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RELAXATION OF MAMMALIAN SINGLE CARDIAC CELLS AFTER PRETREATMENT WITH THE DETERGENT BRIJ-58

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SUMMARY

1. The influence of load and activation on relaxation of heart muscle has been studied.

2. Cardiac cells devoid of functioning sarcolemma were isolated from rat and cat ventricular myocardium. Pretreatment with the detergent Brij-58 destroyed residual sarcoplasmic reticulum function. In order to analyse the mechanical properties of relaxation in these cells, a new miniature transducer was designed which could simultaneously measure lengths (resolution smaller than 0·2 μm), impose loads and measure force by feedback sensing (resolution of 1 μg). Contraction was induced by ionophoretically released calcium ions. Activation, sequestration of calcium and loading conditions could be controlled independently.

3. The time course of relaxation was shown to be governed by the amount of calcium released, and unlike intact preparations from rat or cat heart (but like those from frog), to be independent of load and of alterations in load.

4. We conclude that relaxation of the cardiac contractile system is determined basically by an activation-dependent mechanism, which is masked by load dependence in intact muscle preparations with a well developed calcium sequestering membraneous system.

INTRODUCTION

Relaxation of muscle is generally thought to follow the removal of activating calcium by the membrane pumps (Ebashi & Endo, 1968; Endo, 1977). In cardiac muscle, the mechanical properties of relaxation appear to be governed by the interplay of two mechanisms, i.e. an activation-controlled mechanism, where the removal of calcium ions by the calcium sequestering systems determines the end of the contraction, and a load-dependent decay mechanism, where the onset and subsequent course of relaxation is largely dependent on the load (Brutsaert, De Clerck, Goethals & Housmans, 1978). The relative contribution of these two mechanisms apparently differs with species. Considerable load-dependence is present for example in cardiac muscle preparations of cat, rabbit and pig, but, though present, is less pronounced in rat. However, in frog, load dependence is scarcely found at all, where the activation dependent mechanism thus predominates. The observed differences may be due to known species differences in excitation-contraction coupling mechanisms (Fawcett & McNutt, 1969; Forssmann & Girardier, 1970; Morad & Goldman, 1974). These reported studies were all performed on multicellular whole muscle preparations with intact membrane control.

In order to explore further the possible role of excitation–contraction coupling in these load-dependent influences on activation we have studied the phenomenon in single cardiac cells. These cells were isolated from cat and rat ventricle by manual dissection and pretreated with the detergent Brij-58 which has been shown to destroy both the sarcolemma and sarcoplasmic reticulum (Fabiato & Fabiato, 1975; Orentlicher, Reuben, Grundfest & Brandt, 1974). Contraction was induced by measured pulses of ionophoretically released calcium ions. Relaxation could also be induced by ionophoretical release of EGTA, in an attempt to simulate the *in vivo* calcium sequestering systems.

METHODS

Dissection of preparation

Partially skinned single cardiac cells from the ventricle of rat and cat were obtained by manual dissection as described previously (De Clerck, Claes & Brutsaert, 1977). In order to destroy the sarcoplasmic reticulum in the present study, the strip from which dissection was started was first incubated in a solution containing the following (mM): 132 NaCl, 4 $MgCl_2$, 5 Na_2ATP, 7 glucose, 18 imidazol, 0·5 EDTA (ethyleneglycol-bis-(β-amino-ethyl-ether)N,N'-tetraacetic acid); and 0·5% Brij-58 (polyethylene-20-cetyl-ether). The ATP, EGTA and the non-ionic detergent Brij-58 were obtained from the Sigma Chemical Company and the other reagents were of analytical grade. Treatment with Brij-58 thus eliminated the sarcoplasmic

TABLE 1. Basic characteristics of the preparations used for this study

	Length (μm)	Width (μm)	Peak velocity of shortening of entire preparation (μm/sec)	Peak velocity of shortening of entire preparation lengths/sec	Total isometric force (f_0) μg	Ratio of preload to f_0
Rat						
Mean ± S.E.	89 ± 3	14 ± 1	43 ± 4	0·49 ± 0·04	493 ± 28	0·11 ± 0·01
n	22	22	18	18	15	15
Cat						
Mean ± S.E.	80 ± 4	11 ± 1	24 ± 4	0·31 ± 0·05	325 ± 25	0·16 ± 0·04
n	9	9	9	9	2	2

