*Journal of Physiology* (1988), **404**, *pp.* 53–69
*With 8 text-figures*
*Printed in Great Britain*



# EFFECT OF ACETYLSTROPHANTHIDIN ON TWITCHES, MICROSCOPIC TENSION FLUCTUATIONS AND COOLING CONTRACTURES IN RABBIT VENTRICLE

BY DONALD M. BERS AND JOHN H. B. BRIDGE*

*From the Division of Biomedical Sciences, University of California, Riverside, CA 92521-0121, U.S.A., and *The Nora Eccles Harrison Cardiovascular Research and Training Institute and Department of Medicine, University of Utah School of Medicine, Salt Lake City, UT 84112, U.S.A.*

(*Received 21 May 1987*)

SUMMARY

1. We have measured the effect of the aglycone acetylstrophanthidin (ACS) on twitches, cooling contractures and microscopic tension fluctuations in rabbit ventricular muscle.

2. Both developed twitches and cooling contractures are strengthened by applications of ACS in the range 1–4 $\mu$M. This positive inotropy averages 150–160% of control (zero ACS) in both twitches and cooling contractures. Cooling contracture magnitude is assumed to reflect the availability of sarcoplasmic reticulum (SR) $Ca^{2+}$ for contraction (Bridge, 1986). We infer that ACS increases the availability of SR $Ca^{2+}$ by enlarging SR $Ca^{2+}$ stores and this may contribute to the positive inotropy.

3. However, twitches appear to increase at lower concentrations of ACS than those required to increase cooling contractures. This observation suggests that the initial ACS inotropy may be achieved without an increase in SR $Ca^{2+}$. Furthermore, low doses of ACS produce positive inotropy in the presence of 10·0 mM-caffeine where cooling contractures are abolished. This also suggests that positive inotropy occurs in the absence of SR $Ca^{2+}$ accumulation.

4. Rest decay of both cooling contractures and twitches is significantly slowed in 4 and 8 $\mu$M-ACS. We infer that ACS slows the rate of decline of SR $Ca^{2+}$ available for contraction by slowing the rate at which $Ca^{2+}$ is lost from the cell during rest. This suggests that ACS produces a net slowing of $Ca^{2+}$ efflux during activity which in the absence of altered $Ca^{2+}$ influx will result in net $Ca^{2+}$ gain and presumably enlarged SR $Ca^{2+}$ stores.

5. Increasing the concentration of ACS (6–10 $\mu$M) results in a decline in developed twitch tension, total tension and an increase in rest tension. Measurement of microscopic tension fluctuations indicates that as developed twitches decline, the root mean square (r.m.s.) of the tension fluctuations increases in a reciprocal manner. This supports the suggestion of others that the decline in developed twitch tension and the appearance of tension fluctuations are causally related.

6. Although ACS (6–10 $\mu$M) causes a decline in twitch tension, rapid cooling

* To whom reprint requests should be sent.

contractures remain elevated. We suggest that in the presence of $Ca^{2+}$ oscillations the magnitude of cooling contractures reflects the sum of cytosolic $Ca^{2+}$ and $Ca^{2+}$ that is available for release. If microscopic tension fluctuations do represent $Ca^{2+}$ moving between the SR and cytosol the sum of SR and cytosolic $Ca^{2+}$ and hence cooling contracture might not decline. Thus a net loss of $Ca^{2+}$ available for contraction (i.e. cytoplasmic + SR) is unlikely to be principally responsible for the decline in developed force at higher ACS. A decrease in the fraction of SR $Ca^{2+}$ released upon excitation may, however, contribute to this decline in force.

7. Large $Ca^{2+}$ oscillations (as observed experimentally by Eisner & Valdeolmillos, 1986) could contribute to a decline in developed twitch tension by reducing the SR $Ca^{2+}$ release associated with action potential-induced contractions. Other factors (e.g. series compliance) must also be invoked to damp the resulting microscopic tension fluctuations to the experimentally observed magnitudes.

