the Na-rich solution and the steady-state relation between $[Ca]_o$ and tension in the Na-free solution.

7. The half-time of Ca leak from the sarcoplasmic reticulum in the skinned cardiac muscle was 40–60 s in the presence of 10 mM-EGTA, much shorter than that of the

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Ca leak in the skinned amphibian skeletal muscle, but much longer than that of twitch responses due to step changes in $[Ca]_o$ in the intact cardiac muscle.

8. All these results indicate that the major and rapid component of the twitch tension response of mammalian ventricular muscle induced by a change in $[Ca]_o$ may be synchronous with the change in $[Ca]_o$ at cell surfaces, and may not follow such a slow time course as either the Na–Ca exchange mechanism across the surface membrane or a filling–depletion mechanism in the sarcoplasmic reticulum as each has been observed to date.

INTRODUCTION

It is well known that contraction in cardiac, as in skeletal, muscle is regulated by intracellular Ca ions (Allen & Blinks, 1978; Wier, 1980; Allen & Kurihara, 1982). The twitch responses of cardiac muscle, unlike those of skeletal muscle, are greatly altered when the extracellular Ca ion concentration or other conditions are changed. There is also much evidence that beat-to-beat Ca regulation is influenced by Ca influx during the plateau of the cardiac action potential. However, the proposed mechanisms for Ca regulation in mammalian cardiac muscle are controversial: it is uncertain whether the Ca contributing to the twitch contraction comes from the slow inward current during the plateau of the action potential (see reviews by Fozzard, 1977; Reuter, 1979) or the carrier-mediated Na–Ca exchange (Langer, 1976, 1977), and whether the Ca influxed across the surface membrane acts mainly as a stimulant of Ca-induced Ca release from the sarcoplasmic reticulum (s.r.) (Fabiato & Fabiato, 1978) or by changing the amount of Ca in the s.r. and thereby causing a proportional change in the amount of Ca released by the subsequent action potential (Wood, Heppner & Weidmann, 1969; see review by Morad & Goldman, 1973). These uncertainties result, at least in part, from the slow kinetics in ordinary (thick) preparations of mammalian heart muscle due to the long times of diffusion within the tissues. Isolated cardiac single cells that tolerate a millimolar range of external Ca (Powell & Twist, 1976; Isenberg & Klöckner, 1980) yield a suitable preparation with the shortest possible diffusion time for studies of cardiac muscle. However, enzymatic digestion may possibly affect excitation–contraction coupling (Crevey, Langer & Frank, 1978) and ionic binding to the surface membrane (Lee, Akaike & Brown, 1977; Brown, Lee & Powell, 1981), and the measurement of tension developed by single cells is technically difficult (Brady, Tan & Ricchiuti, 1979; Fabiato, 1981). Therefore, these issues have been re-examined in the present study by determining the effect of changes in extracellular Ca concentration on very thin, mechanically isolated preparations of guinea-pig right ventricles. In these thin bundles, unlike ordinary thick cardiac preparations, diffusion through the extracellular space was rapid enough to be distinguished from supposedly slow processes of Na–Ca exchange across the sarcolemma and filling–depletion of Ca in the s.r. Some of the preliminary results have been briefly reported previously (Kitazawa, 1980).

METHODS

Solutions

The normal external solution contained (mM): NaCl, 150; KCl, 4; Ca methanesulphonate, 2; Mg methanesulphonate, 2; glucose, 5·6; and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-

sulphonic acid), 5, neutralized with Tris (Tris(hydroxymethyl)aminomethane) to give a pH of 7·4 at 25 °C.

High-K solution was obtained by replacing all of the NaCl in the normal external solution with equimolar K methanesulphonate. When the external Na concentration ($[Na]_o$) was lowered, Na was replaced by Tris or Li. When the external concentration of K, $[K]_o$, Mg, $[Mg]_o$, and/or Ca, $[Ca]_o$, were increased, the normal solution was made hypertonic by adding Tris Cl. EGTA (ethyleneglycol-bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid) was added as Tris–EGTA, when necessary. The pH of all of the solutions used was adjusted to 7·4 after any change in the composition.

The external solutions were simply equilibrated with air since the diffusion of the solutes through extracellular spaces was so rapid in preparations used in this study that bubbling with 100% O_2 was not necessary.

The temperature of the solutions was maintained at 25 °C by circulating water.

Dissection

Small bundles (70–120 μm in diameter) dissected from papillary muscles of guinea-pig right ventricles were used throughout this study. Dissecting procedures were essentially the same as those used for obtaining single frog skeletal muscle fibres (Gordon, Huxley & Julian, 1966). Papillary muscles in a slightly stretched state were fixed to a dissecting chamber filled with the normal external solution and dissected at room temperature (20–25 °C) using small knives and fine forceps under a stereo microscope equipped with dark-field illumination. After thick bundles with a diameter of 300–500 μm were obtained, the normal external solution was replaced by the high-K solution, and after several minutes of incubation the dissection was continued. The depolarizing solution was used during dissection in order to avoid the spread of injury from the cells to be cut to the neighbouring cells. Special care was taken to prepare the fibre bundle in a cylindrical shape with as few damaged cells around it as possible, by observing the sarcomeres of the cardiac cells under an inverted microscope.

Procedures for tension measurement

Small bundles obtained were transferred carefully by the use of the fine glass rod from the dissecting chamber to a Perspex experimental trough similar to that designed by Hodgkin & Horowicz (1959), and filled with the same high-K solution. The tendon at one end of the papillary muscle fibre bundle was tied with a monofilament of silk to the rod of tungsten wire (100 μm in width) coated with silicone, which was jutting from one end of the trough. The other end of the bundle was bound to an L-shaped extension of a strain-gauge transducer in the same way. The distance between two knotting points was usually 1 mm (Pl. 1). After reintroduction of normal external solution, contractions of small cardiac bundles were driven at a constant frequency of 12/min to allow stabilization for 30–60 min before the experimental procedures were begun.

The experimental trough and tension transducer were mounted on the mechanical stage of conventional microscope set on a vibration-free desk. Temperature-controlled water at 25 °C circulated below the glass bottom of the trough. Several temperature-regulated syringes were easily attached to and detached from the trough by a tap connecting a short section of thin silicone rubber tube. The trough had a volume of about 0·8 ml. This method allowed the solution to be exchanged very rapidly, at an average flow speed of 5 ml/s.

Isometric tension was measured through a strain-gauge transducer (UL-20, Shinko, Tokyo) connected to a carrier amplifier (RP-5, Nihon Koden, Tokyo) and recorded with a pen recorder (Recticorder, Model RJG-3024, Nihon Koden). The compliance of the transducer was 6·7 μm/mN, and its resonant frequency was about 160 Hz.

To excite the fibre bundle simultaneously along its whole length and to avoid the artifact due to conduction delay, the cardiac fibres were stimulated electrically by 3 ms duration pulses passing transversely through the fibres from a pair of 1 cm long platinum plate electrodes fixed to the side walls of the trough. The strength of the pulses was 2·5 times that of the threshold voltage for twitch contraction, which was not affected by either 10^{-6} M-phentolamine or -propranolol.

Adjustment of sarcomere length

Sarcomere lengths were measured at rest in isolated cardiac fibre bundles using an inverted microscope (Pl. 2) and a laser diffraction technique. Observation of the sarcomere striation was desirable in order to judge the intactness of the preparations not only during dissection, but also

during experiments. Even in microscopically good preparations, the shapes of both the zero and first order of the diffraction pattern were considerably broadened (Krüeger & Pollack, 1975). Average sarcomere length of cardiac fibres during experiments was obtained from the spacing between the peak intensities of the zero and first orders of the diffraction pattern. The maximum twitch tension was usually obtained at a resting sarcomere length of 2·35–2·5 μm. It should be noted that spontaneous sarcomere movements frequently developed when sarcomere length was stretched to more than about 2·6 μm, this phenomenon having previously been described by Chapman & Leoty (1976) and Endo, Kitazawa & Yagi (1980). Such an abnormal condition sometimes caused a large twitch tension even at low stimulus frequencies after restoring the muscle fibres to the shorter sarcomere length or at other times abolished the twitch. Therefore, in all of these experiments the average sarcomere length in the resting state was adjusted to 2·4 μm by using the laser diffraction technique.

Skinning procedure with saponin

Some of the dissected thin cardiac muscle fibre bundles were chemically skinned with saponin as described previously (Endo & Kitazawa, 1978), to measure the leak rate of Ca from the s.r.

The composition of the normal relaxing solution was (mM): K methanesulphonate, 117·5; ATP, 5·2; Mg methanesulphonate, 5; EGTA, 2; and PIPES (piperazine-*N*,*N*′-bis[2-ethanesulphonic acid]), 20; brought to pH 7·0 with KOH. The concentration of EGTA was altered when necessary. In the activating or loading solutions, 10 mM-total EGTA was used and a specified amount of Ca methanesulphonate was added. Free Ca ion concentration was calculated using an apparent association constant of $2{\cdot}5 \times 10^6$ M^{-1} for CaEGTA (Allen, Blinks & Prendergast, 1977; Harafuji & Ogawa, 1980). In all of the above alterations from the normal relaxing solution, ionic strength was kept constant by adjusting the concentration of K methanesulphonate.

Saponin obtained from ICN Pharmaceuticals, Inc. (OH) was dissolved in a relaxing solution shortly before each use.

To compare with the leak rate of Ca from the cardiac s.r., the mechanically skinned skeletal muscle fibres of iliofibularis of *Xenopus laevis* were also used at 4 °C in solutions with compositions (Endo & Nakajima, 1973) slightly different from those described for cardiac muscle.

The responses of intact cardiac fibres to the electrical stimulation, the high-K solution and 30 mM-caffeine in the Na-free solution were first examined. The preparation was then immersed in the normal relaxing solution for several minutes and then treated with saponin (50 μg/ml) for 25 min. The contractile responses after saponin treatments could be controlled by altering free Ca ion concentration in a range of 10^{-7}–10^{-4} M in the external solution. The maximum tension generated in 10^{-4} M-Ca was approximately the same as the maximum contracture produced by 30 mM-caffeine in the Na-free solution before the saponin treatment. If either ATP or Mg was removed from the relaxing solution, the bundle rapidly developed a rigor tension, which was abolished when ATP or Mg was reapplied. At this stage, no small responses originating from the intact fibres which might have remained in the bundle after saponin treatments, were observed. These results suggest that the surface membranes of all fibres in the bundle were probably sufficiently skinned by saponin to allow substances such as Ca, Mg, EGTA and ATP to pass freely.