The dimensions of the preparations along with the ratio of the preload to f_0 were used as criteria for selection of suitable preparations.

n indicates the number of preparations.

reticulum and remaining parts of the sarcolemma (Orentlicher *et al.* 1974). The EGTA concentration used was higher to avoid the contracture otherwise induced by perfusion with Brij-58. After 30–45 min, depending on the dimensions of the strip, perfusion with Brij solution was stopped and cellular preparations were then dissected manually, in a solution containing the first five ingredients listed above, 0·125 mM-EGTA and no Brij-58. Actual experiments were conducted in this solution. These single cardiac cells did not show spontaneous activity when the EGTA concentration in the solution was reduced to 0·025 mM or to zero, but they responded to ionophoretically released calcium ions. Moreover, adding caffeine (25 mM) did not induce contraction, in accord with previous demonstrations in similar preparations (Fabiato & Fabiato, 1975; Orentlicher *et al.* 1974).

Table 1 shows the basic characteristics of the preparations, pretreated with Brij-58, in which satisfactory results were obtained in this study.

Length transducer

In a previous study (De Clerck *et al.* 1977), video processing and television tracking techniques converted the video information into a length signal. The lever system consisted of a miniature

panel meter (electromagnetic torque) and force was measured by feed-back sensing. However, this set-up had some disadvantages in the restricted frequency response: time lag in the camera tube sampling system and inertia of the lever system. These gave rise to a rather high *dynamic* compliance (i.e. displacement of the lever as a function of the rate change of force during an isometric contraction) of 12 μm/mg.s. Television tracking also had to be of exceptional quality to give clean force measurements.

A new miniature transducer was therefore designed which could simultaneously (*a*) measure lengths to 100 μm (with a resolution smaller than 0·2 μm); (*b*) impose loads up to 990 μg and (*c*) measure force by feed-back sensing (with a resolution of 1 μg).

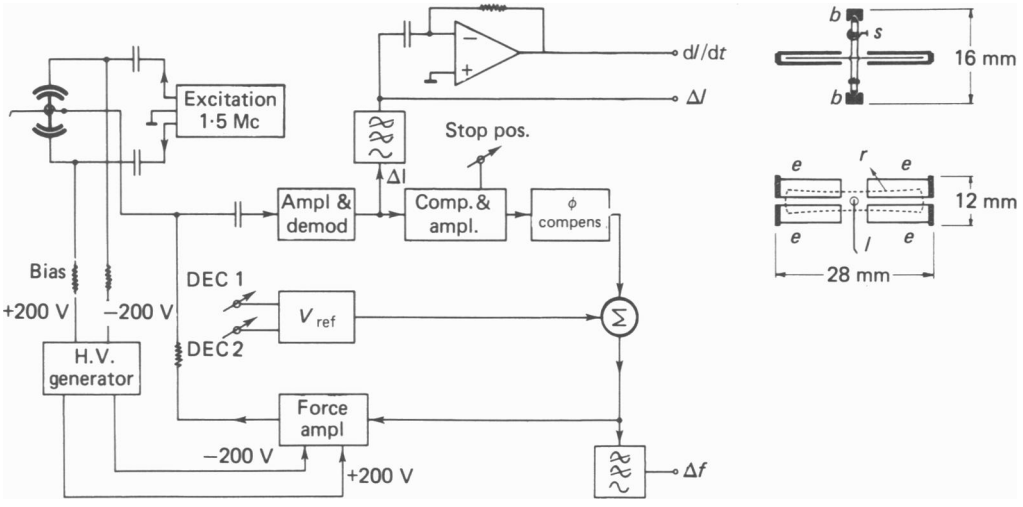

Fig. 1. Block diagram of the force and length sensing system (details see text). Insert upper (front view): *s*, spring; *b*, bearing. Insert lower (bottom view): *e*, stator electrode; *l*, lever; *r*, rotor.