## INTRODUCTION

Cardiac glycosides and aglycones strengthen ventricular muscle contractions (positive inotropic effect). At higher concentrations the magnitude of the developed twitches begins to decline, diastolic tension begins to rise and after-contractions start to appear. These latter effects are said to reflect cardiotoxicity (e.g. Wier & Hess, 1984).

Hypotheses regarding the role of reciprocal $Na^+$ and $Ca^{2+}$ movements in the positive inotropic effect of cardiac glycosides were first postulated by Repke (1964), Glynn (1964), Langer (1965) and Baker, Blaustein, Hodgkin & Steinhardt (1969). Partial inhibition of the $Na^+$ pump by glycosides (or aglycones) increases intracellular $Na^+$ (Langer & Serena, 1970; Ellis, 1977; Sheu & Fozzard, 1982). The relationship between intracellular $Na^+$ and tension is very steep (Lee, Kang, Sokol & Lee, 1980; Lee & Dagostino, 1982; Eisner, Lederer & Vaughan-Jones, 1983). The increase in intracellular sodium activity ($a^i_{Na}$) would reduce extrusion of $Ca^{2+}$ via $Na^+$–$Ca^{2+}$ exchange and may also enhance $Ca^{2+}$ influx via this system (Mullins, 1979; Axelson & Bridge, 1985; Bers, 1987) resulting in a net gain in cellular $Ca^{2+}$. This would be expected to increase sarcoplasmic reticulum (SR) $Ca^{2+}$ content and the magnitude of the twitch by making more $Ca^{2+}$ available for contraction (Allen, Jewell & Wood, 1976). If this scheme is correct, positive inotropic doses of cardiac glycosides should lead to increases in the SR $Ca^{2+}$ available for contraction.

The negative inotropic effects of toxic doses of cardiac glycosides have received less attention than the positive inotropic effects of subtoxic doses. High extracellular $[Ca^{2+}]$ is known to produce negative inotropic effects and these are further increased in the presence of glycoside (Kort & Lakatta, 1984; Eisner & Valdeolmillos, 1986). Since toxic doses of glycoside and high extracellular $[Ca^{2+}]$ produce intracellular $Ca^{2+}$ fluctuations and microscopic tension fluctuations (thought to reflect the oscillation of $Ca^{2+}$ between the SR and cytosol), Eisner & Valdeolmillos (1986) suggested that the presence of these oscillations and negative inotropic effects are causally related.

Extensive investigations by Kurihara & Sakai (1985) and later by Bridge (1986) and Bers, Bridge & MacLeod (1987) suggest that rapid cooling of mammalian heart

muscle causes contractions that are activated by $Ca^{2+}$ from intracellular stores. In particular, the magnitude of these rapid-cooling contractures (RCCs) very likely reflects the availability of SR $Ca^{2+}$ for contraction (Bridge, 1986; Bers *et al.* 1987). Thus RCCs may provide a useful tool to investigate changes in SR $Ca^{2+}$ availability during both the positive and negative glycoside inotropy in heart muscle.

We find that positively inotropic doses of the aglycone acetylstrophanthidin (ACS) strengthen both twitches and RCCs, suggesting that the drug does augment SR $Ca^{2+}$ stores. At higher ACS concentration, twitches are weakened and microscopic tension fluctuations become apparent (presumably produced by $Ca^{2+}$ oscillation). However, despite the decline in twitch tension at higher ACS concentrations, RCC amplitude increases to a plateau level. This result suggests that the negative inotropy may not be due simply to a decrease in SR $Ca^{2+}$ available for release.

## METHODS

### *General*

Papillary muscles or ventricular trabeculae (0·2–1·0 mm in diameter) were obtained from the hearts of adult New Zealand White rabbits after an intravenous injection of pentobarbitone sodium (~ 70 mg/kg). Loops of fine suture were tied around each end of the muscles, which were then mounted in a superfusion chamber (0·15 ml in volume). One end was fixed, and the other was attached to a force transducer contructed from a piezo-resistive element (AE 801, SensoNor). All experiments were performed at 29–30 °C. Temperature variation during any experiment was less than 0·4 °C. Prior to experimental interventions, the muscles were equilibrated for at least 45 min in normal Tyrode solution and stimulated at 0·5 Hz. The normal Tyrode solution contained (mM); NaCl, 140; KCl, 6; $MgCl_2$ 1; glucose, 10; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 5 (pH 7·40). Muscles were stimulated by square pulses (5 ms) applied by platinum field electrodes fixed to the lateral walls of the chamber.