Endo & Iino (1980) showed that the treatment of the mechanically skinned skeletal muscle fibres with saponin (50 μg/ml) for 30 min did not affect their s.r., as well as contractile, responses, such as Ca uptake, Ca-induced Ca release and Cl-induced Ca release. In the saponin-skinned, as well as mechanically skinned, cardiac muscle fibres mitochondria may be alive and functional. I think, however, that Ca uptake by and release from mitochondria have not significantly interfered with the results obtained in this study, as indicated by the slow rate of Ca uptake at about 1 μM-Ca (Kitazawa, 1976) and the lack of release of Ca by caffeine (Jundt, Porzig, Reuter & Stucki, 1975).

Calculation of the time course of twitch tension change predicted by the diffusion of extracellular Ca ions

Change in $[Ca]_o$ greatly modifies the twitch contractions of cardiac muscle. In order to clarify the $[Ca]_o$ effects, it is essential to compare the time course of twitch responses with that of diffusion of the ions through the extracellular space in the tissue as $[Ca]_o$ is changed.

The cardiac muscle bundle is regarded as a long circular cylinder which is filled with a isotropic medium. (The latter assumption is clearly not correct, but can be justified by using the apparent diffusion constant including the so-called tortuosity factor (Safford & Bassingthwaighte, 1977).) Uptake of Ca ions by cells is negligible because less than one-hundredth of the total amount of ions

in the solution are expected to penetrate into the cells during an action potential (Winegrad & Shanes, 1962; Reuter, 1973).

Concentration of the diffusing substances in the circular cylinder is a function only of radius r and time t, and the diffusion equation is represented by

$$\frac{\partial C}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left(rD\frac{\partial C}{\partial r}\right), \tag{1}$$

(Crank, 1956), where C is the concentration of diffusing substance and D the effective diffusion constant. If, in a cylinder of radius a, the initial and boundary conditions are

$$C = C_0, \quad r = a, \quad t \geqslant 0, \tag{2}$$

$$C = C', \quad 0 < r < a, \quad t = 0, \tag{3}$$

where C_0 is the surface concentration and C' the initial concentration in the circular cylinder, and the solution of eqn. (1) is

$$\frac{C-C'}{C_0-C'} = 1-2\sum_{n=1}^{\infty}\frac{\exp\left(-\frac{Dt}{a^2}\alpha_n^2\right)J_0\left(\frac{r}{a}\alpha_n\right)}{\alpha_n J_1(\alpha_n)} \tag{4}$$

(eqn. (45) of Hill, 1928; eqn. (5.22) of Crank, 1956), where J_0 and J_1 are the Bessel functions of the first kind of zero and first order, respectively, and α_n is the nth root of $J_0(\alpha_n) = 0$. The profile of the relative concentration represented by eqn. (4) can be calculated using tables of Bessel functions (Crank, 1956; Weast & Selby, 1966) and drawn as a function of r/a for different values of Dt/a^2 (Fig. 1*A*).

The amount of a diffusing substance which has entered and left the cylinder in time t, M_t is represented by

$$\frac{M_t}{M_\infty} = 1-4\sum_{n=1}^{\infty}\frac{\exp\left(-\frac{Dt}{a}\alpha_n^2\right)}{\alpha_n^2} \tag{5}$$

(eqn. (47) of Hill, 1928; eqn. (5.23) of Crank, 1956), where M_∞ is the total amount after infinite time. Fig. 1*B*, where M_t/M_∞ is plotted as a function of Dt/a^2, shows the only superficial resemblance of the time course of the diffusion to an exponential, the first 60 % of the change being non-exponential in contrast to the exponential change of the remaining late phase.

One can predict the time course of the twitch tension changes on the basis of that of the Ca diffusion (Fig. 1*A*) and of the $[Ca]_0$–twitch tension relation (see Fig. 8*A*), on the assumption that the individual muscle cells have the uniform property. For example, the tension profiles shown in Fig. 2*A* and *B* were obtained when $[Ca]_0$ was increased from 0·5 to 2 mM at stimulus frequencies of 6 and 30/min, respectively. The volume of the solid figure formed by the rotation of the curve about the vertical axis in Fig. 2*A* or *B* gives the total amount of tension development at a certain time. The numerical integration was done on the basis of Simpson's rule. When the calculated time course is matched to the observed one, a value of the effective diffusion constant, D is taken for a series of experiments as a reasonable fit is obtained. The resultant time courses in Fig. 2*A* and *B* will be illustrated in Fig. 8*B* and *D*.

RESULTS

Inotropism produced by change in $[Ca]_0$

Fig. 3 shows a comparison between the changes produced by a sudden alteration in the extracellular concentration of Ca ions on the time course of isometric twitch tension of whole papillary muscle and of dissected small bundles. In whole papillary muscle, the continuous flow of the solution was essential to maintain the steady twitch tension. On the other hand, this was not required for the small bundles, in which a sudden flow of the same new solution equilibrated with air or 100 % O_2 did not cause any change in the following twitches, except when the stimulus was delivered during

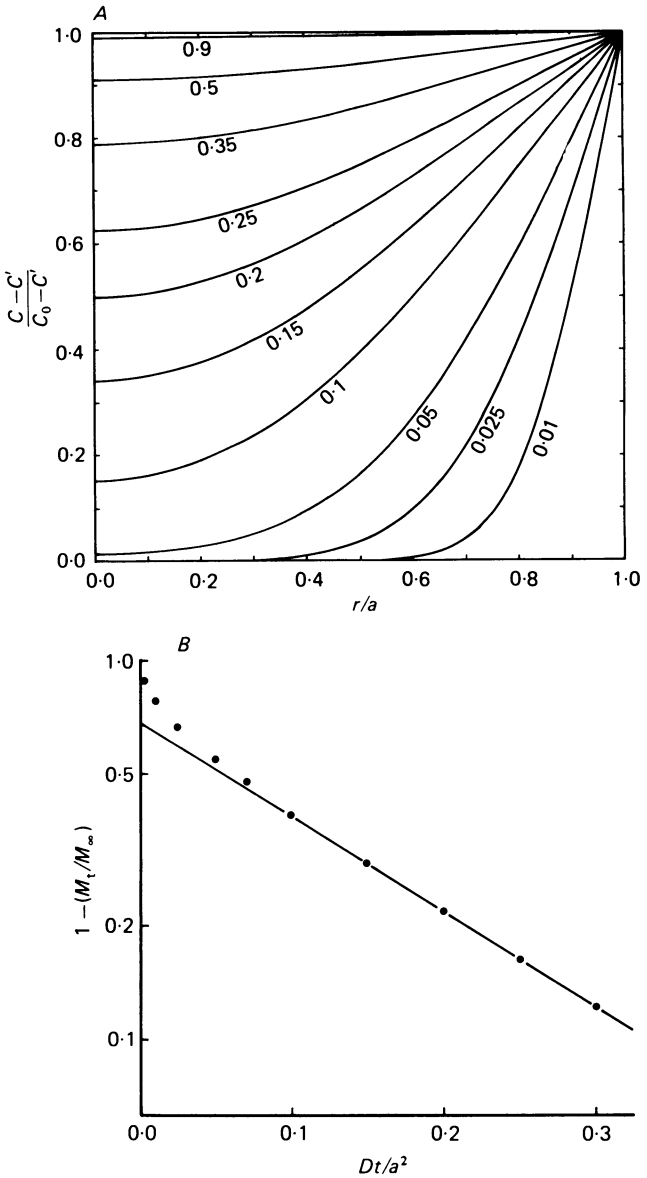

Fig. 1. *A*, concentration distributions of Ca ions in the half-cylinder at various times (Dt/a^2), calculated by use of eqn. (4). Initial concentration is C' in the circular cylinder and surface concentration C_0 remains constant. Numbers on curves are values of Dt/a^2. *B*, time course of total amount of Ca ion which has entered and left the cylinder. Dots are values computed from eqn. (5) and continuous line is exponential.

the flush as shown in Fig. 3*B*. This clearly indicated the good diffusion of the O_2 and solutes in the solution through the extracellular spaces in the dissected thin preparation.

The time to half-maximum (half-time) of the effect of the decrease in $[Ca]_o$ on twitch tension was about 30 s in the whole muscles (about 800 μm in diameter), while the

half-time was 1·0–2·5 s in the small cardiac bundles with a diameter of about 100 μm, as shown in Fig. 3. It was evident that the half-time of the $[Ca]_0$ effect became longer as the fibre size was increased. The values obtained in the small cardiac bundles appear too fast to be the time for accumulation or depletion of Ca in the intracellular store induced by a change in $[Ca]_0$. Therefore, whether or not the contractile changes coincide with the change in $[Ca]_0$ at the cardiac cell surfaces as a result of simple diffusion of Ca through the extracellular spaces in the small cardiac bundles has been investigated.