The transducer consisted of four U-shaped stator electrodes (Fig. 1, insert) and a thin magnesium rotor electrode, mounted on a short aluminium shaft. This was supported by two miniature jewel bearings. A very small spiral spring was soldered to an extension of the upper pivot. The lower pivot supported the lever. The electrical contact between the rotor and the spiral spring was made with Ecolit 313, a one component electrically conductive epoxy-adhesive. Both, the upper pivot with the spiral spring and the lower pivot with the lever (in fact a pointer), the bearing support and the jewel bearings were all parts of a dismantled miniature panel meter. All other parts were hand-made and assembled under a binocular microscope. Particular attention was paid to the clearance between the rotor and stator electrodes, to minimize the risk of corona discharge or even arcing-over: the clearance was 0·4 mm or less (bending of the rotor) and the voltage differences could be as high as 375 V.

Displacement

The diametrical opposing stator electrodes were connected to each other, and the two electrode pairs and the rotor were arranged as a variable capacitor half-bridge connection. As the lever moved, the capacitance between the rotor and one pair of stator electrodes increased, whereas the capacitance between the rotor and the other stator electrode pair decreased. To sense these very small changes in capacitance, the bridge was excited with a rather high frequency of 1·5 Mc/s. The signal was sensed by the rotor, amplified and demodulated. The amplifier was calibrated for 1 V/10 μm at the output. Signal-to-noise ratio was better than 40 dB at this sensitivity. This implied that a resolution better than 0·1 μm could be achieved but mechanical vibrations of the whole system limited resolution to 0·2 μm. Four cross-coupled electrodes were used instead of two (theoretically possible), so that capacitance changes due to small movements other than rotation, were cancelled out giving a more stable electrical system.

Velocity of displacement

The length signal was filtered with a second order Butterworth low pass filter (f_c = 5 c/s). An active differentiatior (RC = 0·5 s) was used to obtain the first derivative of the displacement or the velocity of length changes (dl/dt).

Force generation

Force generation was based on electrostatic attraction and repulsion forces between two charged bodies. The two stator pairs were connected to a +200 V and −200 V high voltage generator with the resting potential of the rotor at 0 V. The forces acting on the rotor were in balance, and no torque was developed. A potential applied to the rotor caused attraction to one stator pair and repulsion from the other resulting in a torque which was linearly proportional to the applied voltage (not a quadratic relationship because of the differential electrode configuration; compare with a differential electrostatic loud-speaker). Calibrated voltage was established by decade switches, amplified by the force amplifier and applied to the rotor. This amplifier could deliver up to ± 175 V, and the maximum force, measured at the tip of the lever, exceeded 2000 μg. The decade switches were calibrated up to 990 μg in steps of 10 and 100 μg. Switching from one voltage level to another provided a means of changing the force in the course of a contraction.

Force measurement

The feed-back force-sensing loop has been fully described previously (De Clerck *et al.* 1977). The current source and coil previously used were substituted by the force amplifier and the electrostatic transducer resulting in lower inertia and lag and thus extending the frequency response. The phase compensation was expanded in the frequency domain, and the *dynamic* compliance was reduced to a negligible quantity (1 μm/mg.s). The higher open loop gain also lowered the *static* compliance to 3 μm/mg. The output was filtered (f_c = 5 c/s) and calibrated for 0·1 V/20 μg.

It was possible with this system simultaneously to measure length and force and to generate force, because of the large differences in frequency used for each parameter. Capacitors coupled the 1·5 Mc/s excitation to the stator electrodes, but blocked the high d.c.-bias voltages. A small capacitor coupled the rotor signal to the 1·5 Mc/s amplifier, and blocked the low frequency components of the force amplifier. Further filtering at the input of the 1·5 Mc/s carrier amplifier reduced the interference between carrier and force signals. The output of the bias voltage generator and the force amplifier was connected to the transducer with high-value resistors, which passed the d.c. low frequency components, and represented a high impedance for both excitation and rotor signals.