All solutions were equilibrated with 100% $O_2$. Caffeine (Sigma) was added as a solid to the Tyrode solution. ACS (Sigma) was dissolved in ethanol to produce stock solutions. The concentrations of ethanol in the superfusate did not exceed 0·1%. Membrane potentials were measured with conventional borosillicate micropipettes filled with 3 M-KCl using standard techniques.

The flow rate was normally ~ 5 ml/min and was increased to ~ 15 ml/min when rapid cooling contractures were being measured. The perfusion lines to the chamber were jacketed (and maintained at −2·0 and 30·5 °C) before going into solenoid switch valves, with the outlets connected directly to the chamber inlet. The solenoid valves allowed rapid changes in bath temperature from 30 to 0 °C. The bath temperature was generally below 5 °C in less than 750 ms. Details of the cooling method have already been described (Bridge, 1986).

Some of the experiments were performed with a transducer that was both AC and DC coupled. The AC-coupled response was observed at high gain and was filtered (single-pole low cut-off = 2 Hz, high cut-off = 10 Hz). The data were collected digitally for 60 s at a sampling rate of 25 Hz. The digitized data were subjected to spectral analysis by fast Fourier transform or computation of root mean square (r.m.s.) amplitude of the microscopic tension fluctuations.

Results from our mathematical model (see Appendix) were calculated on an IBM PC XT with a maths coprocessor (8087). The model program was written in BASIC and compiled for rapid execution (87BASIC/INLINE, Microway, Inc.).

## RESULTS

### *The positive inotropic effect of acetylstrophanthidin*

If isolated rabbit papillary muscles are rapidly cooled immediately after electrical stimulation they develop a contracture that rises to a peak in 5–15 s and subsequently

declines much more slowly. Available evidence (Bridge, 1986) suggests that these contractures are activated by intracellular stores of $Ca^{2+}$ and that their magnitude provides a relative measurement of the quantity of SR $Ca^{2+}$ available to activate tension. We assume that, in general, if SR $Ca^{2+}$ availability increases it is because SR $Ca^{2+}$ content is enlarged (Allen *et al.* 1976). The effect of ACS on intracellular $Ca^{2+}$ stores was measured using this approach. Stimulation was stopped and control RCCs were obtained by rapidly cooling the muscle immediately after the last stimulated

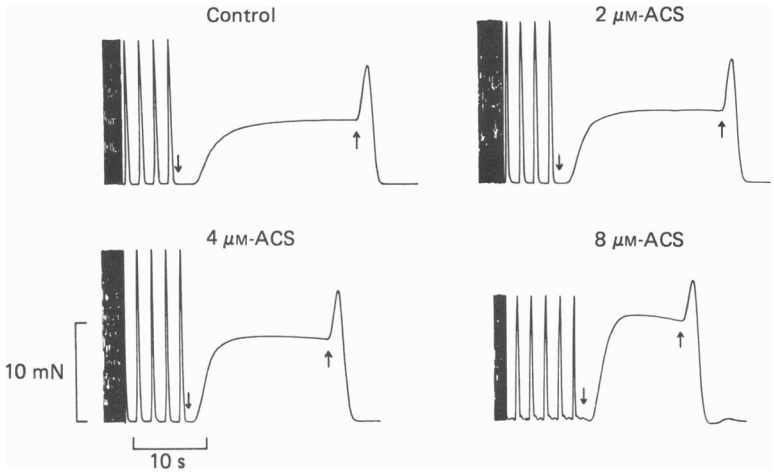


Fig. 1. A single rabbit papillary muscle (0·45 mm diameter) was cooled (first arrow) after stopping electrical stimulation (0·5 Hz). This produced a cooling contracture which relaxed upon rewarming (second arrow). This figure illustrates the increase in twitch and RCC magnitude after treating the muscle with increasing concentrations of ACS (0–8 μM in this case). Cooling contractures were always elicited after steady-state twitches were obtained.