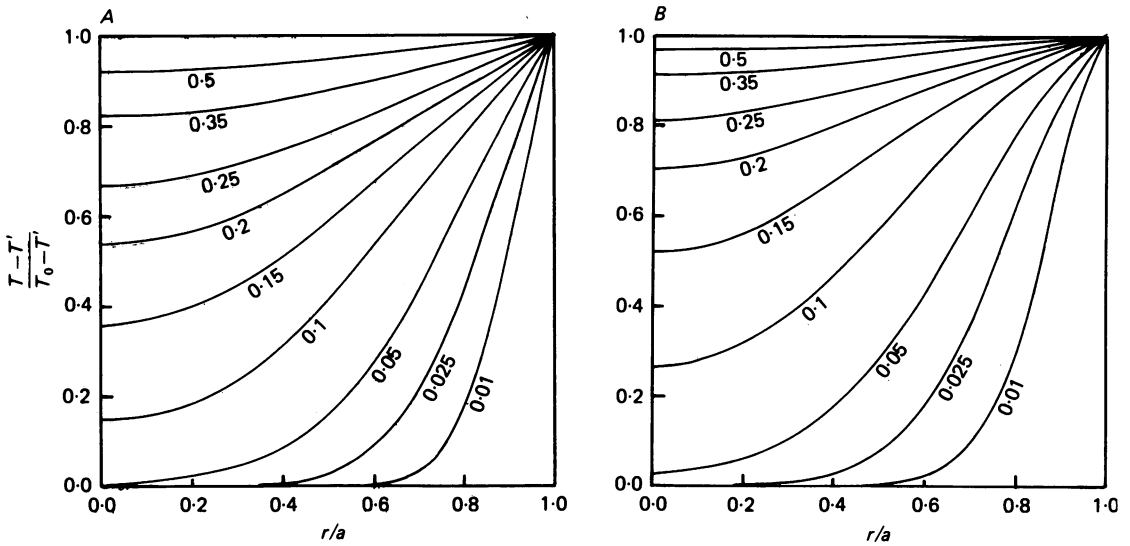

Fig. 2. Tension profiles in the half of the muscle cylinder at various times (Dt/a^2) when $[Ca]_0$ was increased from 0·5 to 2 mM at a stimulus frequency of *A*, 6 and *B*, 30/min. T_0 is the steady twitch tension at the higher $[Ca]_0$ (here 2 mM), T' the steady twitch tension at the lower $[Ca]_0$ (0·5 mM), T the twitch tension during the contractile change due to $[Ca]_0$ change. Note the tension profile in *A* is similar to the Ca concentration profile in Fig. 1*A*, but not in *B*. See Methods for more details.

Rapidness of contractile change due to $[Ca]_0$ change

Assuming the shape of the small cardiac bundles to be a circular cylinder with a diameter of 100 μm, from the line in Fig. 1*B* the half-time of the change in average concentration of Ca ions in the inside is about 0·2 s, when the Ca concentration in the outside is instantaneously changed at zero time and $7{\cdot}8 \times 10^{-6}$ cm^2/s (Wang, 1953) is taken as the diffusion constant of Ca ions in the aqueous solution. If the tortuous geometry of the cardiac muscle (see Safford & Bassingthwaighte, 1977) and the presence of the damaged cells and of a large number of the cation binding sites in the glycocalyx of the surface membrane are taken into account, the experimental values (1·0–2·5 s) for the half-time of tension change of the small cardiac bundles (70–120 μm in diameter) due to the change in $[Ca]_0$ lie roughly within the range of values to be expected from a simple diffusion process (see Discussion for more details). In fact, the half-time obtained in small cardiac bundles was within the range of the

half-times of diffusion of Na and K in the T-system of frog skeletal muscles having almost the same diameter (Nakajima, Nakajima & Bastian, 1975).

It is obvious, however, that the half-time for the contractile change, even if it is not affected by slow responses such as accumulation or depletion of Ca in the intracellular store, does not always correspond to that for the changes in $[Ca]_o$ because

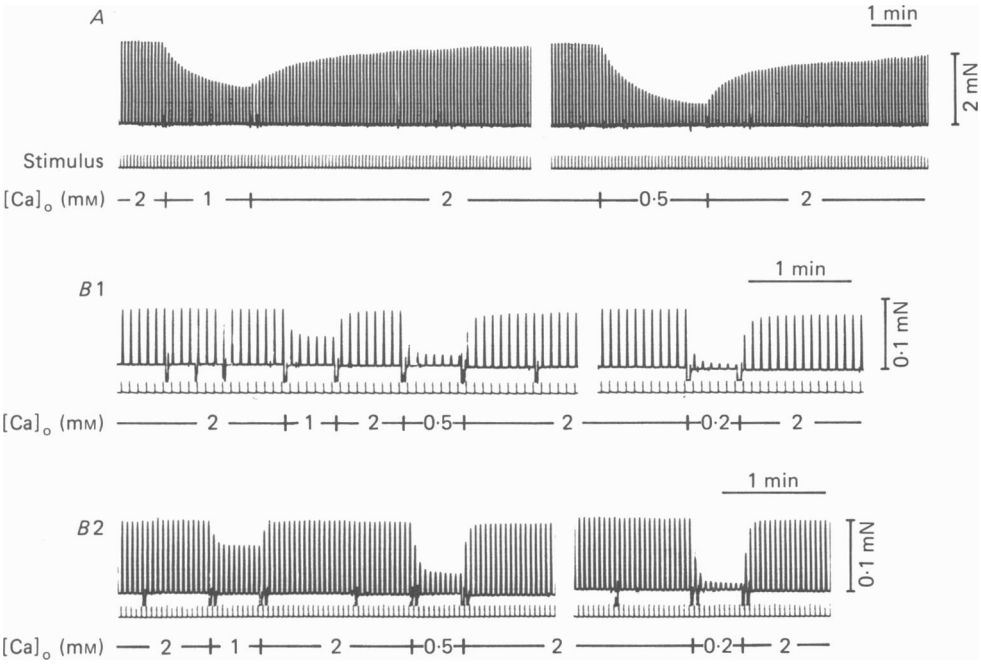

Fig. 3. Changes in isometric twitch tension induced by changes in $[Ca]_o$. In *A*, a whole papillary muscle with a diameter of 800 μm was continuously perfused with the normal external solution and stimulated at 12/min by electrical pulses. In *B*, a small dissected papillary muscle with a diameter of 110 μm was stimulated at B_1, 12 or B_2, 20/min. Each downward deflexion of the tension traces represents the exchange of the solutions. Note the different time scales in *A* and *B*.

of the non-linearity of the relation between $[Ca]_o$ and steady tension in many cases. Therefore, the time course of the twitch tension change measured under various conditions was compared with that predicted from the Ca diffusion process and the Ca–tension relation measured in the same preparation and under the same conditions (see Methods for details).

Effect of direction of $[Ca]_o$ change on the time course of contractile change

When $[Ca]_o$ in the external solution containing 2 mM-Mg was suddenly changed from 2 to 1 mM, the isometric twitch tension of small cardiac bundles quickly decreased to a new steady level (Fig. 4). After the cardiac small bundles had been soaked in the 1 mM-Ca solution for 60 s, a normal $[Ca]_o$ was suddenly re-introduced, and the rapid recovery of twitch tension was observed. The diffusion equation (eqn. 4) shows that the Ca concentration change inside the circular cylinder has the

same time course in either direction of a change in $[Ca]_o$, decrease or increase. In fact, the expected time course of twitch change calculated using the same effective diffusion constant ($1{\cdot}20 \times 10^{-6}$ cm^2/s) is well fitted to the observed one, both during decline and recovery (Fig. 4) when the slightly non-linear relation between $[Ca]_o$ and the steady twitch tension of the same cardiac bundle is taken into account (see Methods for more details). The time course of twitch decline shows a tendency, but not significant, to have a very slow and small decrease following a rapid and large one.

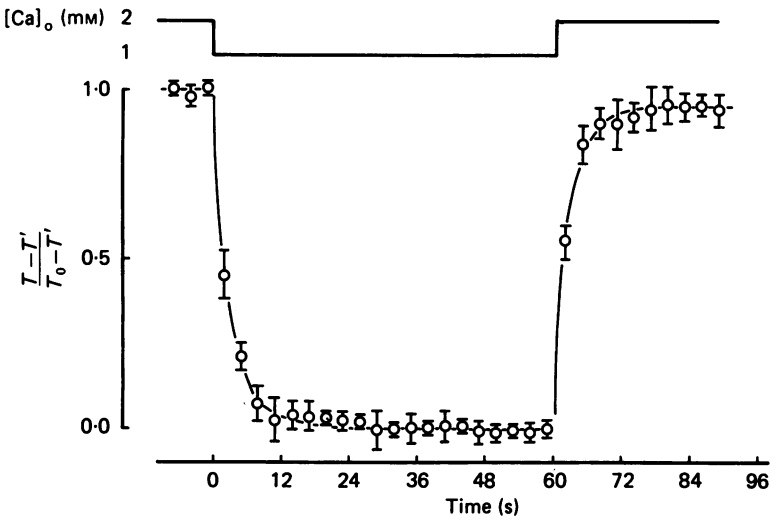

Fig. 4. Response of isometric twitch tensions of a small cardiac bundle to a change in $[Ca]_o$. $[Ca]_o$ was decreased from 2 to 1 mM at zero time and restored to the original concentration after 60 s. Each point is the mean ± S.D. for six observations. Continuous curve represents the calculated time course of the twitch responses expected from the steady-state relation between $[Ca]_o$ and twitch tension and the time course of the diffusion of extracellular Ca ions in the bundle, with a radius of 49 μm, assuming the effective diffusion constant D to be $1{\cdot}20 \times 10^{-6}$ cm^2/s, which was taken as the calculated curve was fitted to the observed time course of twitch decline. The steady-state level of twitch tension at 1 mM-external Ca was chosen at 60 s for the curve fitting. See Methods and text for more detail. Stimulus frequency 20/min.

Effect of the magnitude of $[Ca]_o$ change

As expected from the simple diffusion theory, an almost identical time course of the twitch tension changes was obtained for twitch decline when $[Ca]_o$ was decreased from 2 to 1 or 0·5 mM in the presence of 2 mM-external Mg (Fig. 5), in accordance with the continuous curves indicating the predicted time course with an effective Ca diffusion constant of $1{\cdot}50 \times 10^{-6}$ cm^2/s. However, when $[Ca]_o$ was decreased to lower than 0·5 mM, the actual time course of twitch tension change deviated very slightly, but significantly, from the predicted line and a small lag was observed.