Ionophoretic current sources

Ionophoretic calcium pulses were used to activate the preparations. Relaxation then occurred either spontaneously after activation induced by these calcium pulses in high EGTA or was induced by ionophoretic EGTA pulses when the cell had been activated by the calcium pulses. Calcium ions or $EGTA^{2-}$ pulses were applied to the preparation by ionophoresis from micropipettes filled with calcium chloride (1·0 M or 0·1 M) or Na_2 EGTA (0·1 M). The pipettes were connected to a positive (calcium ion) or negative ($EGTA^{2-}$) current source. These sources had the same electrical specifications as described previously (De Clerck *et al.* 1977).

RESULTS

I. Lack of load dependent changes in relaxation

Fig. 2 shows the length and force traces of a series of increasingly afterloaded contractions from an isolated Brij-pretreated cardiac cell of cat (panel *A*) and rat (panel *B*). Each contraction was activated similarly with the same sized pulses of calcium ions. The afterload was increased with each contraction until the preparation

contracted isometrically. The time course of relaxation of these isolated cells was found to be unaffected by load; regardless of the loading conditions both in cat and in rat, all contractions tended to coincide during the isometric phase and, hence, were of about the same over-all duration.

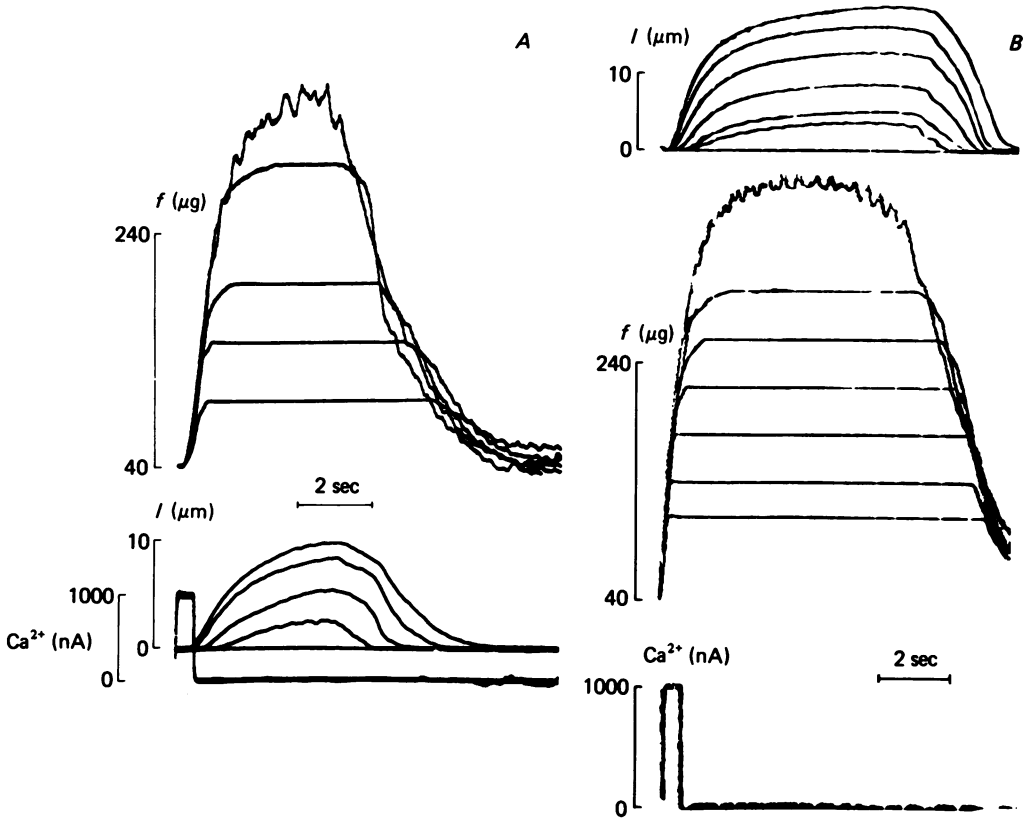

Fig. 2. Force (f) and length (l) traces of a series of afterloaded contractions in single Brij-pretreated cardiac cells of cat (panel A) and rat (panel B). The activating calcium pulses (Ca^{2+}) are also indicated.