contraction (Fig. 1). After development of peak contracture tension the muscle was rewarmed and stimulated until stable twitches were obtained. The muscle was then superfused with a modified Tyrode solution containing a positive inotropic dose of ACS. When stable twitches were measured, another RCC was induced. This procedure was repeated for increasing concentrations of the aglycone. Increasing concentrations of ACS (1–4 μM) increased twitch tension. This was usually accompanied by increases in RCC magnitude. The increase in cooling contracture magnitude was most pronounced in those cases in which the strengthening of twitch tension was substantial. The averaged response of both twitches and cooling contractures varied with the concentration of ACS (Fig. 2).

At concentrations higher than 4 μM the muscles usually began to exhibit effects which are characteristic of glycoside toxicity (e.g. decreased twitch force, after-contractions and increased resting force). For the present we will consider results at ACS concentrations which are below that at which toxic effects are observed.

In explaining these results we assume that ACS has no direct effect on the contractile elements that would produce the observed inotropy (Fabiato & Fabiato, 1973; Nayler, 1973). We further assume that the inotropic effect is due to a change

in the magnitude and/or time course of the $Ca^{2+}$ transient or an increased diastolic $[Ca^{2+}]_i$. If the duration of the $Ca^{2+}$ transient is prolonged the tension-generating mechanism might come closer to equilibrium and produce more tension. An increase in the diastolic or systolic $Ca^{2+}$ concentration in the vicinity of the myofilaments would also lead to the increased tension. A partial explanation of the inotropic effect of ACS is suggested by the increase in cooling contracture magnitude. Based on previous interpretations of cooling contractures, ACS appears to cause an increase in the SR $Ca^{2+}$ that is available for contraction. If more SR $Ca^{2+}$ is available for

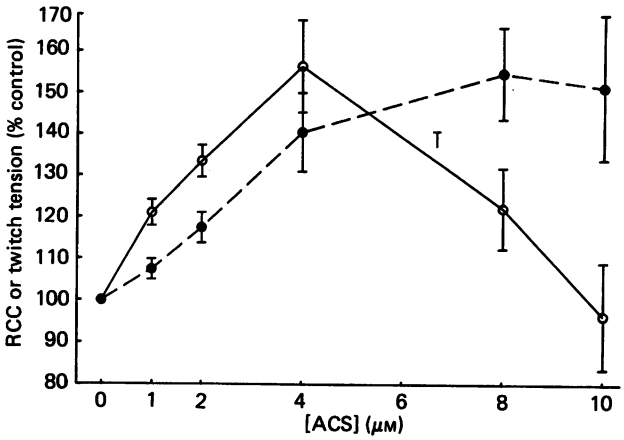


Fig. 2. Dose–response curve for both twitch and cooling contracture magnitude as a function of ACS concentration (mean ± S.E.M., $n = 14$ muscles). ○ represent twitches and ● represent cooling contracture magnitude. The results are expressed relative to the control value (0 mM-ACS = 100%).

contraction then changes in both the magnitude and/or time course of the $Ca^{2+}$ transient might produce the inotropy. However, this cannot be the only explanation of the inotropic effect of this drug because occasionally increases in twitch tension are not accompanied by increases in cooling contractures (see below and Bers, 1987).

*The inotropic effect of acetylstrophanthidin in the presence of caffeine*

In this section we have investigated the hypothesis that positive inotropy may be produced by ACS in the absence of SR function. There is considerable evidence that caffeine prevents the SR from sequestering $Ca^{2+}$ (Weber & Herz, 1968; Blaney, Thomas, Muir & Henderson, 1978) and RCCs which are thought to reflect the availability of SR $Ca^{2+}$ are also abolished by caffeine in a dose-dependent manner (Fig. 3). The monotonic nature of the decline in RCC as a function of caffeine concentration is of interest since low concentrations of caffeine were reported to enhance or allow observation of RCCs in skeletal muscle and frog heart (Sakai, 1965; Chapman & Ellis, 1974).