Fig. 6*A* shows the recovery time course of twitch tension when the normal external solution containing 2 mM-Ca was re-introduced after the small cardiac bundles had been incubated in a solution of various lower $[Ca]_o$ for various periods of time (3 min

in the Figure) in the absence of Mg ions. The twitch tension during the recovery phase from the $[Ca]_o$ lower than 0·5 mM transiently became much larger than the steady control tensions in normal-Ca solution. The lower the concentration of $[Ca]_o$ in which the fibres had been soaked and the longer the duration in low-Ca solutions, the larger the twitch tension became during the recovery phase, as shown in Fig. 6*A*. Finally,

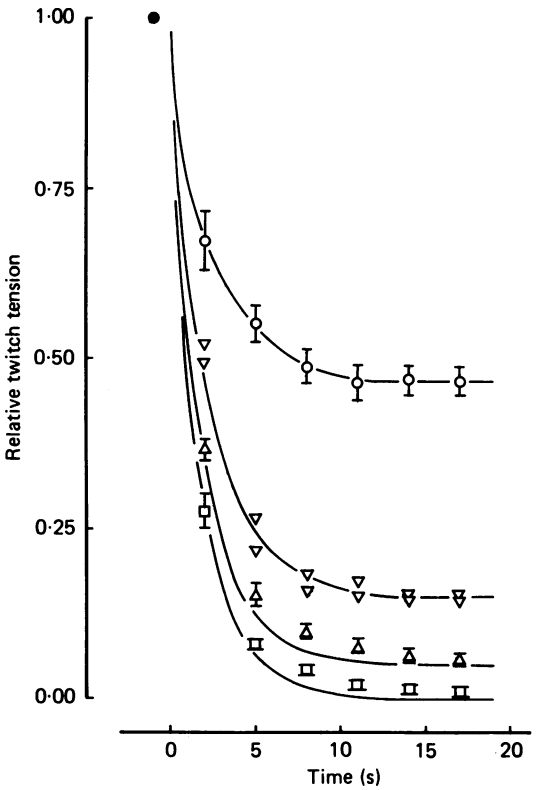

Fig. 5. Responses of twitch tension to various reductions of $[Ca]_o$. $[Ca]_o$ was, respectively, 2 mM (●) as control, 1 mM (○), 0·5 mM (▽), 0·2 mM (△) and 0·0 mM (□). Numbers of experiments at each $[Ca]_o$ were 15, 2, 9 and 10, respectively. Stimulus frequency 20/min. The same preparation as in Fig. 3 was used. Continuous curves are the calculated results taking D as $1{\cdot}50 \times 10^{-6}$ cm²/s, which was chosen so that the predicted and measured time courses of the twitch decline due to $[Ca]_o$ decrease to 1 mM could be matched.

transient contractures, the so-called Ca-paradox (see review by Grinwald & Nayler, 1981), occurred with disappearance of the twitch, followed by restoration of normal twitches. At the time when abnormally large twitch tensions or contractures developed in the absence of Mg ions, spontaneous sarcomere oscillation (see Methods) appeared. The half-time of twitch recovery from $[Ca]_o$ lower than 0·5 mM was almost the same as that from 1 mM. The potentiation after a treatment with low-Ca solution was usually not observed in the presence of 2 mM-external Mg (Fig. 6*B*). The treatment of the small cardiac bundles with a solution containing 1 mM-EGTA and no added Ca overcame the repressive effect of 2 mM-external Mg on the potentiated

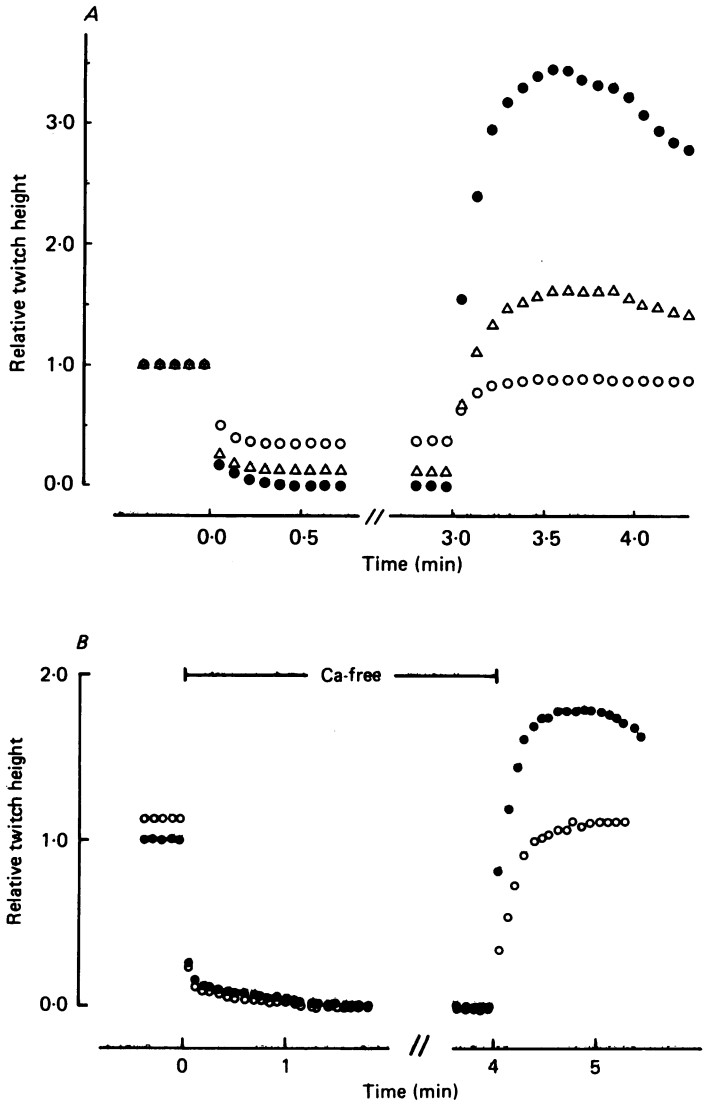

Fig. 6. Effects of duration in low $[Ca]_o$ on twitch recovery in *A*, the absence and *B*, the presence of 2 mM-external Mg. In *A*, $[Ca]_o$ was changed in the sequence of 2→1→2 mM (○), 2→0·2→2 mM (△) or 2→0·0→2 mM (●). Stimulus frequency 12/min. In *B*, twitch responses evoked by reduction of $[Ca]_o$ to 0 mM and thereafter by re-introduction to 2 mM-external Ca were compared in the absence (●) and presence (○) of 2 mM-external Mg. Stimulus frequency 12/min.

twitch tension during recovery, but not that at 10 mM-external Mg. The time course of twitch decline on decreasing $[Ca]_o$ was not influenced by Mg ions (Fig. 6*B*). At the time when the abnormally large twitchs or contractures appeared in Mg-free solutions, the normal relationship between twitch tension and $[Ca]_o$ or stimulation frequency was no longer present. Such preparations, unlike normal cardiac muscle,

responded to caffeine with large contractures even in the presence of high concentrations of Na (see Endo *et al.* 1980). Therefore, the time courses of twitch recovery from various lower levels of $[Ca]_o$ could not be compared with each other in the absence of Mg ions.

On the other hand, in the presence of 2 mM-external Mg, the time course of the twitch recovery was apparently also influenced by the solution containing a $[Ca]_o$ lower than 0·5 mM with which the small cardiac bundles had been pre-treated. The

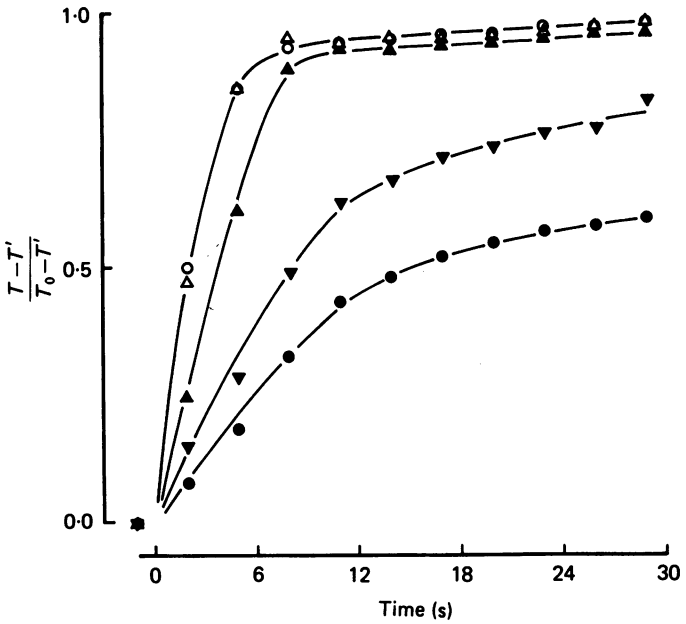

Fig. 7. Twitch recoveries in the presence of 2 mM-external Mg after various periods of exposure to different low $[Ca]_o$. Symbols represent the time courses of twitch tension change when 2 mM-external Ca was re-introduced from 1 mM (○, △) or 0 mM (●, ▲). The fibres had been pre-treated with each low $[Ca]_o$ for 9 s (▲, △), 60 s (▼) and 120 s (●, ○), respectively. Stimulus frequency of 20/min was used. Continuous lines were drawn by eye.

slow recovery of twitch tension followed the rapid rise when $[Ca]_o$ was increased from 0 to 2 mM, and was more obvious when the small cardiac bundles had been pre-treated in low-Ca solution for a longer period (Fig. 7). The apparent half-time of the twitch response, without distinguishing the fast and slow phases, increased almost linearly with the incubation period for up to 3 min in Ca-free solution, while the twitch recovery due to an increase of $[Ca]_o$ from 1 to 2 mM had a constant half-time regardless of the length of the incubation period, at least from 9 s to 10 min. However, the time course of twitch recovery after a short incubation (9 s) of the fibres in Ca-free solution, approached that in 1 mM-external Ca (Fig. 7). This means that the time course of fast twitch recovery in Mg-containing solution, at least with a short pre-treatment, is almost independent of the concentration of extracellular Ca ions immediately before re-introduction of 2 mM-external Ca, in spite of the big difference in twitch height.