The possibility that load might influence the onset and time course of relaxation was further tested by imposing abrupt step changes in load (load clamps) during the isotonic phase (Brutsaert, Cleas & Sonnenblick 1971). Such changes in load were similarly without effect on relaxation (Fig. 3). Contraction simply proceeded in the manner appropriate to the instantaneous load, independently of any differences in loading conditions earlier in that same contraction, and without any effect of instantaneous or preceding load on the time course of contraction and relaxation. Similar findings were obtained in two cells isolated from rabbit ventricle. Accordingly, in isolated cells, deprived of sarcoplasmic reticulum by Brij-pretreatment, no load dependence occurred; relaxation seemed determined mainly by the decay of activation. In contrast to the results in the isolated cells, a marked load dependence was observed in intact papillary muscle of cat, rabbit and rat (Brutsaert *et al.* 1978).

II. Activation dependence of relaxation

The influence of activation on relaxation was examined by comparing contractions activated by different sized calcium pulses, but with the same load (Fig. 4). In Fig. 4*A*, a series of isotonic, increasingly more highly activated contractions was

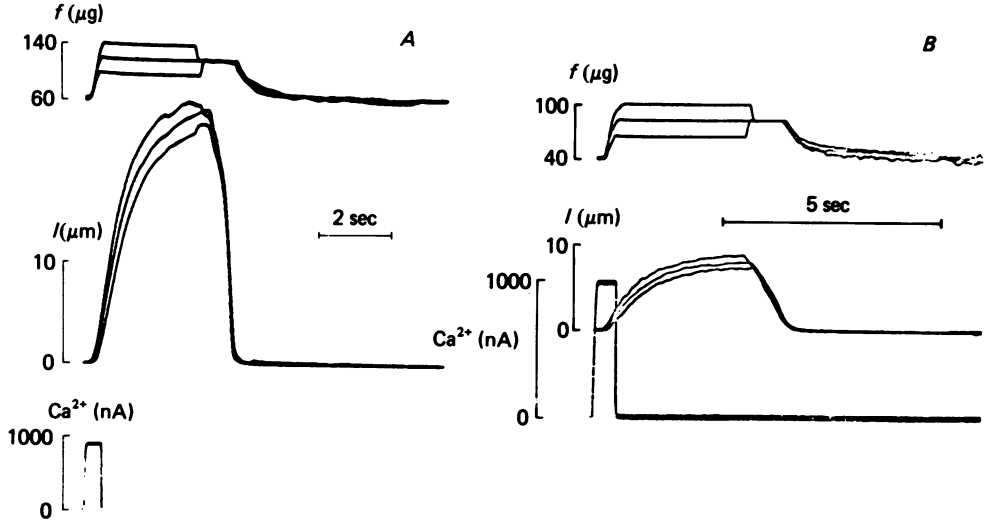

Fig. 3. Force (f) and length (l) traces of a loading and an unloading step of the same magnitude and imposed at about the same time during the contraction in single Brij-pretreated cardiac cells of cat (panel *A*) and rat (panel *B*). The activating calcium pulses (Ca^{2+}) are also indicated. Both load-clamped contractions were clamped to the same afterload. The control afterloaded isotonic contractions to which the preparation was clamped is also shown.