The influence of caffeine on the SR offers the possibility of investigating inotropy induced by ACS under circumstances where the SR is unlikely to contribute to the inotropic effect. Indeed, Bers (1987) has observed that pre-treatment of rabbit

ventricular muscle with caffeine or ryanodine does not prevent the inotropy induced by ACS, $[K^+]_o$ reduction or low $[Na^+]_o$. An extension of these findings (Fig. 4) indicates that low ACS concentrations produce inotropy which is similar in the presence or absence of 10 mM-caffeine. However, in the presence of caffeine cooling contractures are not observed at any concentration of ACS. Caffeine also increases myofilament $Ca^{2+}$ sensitivity (Fabiato, 1981*a*; Wendt & Stephenson, 1983), such that a smaller increase of intracellular $Ca^{2+}$ accumulation might produce a significant

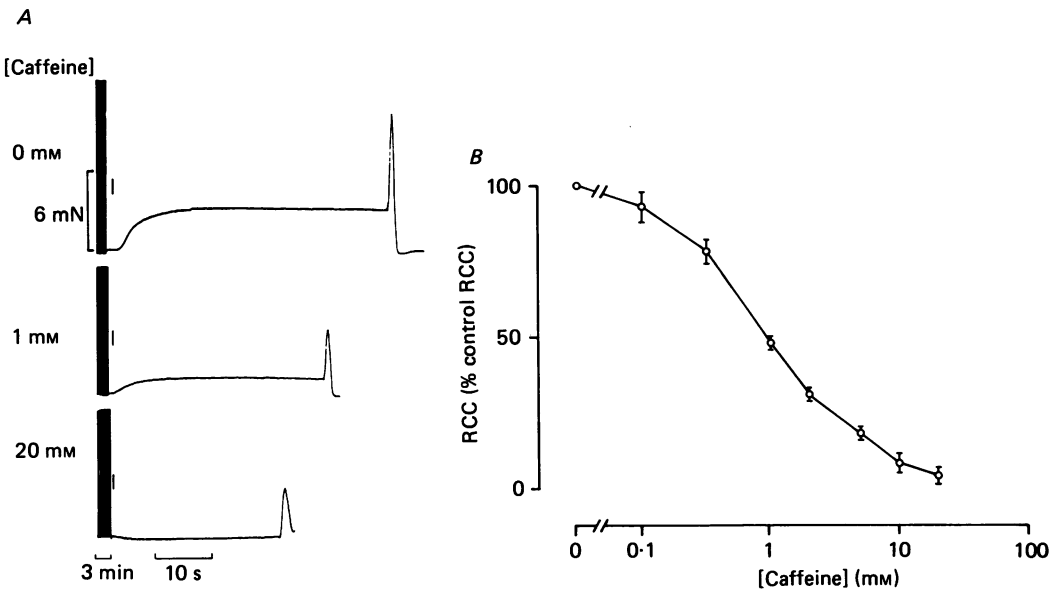


Fig. 3. The effect of caffeine on cooling contractures. *A*, after obtaining steady-state twitches in the presence of 0, 1 and 20 mM-caffeine the muscle was abruptly cooled. One millimolar caffeine partially and 20·0 mM-caffeine completely abolished cooling contractures. *B*, dependence of cooling contractures magnitude on caffeine concentration. Cooling contracture magnitude is 50% inhibited by 1·0 mM-caffeine.

tension increase. In muscles equilibrated with 10 mM-caffeine, the decline in developed tension at higher [ACS] is always accompanied by increased resting force. This is in contrast to control muscles where substantial reduction in developed twitches usually occurred prior to increased resting force.

Figure 4 also shows that in the absence of caffeine, 1 $\mu$M-ACS is sufficient to produce an increase in contractile force (□) without an increase in cooling contractures (△). This trend is also apparent, but less clear in Fig. 2. This suggests that even *with* a functional SR an inotropic effect is observable which does not depend on an increase in SR $Ca^{2+}$ stores.

We suggest that the inotropic effect of ACS (which does not depend on SR $Ca^{2+}$ load) is due to either an increase in diastolic $[Ca^{2+}]_i$ and/or a change in duration or magnitude of the $Ca^+$ transient. This could come about if ACS altered the balance of transsarcolemmal $Ca^{2+}$ fluxes which presumably play a major role in determining the $Ca^{2+}$ transient, especially in the absence of SR function.