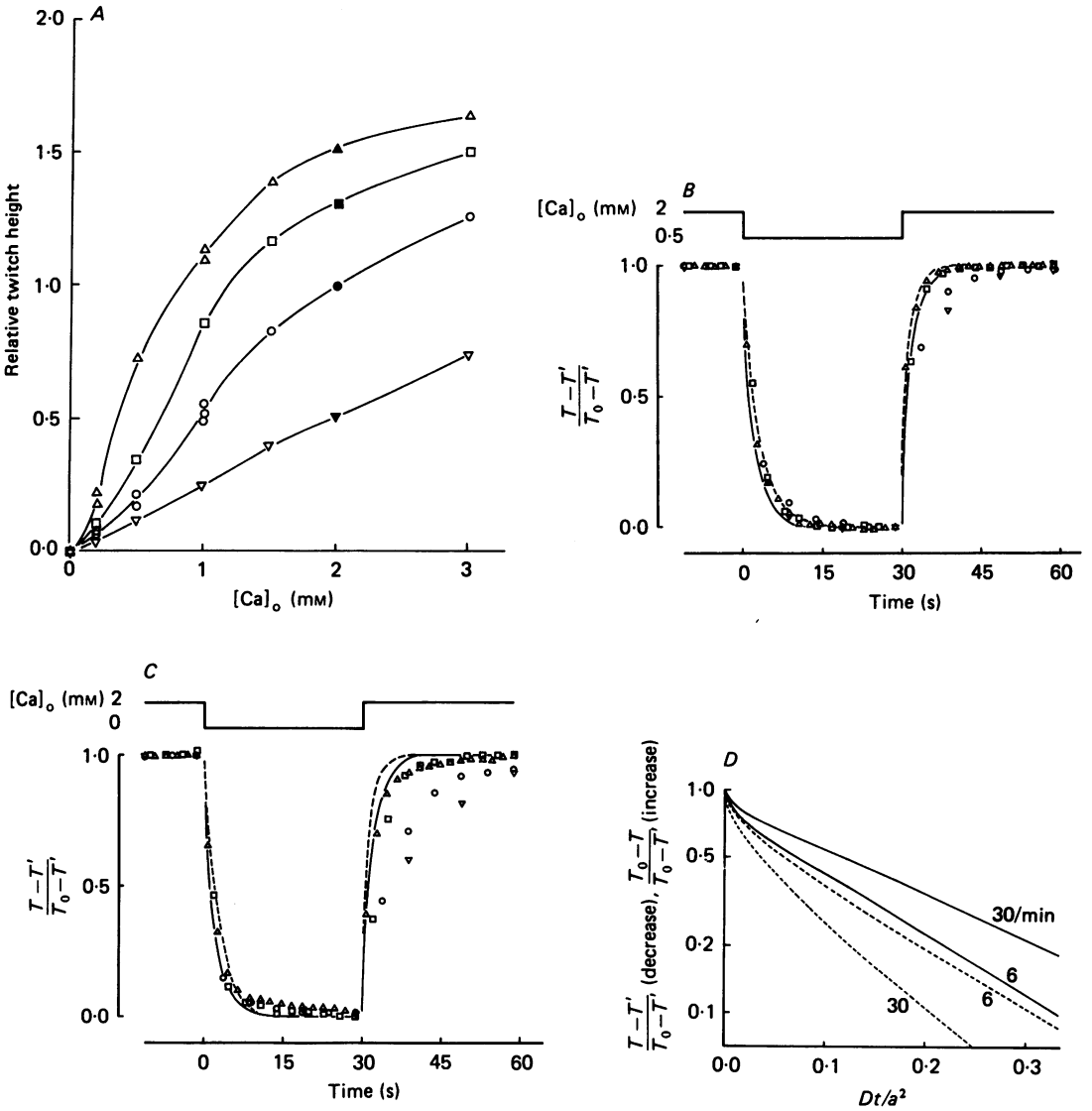

Fig. 8. Effect of stimulus frequency on twitch response produced by changes in $[Ca]_o$. *A*, influence of various stimulus frequencies on the relation between $[Ca]_o$ and steady twitch tension. △, 30/min; □, 20/min; ○, 12/min; ▽, 6/min. *B* and *C*, time course of changes in the twitch tension. Symbols indicate the same stimulus frequencies as in *A*. Continuous curves in *B* and *C* represent the calculated time course of twitch response predicted from the $[Ca]_o$–twitch tension relation at 6/min and the time course of the diffusion of Ca, dashed lines correspond to that at 30/min. Same effective diffusion constant, $1{\cdot}54 \times 10^{-6}$ cm^2/s taken for matching the curves of tension decline due to $[Ca]_o$ change from 2 to 0·5 mM, was used in all cases. $a = 48$ μm. *D*, semilogarithmic plot of the same theoretical time course of twitch response as in *B*. Continuous curves represent the time course when $[Ca]_o$ was decreased from 2 to 0·5 mM; dotted lines when $[Ca]_o$ was increased from 0·5 to 2 mM.

Effect of a change in stimulus frequency

A change in stimulus frequency strongly influences the contractility of cardiac muscle and the relationship between $[Ca]_o$ and steady twitch tension (Fig. 8*A*; for references, see Allen, Jewell & Wood, 1976). However, if the time course of the twitch response due to a step change in $[Ca]_o$ is solely determined by the diffusion rates of the ions, there should not be any difference between the time courses at different stimulus frequencies beyond that expected from the diffusion equation and the non-linear relation between $[Ca]_o$ and steady twitch tension. In fact, this was the case for the twitch decline (Fig. 8*B* and *C*) and the predicted time courses could be fitted to the observed ones by using the same effective diffusion constant ($1{\cdot}54 \times 10^{-6}$ cm^2/s), although a similar slow phase was seen at each stimulus frequency when $[Ca]_o$ was decreased from 2 to 0 mM (Fig. 8*C*). On the other hand, twitch recoveries due to re-introduction of normal $[Ca]_o$ were varied by changes in stimulus frequency, slower recovery occurring at lower frequencies (Fig. 8*B* and *C*). It was also more obvious when the cardiac muscle fibres had been treated with lower $[Ca]_o$ for a longer period (see also Fig. 7). However, when $[Ca]_o$ was increased from 1 to 2 mM or increased to 2 mM after a very short incubation (e.g. 10 s) in Ca-free solution, the dependence of the recovery upon the stimulus frequency almost disappeared (not shown). It should be further noted that different stimulus frequencies alter the $[Ca]_o$–tension relation and therefore give different predicted time courses of contractile changes. Slower decline and faster recovery due to $[Ca]_o$ change can be expected at a higher frequency as a result of the more sigmoidal relation between $[Ca]_o$ and tension, clearly shown in Fig. 8*D*. This was the case, and in the observed time course at 30/min (Fig. 8*B*), for example, the first twitch contraction after $[Ca]_o$ was decreased to 0·5 mM corresponded to only 30 % of the change, while it corresponded to 60 % when $[Ca]_o$ was increased. These confirmed the result obtained by Toll (1978) that a non-linear relation between $[Ca]_o$ and steady twitch tension causes the direction-dependent dynamics.

Effects of change in $[Na]_o$ on the time course

From the simple diffusion theory, the half-time for the effect of $[Ca]_o$ on twitch tension should be of the same order of magnitude as that for the effects of other ions that are considered to exert their actions from outside the cardiac cells.

Lüttgau & Niedergerke (1958) showed that Na ions affected the contractility of frog cardiac muscle in an antagonistic way with Ca ions, and suggested that two Na ions compete with one Ca ion for the binding site, probably at the cell surface. On the other hand, Tillish, Fung, Hom & Langer (1979) demonstrated by using whole papillary muscle of rabbit heart, that a change in $[Na]_o$ resulted in a transient change in twitch tension followed by a return towards the control level, and only the initial fast component of the response was dependent on the ratio $[Ca]_o/[Na]_o^2$.

Experiments in the small bundle dissected from guinea-pig papillary muscle essentially confirmed the results of Tillish *et al.* (1979) except for a major difference in the time course of the response. As shown in Fig. 9*A*, when normal $[Na]_o$ was suddenly decreased to 75 mM with osmolarity maintained by adding Tris, twitch tension was rapidly increased with a half-time of about 2 s, and then partially