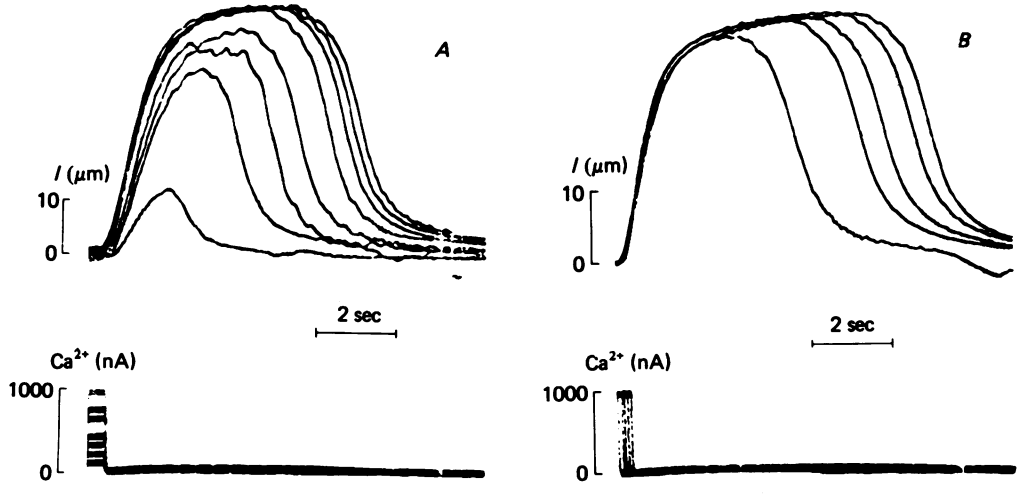

Fig. 4. Length (l) traces of a series (preloaded without afterload) isotonic contractions of single Brij-pretreated cells activated by ionophoretic pulses (Ca^{2+}) of increasing magnitude and constant duration (panel *A*) and of increasing duration at constant amplitude (panel *B*). Amplitude and duration of contraction is solely determined by the amount of released calcium ions.

superimposed. A corresponding increase was seen in the extent of shortening of the preparation, up to a maximal value or ceiling. The amplitude of the response, as a function of the amount of calcium ions released in the activating ionophoretic pulse, represents the dose–response curve of the preparation as previously described (De Clerck *et al.* 1977). Prolongation of a calcium pulse, which would induce a maximal response resulted in no further increase in amplitude, but a prolongation of the mechanical response with delay of subsequent relaxation as shown in Fig. 4*B*.

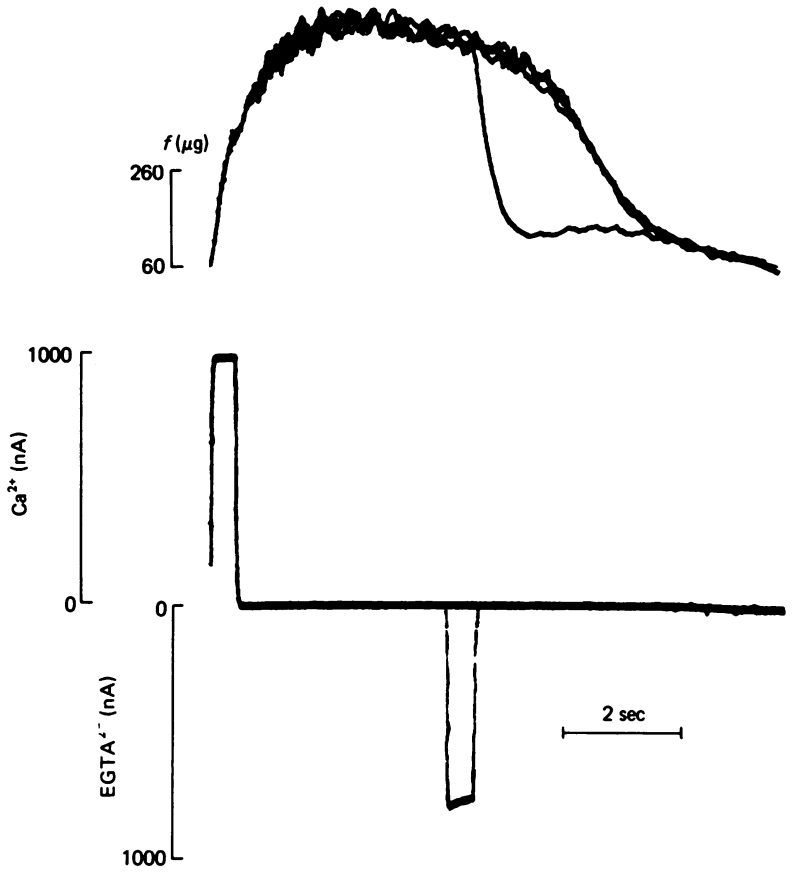

Fig. 5. Force (*f*) traces of an isometric contraction of a single Brij-pretreated rat cell in which relaxation was induced by an EGTA-pulse ($EGTA^{2-}$) and of three control contractions activated by the same calcium pulse (Ca^{2+}), but where relaxation occurred spontaneously.