*Rest decay of cooling contractures in the presence of acetylstrophanthidin*

If cooling contractures are preceded by increasing periods of rest they are weakened. The longer the period of rest the weaker the contracture (Bridge, 1986). The rest decay of cooling contractures has been explained by supposing that at rest $Ca^{2+}$ leaks from the SR into the cytosol and is then transported across the sarcolemma by the $Ca^{2+}$ pump and/or $Na^{+}$–$Ca^{2+}$ exchange. If this explanation is correct, the rest decay of cooling contractures provides an indirect indication of the rate of net $Ca^{2+}$ loss from quiescent cells (and SR). Twitches also exhibit rest decay. This may also be explained by SR depletion (Allen *et al.* 1976).

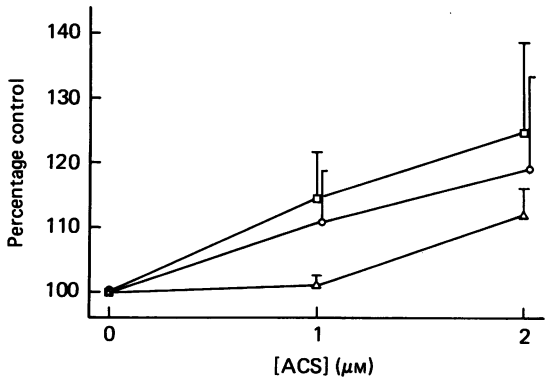


Fig. 4. Effect of ACS on cooling contractures (△) and twitches (□) in the absence of 10·0 mM-caffeine. The response of twitches to ACS in the presence of caffeine (○) is very similar. When the concentration of ACS (in the presence of caffeine) is increased beyond 2 μM toxic effects begin to appear. No cooling contractures are elicited in the presence of 10 mM-caffeine.

Rest decay of twitches and RCCs was measured in isolated single papillary muscles at 30 °C (Fig. 5). In the absence of ACS, muscles exhibited decay half-times of 95 and 60 s for twitches and cooling contractures respectively. The same muscles were treated with ACS (4 and 8 μM) and rest decay was then measured on the treated muscle. ACS produced increases in twitch tension and cooling contractures and significantly slowed both rest decays (half-times of decay were > 300 s for both). Insofar as the rest decay curves reflect the rate at which the SR is depleted by rest, our result suggests that inotropic doses of ACS significantly retard this depletion. If ACS reduces time-averaged $Ca^{2+}$ efflux or increases time-averaged $Ca^{2+}$ influx (via $Na^{+}$–$Ca^{2+}$ exchange) during the rest, then slowing of the rest decay of SR $Ca^{2+}$ content may be expected (Sutko, Bers & Reeves, 1986).

*The negative inotropic effect of ACS*

When ACS concentration exceeds 4 or 6 μM the magnitude of developed twitches begins to decline (Figs 1 and 2). Action potential duration declines both at positive, and to a lesser degree, at negative inotropic ACS concentrations (not shown). It

seems unlikely that these effects are directly responsible for the negative inotropy. As the concentration of ACS is increased, diastolic tension increases and the negative inotropic effect (reduced developed tension) is more pronounced and slow after-contractions often occur. Moreover, total twitch tension (developed and diastolic) also declines. Unexpectedly, the magnitude of RCCs continued to increase while the twitch force declined. Thus, we might infer that with increasing glycoside the SR content enlarges. This seems inconsistent with the finding that twitch tension simultaneously declines.