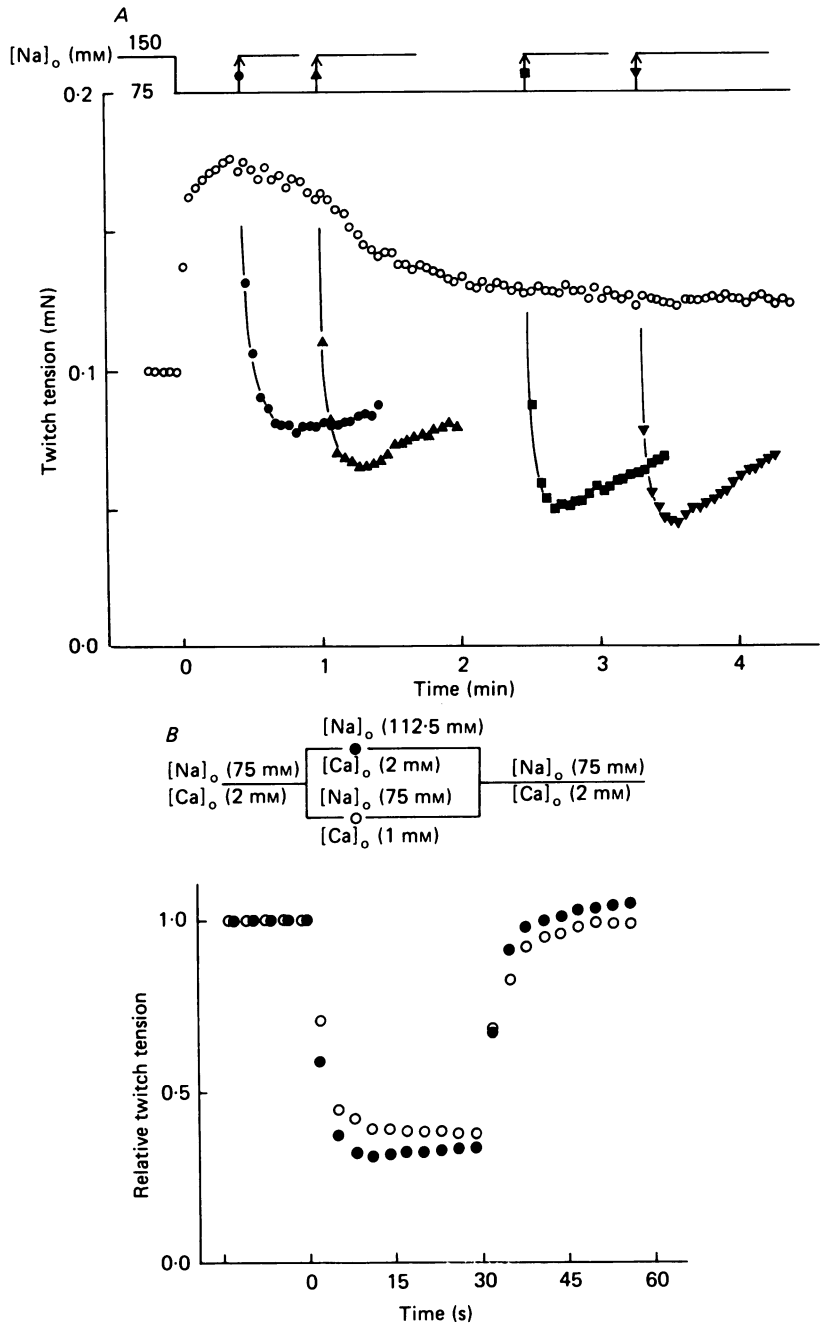

Fig. 9. Time course of twitch responses induced by decrease in $[Na]_o$ followed by return to the control level after exposure of different durations to a low concentration (*A*), and comparison between $[Ca]_o$-induced and $[Na]_o$-induced twitch responses (*B*). In *A*, ○ represents the transient effect of decrease in $[Na]_o$. At 0·5 min (●), 1 min (▲), 2·5 min (■) and 3·3 min (▼), respectively, $[Na]_o$ was re-introduced from 75 to 150 mM. In *B*, the control $[Na]_o$ had been already decreased to 75 mM with maintained osmolarity by adding Tris. Stimulus frequency 20/min.

returned toward the original level with a half-time of about 60 s, regardless of the maintenance of low $[Na]_o$. After various durations in low $[Na]_o$, $[Na]_o$ was returned to normal. A transient overshoot in twitch tension below the control level was observed with a half-time of about 2 s, followed by recovery towards control levels with a half-time of about 60 s, irrespective of the duration in low-Na solution. Minimum twitch tension during transient depression was gradually decreased with the same half-time of about 60 s as described above. The strength of the initial rapid change in twitch tension produced by a change in $[Na]_o$ in a particular condition was apparently proportional to the ratio $[Ca]_o/[Na]_o^2$, in the range 75–150 mM-external Na.

When, after the small cardiac bundles had been pre-treated with 75 mM-Na solution until steady development of twitch tension, $[Na]_o$ was suddenly increased to 112·5 mM, the twitch tension was rapidly reduced at a rate similar to that induced by a step change in $[Ca]_o$ (Fig. 9*B*). The time course of the twitch decline caused by $[Ca]_o$ decrease expected from the rapid twitch decline caused by $[Na]_o$ increase more closely coincided with the observed one when the steady-state relation between $[Ca]_o/[Na]_o^2$ and twitch tension and the difference between the diffusion constants of Na ($1{\cdot}48 \times 10^{-5}$ cm^2/s) (Robinson & Stokes, 1959) and Ca ions in aqueous solution were taken into account.

Replacement of all of the Na ions in the external solution with Li ions still allowed the cardiac muscle to generate twitch tension in response to the same external electrical stimulations. Even after 60 min exposure to the Na-free solution, a step change in $[Ca]_o$ still caused a rapid change in twitch tension (Fig. 10*A*). The time course of the twitch changes in the Na-free solution, however, cannot be compared with that in the high-Na solution directly, since the response of cardiac muscle to $[Ca]_o$ is greatly influenced by $[Na]_o$ (Fig. 10*B*). The observed time courses of the twitch responses in Na-free or high-Na solutions were in accordance with the time courses calculated by using the same effective diffusion constant ($1{\cdot}39 \times 10^{-6}$ cm^2/s) and the steady-state relation between $[Ca]_o$ and tension in each condition (Fig. 10*C*). This suggests that even in the Na-free condition the time course of twitch decline due to $[Ca]_o$ decrease follows that expected from the diffusion of Ca ions as well as in the high-Na condition, in spite of a mechanism of Na–Ca exchange.

Time course of leak of Ca from the s.r. in skinned cardiac muscle fibres

A comparison between the time course of the leak of Ca from the cardiac s.r. and that of the twitch decline in the intact muscle on decrease in $[Ca]_o$ gives important information on the excitation–contraction coupling mechanism in cardiac muscle. In order to measure the leak rate of Ca from s.r. in skinned muscle fibres, a high concentration of caffeine, e.g. 30 mM for cardiac muscle, 25 mM for skeletal, was applied first, to deplete the s.r. of Ca. Then the fibre was incubated with a solution containing 10^{-6} M-Ca buffered with 10 mM-EGTA for 2 min for cardiac muscle, or 5×10^{-7} M-Ca for 3 min for skeletal muscle, to load the s.r. with a certain amount of Ca (a in inset of Fig. 11), and washed at b by 10 mM-EGTA without added Ca for 10 s. The fibre was immersed at c for various periods in the relaxing solution containing 10 mM-EGTA, which was followed by decrease in the concentration to 0·15 mM for cardiac muscle, or 2 mM for skeletal muscle, at d to observe the tension

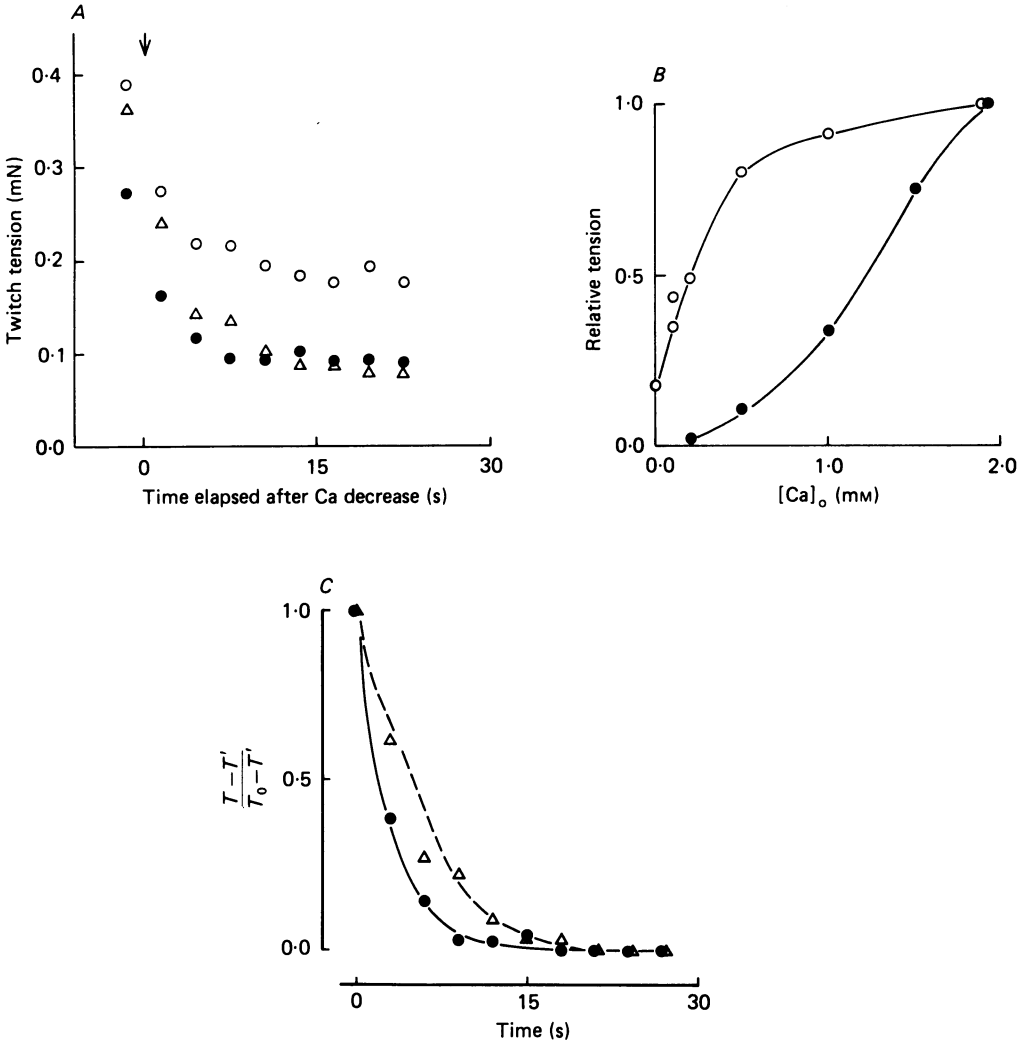

Fig. 10. Twitch reduction induced by decrease in $[Ca]_o$ in the Na-free (Li) solution. Experiments were carried out more than 60 min after complete replacement of Na with Li. In *A*, twitch responses induced by a decrease in $[Ca]_o$ from 2 to 0·1 mM (○) and to 0·0 mM (△) in Na-free (Li) solution were compared with those induced by a decrease in $[Ca]_o$ from 2 to 1 mM (●) in the high-Na normal solution. Arrow indicates when $[Ca]_o$ was decreased. *B* shows the steady-state relation between $[Ca]_o$ and twitch tension in the presence (●) and absence (○) of $[Na]_o$ using the same small cardiac bundle as in *A*. *C*, continuous and dashed lines represent the time course of twitch responses calculated on the basis of the steady-state relation between $[Ca]_o$ and twitch tension in the high-Na and Na-free solutions, respectively. Same symbols were used as in *A*. Same effective diffusion constant, $1{\cdot}39 \times 10^{-6}$ cm^2/s taken for matching the curves of twitch decline in the high-Na solution was also used in the Na-free solution. $a = 50$ μm.

transient produced by subsequent application of high concentrations of caffeine at e. The area of the tension transient due to caffeine indicated the amount of Ca remaining in the s.r. after treatment with 10 mM-EGTA (Endo, 1977). As shown in Fig. 11, prolongation of the treatment decreased the Ca in the s.r. and the rate of loss was much more rapid in cardiac muscle, with a half-time of about 40–60 s. The