An attempt was made to simulate the rapid sequestration of calcium normally effected in intact muscle by the sarcoplasmic reticulum (Endo, 1977; Fabiato & Fabiato, 1977). This is illustrated in Fig. 5, which shows the effect of ionophoretically releasing EGTA from a micropipette to a cellular preparation activated by an earlier pulse of calcium. An EGTA pulse of sufficient magnitude induced relaxation; this induced relaxation was only partial and transient with smaller pulses of EGTA.

Load dependent effects on the onset and time course of relaxation were sought also under different activation conditions (Fig. 6). A load step of the same magnitude

and at approximately the same time was imposed during two contractions, one of which was activated by a long calcium pulse and the other one by a short pulse. Both calcium pulses were of sufficient magnitude to assure maximal activation. Again, in each case, the change in load had no effect on relaxation, and the clamped contraction coincided in each case with its respective control contraction. An abrupt change in load was also shown to have no effect on relaxation in contractions where the contraction was abbreviated by pulsed EGTA. These findings indicate that

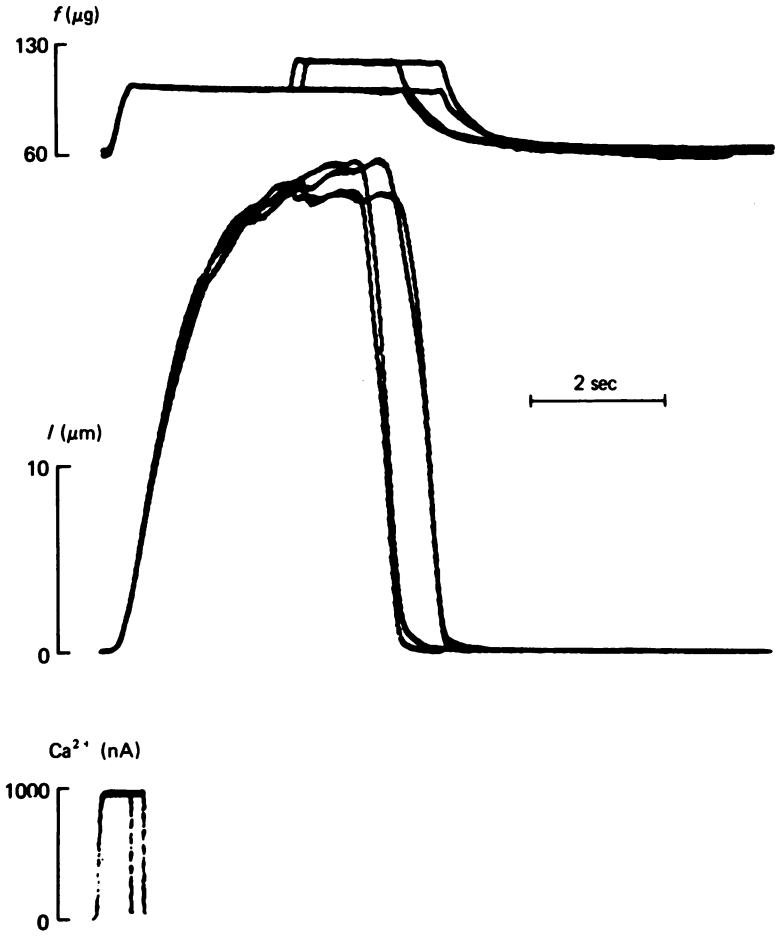

Fig. 6. Force (f) and length (l) traces of two load-clamped and two afterloaded control contractions of single Brij-pretreated cells, activated by two calcium pulses (Ca^{2+}) of different duration.

relaxation in these single cell preparations remains mediated by activation-dependent mechanisms, and no experimental conditions were found where this could be overridden by any load-dependent mechanism, in contrast to the situation in whole muscle from these mammalian species.