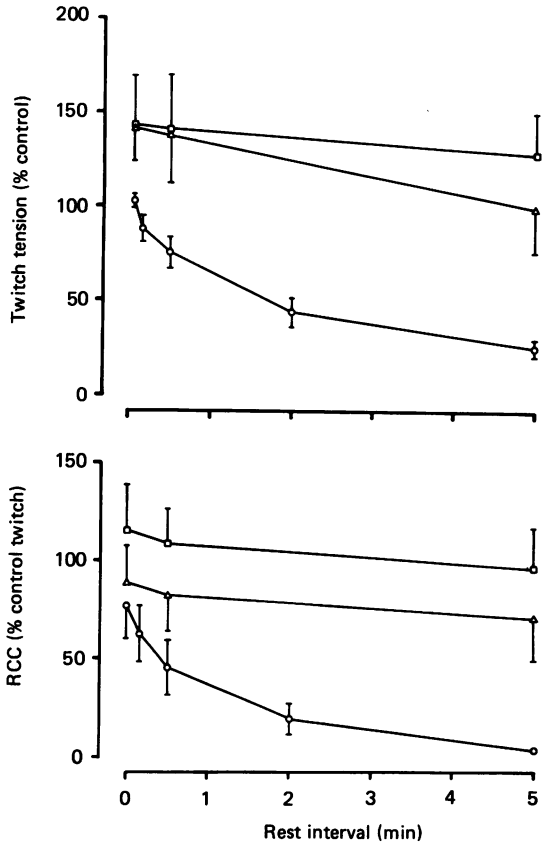


Fig. 5. Upper panel shows rest decay twitches in zero ACS (○) in the presence of 4 $\mu$M-ACS (△) and in the presence of 8 $\mu$M-ACS (□). Lower panel shows rest decay of cooling contractures in zero ACS (○) in the presence of 4 $\mu$M-ACS (△) and in the presence of 8 $\mu$M-ACS (□). Both twitches and cooling contractures were examined after steady-state contractile activity was achieved at each ACS concentration.

Allen, Eisner & Orchard (1984) and Nieman & Eisner (1985) observed oscillation of $[Ca^{2+}]_i$ and tension in the presence of strophanthidin and at high $[Ca^{2+}]_o$, respectively. Allen *et al.* (1984) and Allen, Eisner, Pirolo & Smith (1985) have suggested that these $Ca^{2+}$ oscillations might interfere with twitch development. We have examined these microscopic tension oscillations as a function of ACS concentration with the same protocol used to assess RCCs (Fig. 6).

Increasing concentrations of ACS were applied to contracting muscle. At each concentration (0–10 $\mu$M) steady-state (or nearly steady-state) twitches were obtained. When electrical stimulation of the muscles was discontinued the AC-coupled tension response was observed at approximately 1000 times the gain of the DC response. After electrical stimulation the AC-coupled transducer was allowed about 5 s to settle then the AC-coupled signal was sampled for 60 s. Root mean square (r.m.s.)