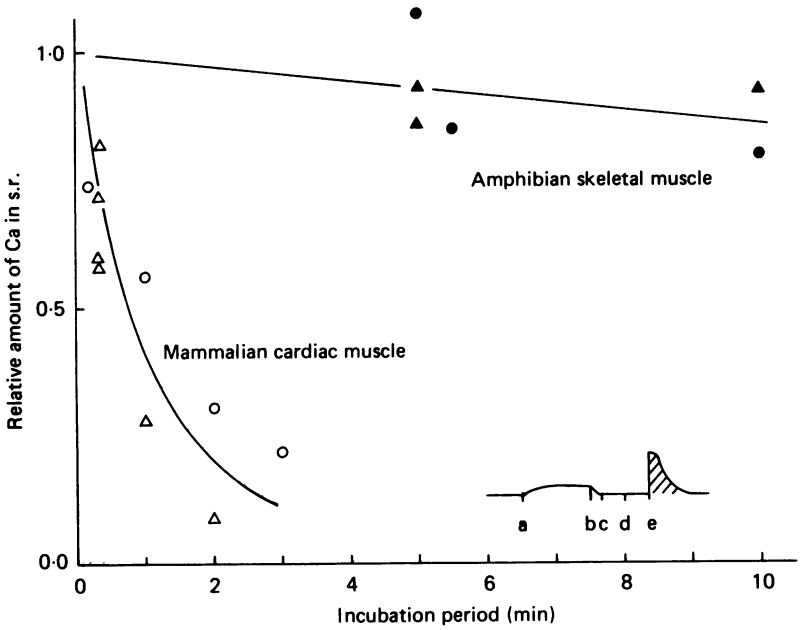

Fig. 11. Time course of Ca leak from s.r. in skinned cardiac and skeletal muscle fibres. The relaxing solution for measurements of the leak of Ca ions from the s.r. in the skinned guinea-pig cardiac muscle, as well as *Xenopus* skeletal muscle, contains 10 mM-EGTA and no added Ca. Procedure to measure the Ca leak from s.r. was described in text.

striking difference has been noted that most of the Ca in the mammalian cardiac s.r., as well as amphibian cardiac s.r. (unpublished observations), was lost in several minutes, while most of the Ca was retained in the s.r. of amphibian skeletal muscle. The high rate of Ca leak in the cardiac s.r. would not result from the use of saponin because the treatment with the drug (50 μg/ml) did not affect the skeletal s.r. responses (Endo & Iino, 1980), and the rate of Ca leak from the s.r. in the cardiac muscle fibres skinned with saponin (50 μg/ml) for 25 min was not affected by further treatment with the same dose of the drug for 20 min.

DISCUSSION

The most interesting finding of this study was that the major and rapid component of the twitch tension response of mammalian ventricular muscle produced by a change in $[Ca]_o$ may be synchronous with the change in extracellular Ca ion concentration at the cell surfaces, and not follow the time course of either the Na–Ca exchange mechanism at the surface membrane or a filling–depletion mechanism in the s.r. On

the other hand, a much slower and smaller phase following the large and rapid change was also observed when $[Ca]_o$ was decreased to and increased from less than 0·5 mM.

In the dissected thin papillary muscle fibres used in this study, the half-time of the rapid twitch response caused by a step change in $[Ca]_o$ was 1·0–2·5 s, less than one-tenth of the value obtained by using routinely isolated or perfused mammalian ventricular preparations (cf. Fig. 3; Shine, Serena & Langer, 1971; Saari & Johnson, 1971; Bailey, Ong & Queen, 1972; Rich & Langer, 1975). This response is close to that expected from the simple diffusion of Ca ions in the extracellular spaces of a tissue 100 μm in diameter. Assuming that the time course of the rapid twitch response is determined solely by the diffusion rate of the Ca ions, the average effective diffusion constant of Ca inside the dissected bundles, including the T-system, estimated from the twitch tension decline when $[Ca]_o$ was changed from 2 to 1 mM, was $1{\cdot}4 \pm 0{\cdot}2 \times 10^{-6}$ cm^2/s (mean ± S.E. of mean, $n = 9$). This value was in agreement with that directly measured in thin mammalian right ventricular sheets using ^{45}Ca (Safford & Bassingthwaighte, 1977) and very similar to that of Na or K ions in the T-system of frog skeletal muscle fibres (Nakajima *et al.* 1975). However, the effective diffusion constant expected from the rapid component of the $[Ca]_o$ effect was one-sixth of that in aqueous solution (Wang, 1953). Most of this difference might well be explained by the structural tortuosity inside the tissue, instead of by Ca binding to cellular surface sites, if the tortuosity factor was assumed to be 2·4 as measured by Safford & Bassingthwaighte (1977), and since the effective diffusion constant varies inversely with the square of the factor. This conclusion was supported by the fact that the extent of the $[Ca]_o$ change had almost no influence on the time course of the rapid twitch response (Fig. 4), and also by the observations that the twitch response due to $[Ca]_o$ change followed the same time course as that of the rapid effects of addition of Co, Mn and K (unpublished observations) or changes in Na (Fig. 9*B*) that must have different affinities for the cation-binding sites on the surface membrane. However, this tortuosity factor might be over-estimated in the dissected preparations that did not contain endothelial cells, which were considered as the main barrier to the diffusion of solutes in frog ventricular muscle (Page & Niedergerke, 1972). The binding of Ca ions to and their dissociation from sites on the cardiac sarcolemma and its surface coats (Bers & Langer, 1979) might possibly delay the rate of diffusion of the ions to give rise to the slow phase of twitch decline observed when normal $[Ca]_o$ was reduced to less than 0·5 mM (Fig. 4 and 8*C*), since it was not significantly influenced by a change in stimulus frequency.

A change in free extracellular Ca concentration should affect, at least, Ca binding to the sarcolemma including the coats, the slow inward Ca current and Na–Ca exchange. It was demonstrated that the amount of sarcolemmal Ca binding is parallel to tension development in cardiac muscle (see review by Langer, 1980). Clearly, it seems unlikely that all of the extracellular Ca-binding sites, including phospholipid, sialic acid, Na–Ca exchange carrier, Ca channel and connective tissue, correlate with excitation–contraction coupling. Furthermore, low concentrations of extracellular Mn and Co (unpublished observations), as well as Mg (see Fig. 6*B*) ions which would be expected to decrease Ca binding to the surface membrane, significantly potentiated, rather than reduced, twitch tension. This finding was consistent with the evidence that the Ca inward current of *Helix* neurones was increased by low concentrations of these divalent cations (Akaike, Lee & Brown, 1978).

Recently, it has been postulated that the Na–Ca exchange system on the surface membrane regulates not only the cytoplasmic free Ca concentration in the resting state, but also the twitch height (Langer, 1976; Katz, 1977; Lee, Uhm & Dresdner, 1980). Of course, one can assume that the alteration of the resting, cytoplasmic free Ca level affects the amount of Ca accumulated by the s.r., if the store is not saturated with Ca and the increased free Ca is below the threshold concentration for Ca-induced Ca release. For reasons given below, it is unlikely that major changes in twitch height are caused by a change through the Na–Ca exchange system. The time course of the rapid twitch response caused by a step change in $[Ca]_o$ or $[Na]_o$ could be explained by the diffusion rate of these ions in the extracellular spaces. If these twitch changes are assumed to be regulated by that exchange system, then the rate of its mechanism should be faster than that of diffusion of these ions in the extracellular space. However, the rate of change of cytoplasmic free Ca and Na concentration through the Na–Ca exchange appears to be one order of magnitude slower than that of twitch responses, from the following evidence: (1) Na-free contractures, even in the dissected thin papillary muscle fibres with a diameter of 60 μm, developed with a half-time of 10–20 s at a pre-stimulation rate of 12–30/min (unpublished observations); (2) activity changes in intracellular Na or Ca following changes in $[Na]_o$ or $[Ca]_o$ were much slower, although the ionic activities measured by the ion-selective micro-electrodes must have been recorded from surface cells (Deitmer & Ellis, 1978; Lee *et al.* 1980; Marban, Rink, Tsien & Tsien, 1980); (3) both Na depletion and Ca accumulation rates of isolated cardiac sarcolemmal vesicles or myocytes are also slow (Pitts, 1979; Philipson & Nishimoto, 1981; Désilets & Horackova, 1982); (4) the half-time of Ca leak from the s.r. in skinned cardiac muscle fibres was 40–60 s even in the presence of 10 mM-EGTA with no addition of Ca (Fig. 11). In addition, the fact that the time course of twitch decline due to $[Ca]_o$ decrease in the Na-free (Li) solution was identical to that predicted from the results in the high-Na solution (Fig. 10*C*) is difficult to explain with such a Na–Ca exchange mechanism as we have considered to date. Extracellular Na ions have been shown to compete with Ca ions passing through the slow inward channel (Reuter & Scholz, 1977).

A change in the extracellular Ca concentration alters the amount of Ca influxed during the slow inward current of the action potential (Beeler & Reuter, 1970; New & Trautwein, 1972). The time course of the change in the Ca current by a step change in $[Ca]_o$ should be predicted to be solely determined by the diffusion rate of the ions in the extracellular spaces. One can predict, however, that the amount of Ca influxed per beat measured isotopically (Winegrad & Shanes, 1962) and electrically (Reuter, 1973) is too small to activate the contractile system directly, even if troponin is assumed to be the only Ca-binding protein during physiological contraction (Kitazawa, 1976). Furthermore, the Ca transient preceding the contractile response (Allen & Blinks, 1978) and the presence of another Ca-binding protein, calmodulin (Chafouleas, Dedman, Munjaal & Means, 1979) and Ca-ATPase in the s.r. should increase the amount of Ca required for the same tension development. It is very likely that in the mammalian cardiac muscle the major fraction of the Ca ions producing the physiological contractions originate from the release of the ions from the intracellular store, although Niedergerke, Ogden & Page (1976) have argued that the measured amount of influxed ^{45}Ca was underestimated because of retarded diffusion of the ions

in the cardiac tissues. The s.r. has sufficient Ca transporting activity to relax cardiac muscle contracting at a physiological level, while the role of mitochondria as a Ca store has been denied (Kitazawa, 1976).