DISCUSSION

In the present study, treatment with the detergent Brij-58 was assumed to destroy the sarcoplasmic reticulum (Fabiato & Fabiato, 1975; Orentlicher *et al.* 1974) and any residual sarcolemma. Single cardiac cells thus obtained, therefore represent simple mechanical systems which differ from intact preparations in that they are deprived of any membrane control of the levels of calcium. The removal of calcium and hence relaxation, can be influenced only by the remaining calcium sequestering system, i.e. the EGTA in the perfusing solution. Thus the load bearing capacity could be analysed under rigorous control of activation and of the subsequent sequestration of the activating calcium ions.

In contrast to demonstrations of load-dependent effects on the time course of relaxation in intact muscle preparations of rat and cat (Brutsaert *et al.* 1978) no such effects were demonstrated in these isolated cell preparations (Figs. 2 and 3). After destruction of the membraneous systems, the activating sarcoplasmic calcium level at any time would be determined solely by the amount released by the ionophoretic calcium pulse and by the buffering capacity of the EGTA which competes with the troponin sites for calcium. Evidence that relaxation in these isolated cells is related to the decline in the amount of free calcium ions is provided by experiments showing that graded responses were obtained, as previously described (De Clerck *et al.* 1977) when the amplitude of the activating calcium pulse was increased, but that relaxation was correspondingly postponed when the duration of the pulse necessary for maximal response was increased (Fig. 4). Alternatively, contraction could be abbreviated by additional EGTA, iontophoretically released, simulating the sequestering function of the sarcoplasmic reticulum in intact muscle (Fig. 5).

In isolated cells without a functional sarcoplasmic reticulum, relaxation thus appears to be determined by the decaying amount of calcium ions, resulting in a progressively diminishing number of force-generating sites and additional load changes do not influence this activation-controlled relaxation. Some small time differences were seen at the end of the isotonic relaxation but these were most pronounced at lower loads and are probably attributable to friction or viscous forces. In intact muscle preparations load-dependent effects on relaxation would seem to be superimposed on the activation-dependent relaxation seen in the simpler preparations of the isolated contractile system and in intact preparations of frog myocardium. We postulate that load-dependent mechanisms become apparent when the decline in calcium concentration in the surrounding myoplasma is relatively fast so that it is no longer the rate-limiting step. Species differences (Briggs, Poland & Solaro, 1977; Fabiato & Fabiato, 1977; Fawcett & McNutt, 1969; Forssmann & Girardier, 1970) in the efficacy of calcium sequestration by sarcoplasmic reticulum might therefore be reflected in the relative predominance of load-dependent mechanisms during relaxation. It has been shown that the load-dependent changes in the time course of mechanical relaxation are very marked in intact ventricular muscle preparations from cat heart, less so in rat myocardium and about absent in frog (Brutsaert *et al.* 1978). The differences between cat and frog are consistent with recognized differences in the importance of the sarcoplasmic reticulum in the two types of mucle. In cat myocardium the sarcoplasmic reticulum is well developed

(Fawcett & McNutt, 1969) whereas frog heart has a less developed sarcoplasmic reticulum and a relatively larger cell surface (Morad & Goldman, 1974; Winegrad, 1973). Physiological studies have also implied a greater role for sarcolemma than for the sarcoplasmic reticulum in frog (Vassort, 1973). The evidence therefore supports the idea that activating calcium can be removed more rapidly in cat than in frog heart muscle. In intact rat heart preparations load-dependent effects on relaxation are less marked than in cat, implying relatively less rapid removal of activating calcium from the myoplasm. Yet, rat heart muscle has not only adequate (Forssmann & Girardier, 1970) but well filled internal calcium stores (Fabiato & Fabiato, 1972). Thus, this seeming discrepance may be due to the fact that in rat heart muscle a large amount of calcium is released during contraction and will impose a large calcium load on the sarcoplasmic reticulum during relaxation so that it may take relatively longer to reduce the concentration of calcium to subthreshold levels. However, cat cardiac muscle must be regarded as only partially activated under normal conditions (Katz, 1970; Bassingthwaighte & Reuter, 1972; Fozzard, 1973; Fabiato & Fabiato, 1977), which could account for the striking preponderance of loading conditions causing differences in relaxation.

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