The following mechanisms have been proposed through which a change in a small amount of Ca influxed from the extracellular space during the action potential alters the twitch contraction, that is, the large amount of Ca released from the s.r.: (1) a small increase in the cytoplasmic free Ca may enhance Ca release from the s.r. through the Ca-induced Ca release mechanism (Fabiato & Fabiato, 1978), or (2) a change in Ca influxed may replenish or deplete the store of intracellular Ca available for release by the subsequent action potential (Morad & Goldman, 1973; Fozzard, 1977), or (3) both. The results obtained in this study clearly indicate that most of the change in twitch tension due to the change in $[Ca]_o$ does not result from a change in intracellularly stored Ca. First, the change in the rapid twitch response seems to be synchronous with the change in $[Ca]_o$ and to have no such a delay as is expected from the filling–depletion mechanism of the store. Secondly, the effect of a decrease in $[Ca]_o$ at the low stimulation rate (6/min in Fig. 8*B* and *C*) was almost completed only during the diastolic phase. Assuming the filling–depletion mechanism of the store, Ca should be leaked out of it to reach a new low level within the diastolic phase if the s.r. is unable to pump Ca from its lumen to extracellular space directly. However, the leak rate of Ca from the s.r. in the presence of 10 mM-EGTA was one order of magnitude less than that of the twitch response. Thirdly, at a higher stimulation rate which might cause more Ca influx per minute, twitch decline due to a decrease in $[Ca]_o$ should be more delayed than that at lower frequency, according to the filling–depletion mechanism. This is not the case. Fourthly, the recovery time course of the fibres incubated in 1 mM-external Ca for 10 min was the same as that for those incubated for 10 s, although, according to the filling–depletion mechanism, the former should be much slower. Fifthly, the peak contracture produced in Na-free, Ca-free conditions by a high concentration of caffeine (which can release most of the Ca stored by the s.r.; Endo & Kitazawa, 1978), was independent of the $[Ca]_o$ in which the muscle fibres had just been equilibrated in the high-Na solution, although the twitch height was quite dependent upon $[Ca]_o$ (briefly reported in Kitazawa, 1980, but details unpublished). This result and others suggest that the major, rapid change in the twitch tension due to $[Ca]_o$ change is not caused by the change in the amount of Ca in the s.r., which, even under normal conditions, is enough to produce a nearly maximum contraction of cardiac muscle. Therefore, the present results support the hypothesis that the change in the $[Ca]_o$ alters the Ca influx that may act as a stimulant of Ca-induced Ca release from the s.r. and so modify the twitch contraction with a rapid time course. However, these arguments do not imply the absence of the filling–depletion mechanism of the Ca store, but lead to the conclusion that this mechanism has little effect on the rapid component of tension change. Despite this, it should be mentioned that we still do not have any reliable evidence of Ca release from the s.r. corresponding to the twitch contraction in the cardiac muscle, irrespective of the mechanism. On the other hand, in the frog heart muscle, Niedergerke, Page and co-workers (Lammel, Niedergerke & Page, 1975; Niedergerke *et al.* 1976) using single atrial trabeculae with widths of 50–150 μm showed that the rapid tension change attributable to extracellular diffusion of Ca occurred with a

half-time of about 2·7 s, and Morad, Goldman & Trentham (1983) found that rapid photochemical inactivation of photolabile Ca antagonists brought about the immediate and full recovery of twitch tension in frog ventricular muscle which had been suppressed by the drugs. It is interesting that they concluded, from similar results to those obtained in this study, that in the frog heart, twitch contraction is induced, directly, by Ca ions which enter the heart cells during the action potential.

In contrast to the fast response, the slow time course of the response following it may not be explained by a simple diffusion of the ions in the extracellular space, but possibly by Ca binding to the sarcolemma and its coats or by slow processes of cellular functions. Specially, the dependence of the time course of the twitch recovery after re-introduction of normal $[Ca]_o$ after Ca-free conditions on the incubation period (Fig. 7) or on the stimulus frequency (Fig. 8*C*) may be favourable for the mechanism of depletion–replenishment of the intracellular store of Ca. In addition, the half-time of Ca leak from the s.r. was roughly in the range of that of the slow twitch change. As expected from the filling–depletion mechanism, the time course of the contractile change differed in the different direction of the same magnitude of the change in $[Ca]_o$, for example, slower decline and faster recovery at higher frequency (Fig. 8*B*). However, this phenomenon in the rapid twitch change can also be explained by the simple diffusion models, as already mentioned by Toll (1978); the calculated time course (Fig. 8*D*) shows that the half-time of the twitch recovery at a frequency of 30/min is expected to be about three times smaller than that of the decline in the preparation, but not significantly different at 6/min. Winegrad & Shanes (1962) have mentioned that even the wash-out curve of [^{14}C]sucrose in atria and of ^{45}Ca loss from the Achilles tendons showed two components, rapid and slow. Anyhow, these critical predictions and assumptions about the intracellular Ca store deduced from physiological experiments, including this study, should be verified by using direct and quantitative methods like the electron probe analysis in skeletal muscle (Somlyo, Gonzalez-Serratos, Shuman, McClellan & Somlyo, 1981). One should determine whether Ca in the junctional s.r. is increased or decreased in a positive or negative inotropic state, how many Ca ions are released from the s.r. during the twitch, etc.

The preparation used in this study has some advantages, e.g. rapid diffusion within the tissue corresponding to that in the frog skeletal T-system, avoidance of enzymatic digestions and the easy measurement of tension developed by fibres isolated from a well-defined portion of the heart. While having some disadvantages for the analysis of electrical or isotopic ion fluxes, due to the presence of damaged fibres, the mechanically dissected, thin cardiac muscle fibre bundles are suitable for the kinetic measurement of tension responses due to changes in extracellular composition or drugs.

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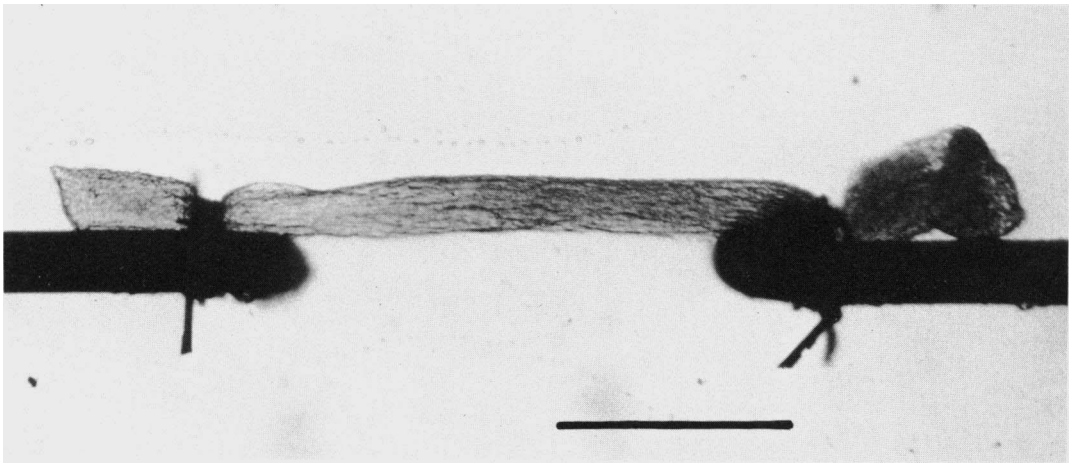

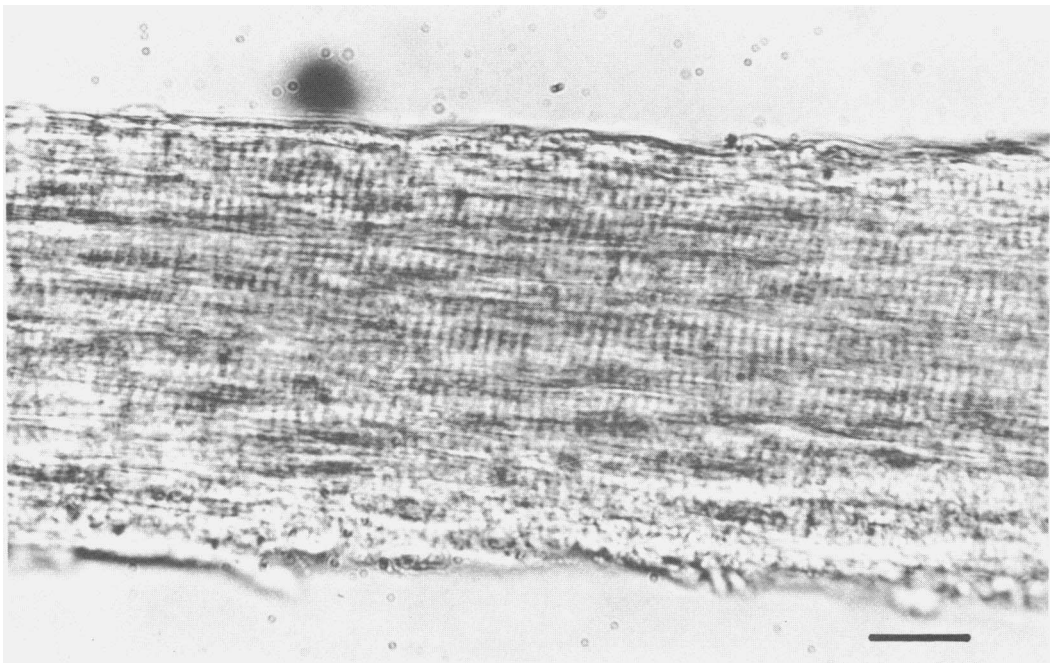

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EXPLANATION OF PLATES

PLATE 1

Light photomicrograph of a small bundle dissected from guinea-pig papillary muscle at low magnification. Both ends of the bundle were tied with a silk thread to the 100 μm wide tungsten rod. Transparent part, left side of the bundle was the tendon, muscle cells consisted in opaque portion. Scale 0·4 mm.

PLATE 2

High magnification of the centre part of the cardiac bundle in Pl. 1. The striation spacing was 2·5 μm. Irregular portion of the lower side was due to the damaged cells with no striation. Scale 20 μm.