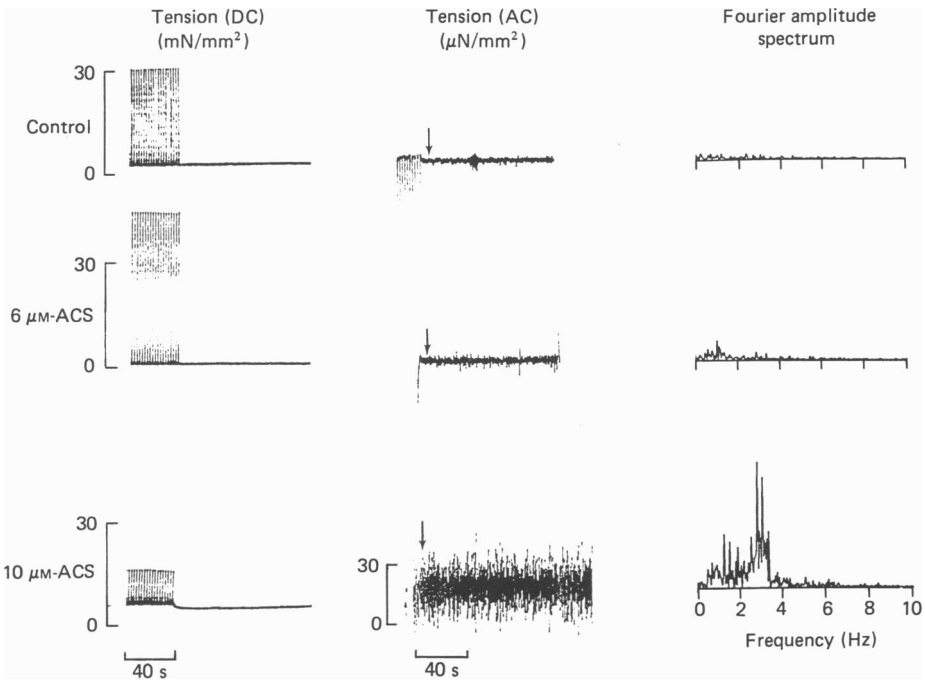


Fig. 6. Upper panel shows control twitches, microscopic tension fluctuations and Fourier spectrum of the fluctuations in modified standard Tyrode solution. Middle panel shows the effect of 6 $\mu$M-ACS on these parameters. The amplitude of the tension fluctuations increased slightly and a peak at low frequency is just apparent in the Fourier spectrum. Lower panel shows the effect of 10 $\mu$M-ACS. Twitch tension is significantly reduced. The amplitudes of microscopic tension fluctuations are considerably increased and they exhibit a pronounced spectral peak at approximately 3 Hz. The arrows indicate the point at which sampling of the tension fluctuations began (60 s at 25 Hz).

values of tension fluctuation amplitude and Fourier spectra were calculated from the samples. At positive inotropic doses of ACS tension fluctuations remain small and stable and periodic components to these fluctuations appear negligible (e.g. 6 $\mu$M in Fig. 6). As the ACS concentration increased there was a striking increase in tension fluctuations, and decline in developed tension. Peaks (between 2 and 4 Hz) became apparent in the Fourier spectrum. Also, as the r.m.s. values increased the spectral peaks shifted to higher frequencies. An example of muscle behaviour in 10 $\mu$M-ACS (Fig. 6, bottom) indicates significantly reduced developed twitches, increased rest tension, large tension fluctuations the Fourier spectrum of which exhibits a

pronounced peak. The appearance of spectral peaks exactly coincides with an increase in r.m.s. (not shown). As the dose of ACS becomes high enough to suppress developed tension there is a reciprocal increase in r.m.s. tension fluctuation amplitude (Fig. 7) which was observed in ten muscles that were studied. Five muscles which could be directly compared indicated a linear reciprocal relationship (correlation coefficient = 0·88) between the relative decline in developed tension and the relative increase in r.m.s. tension fluctuations.

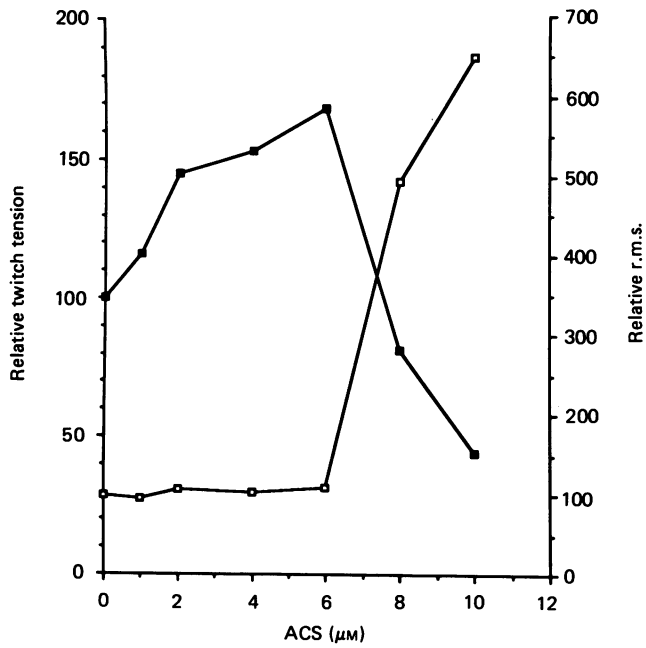


Fig. 7. The effect of increasing concentrations of ACS on relative developed twitch tension and relative r.m.s. fluctuation amplitude. The control condition (zero ACS in standard Tyrode solution) was set at 100 % and the results were expressed relative to this. It is clear that as the concentration of ACS increases beyond 6 μM, developed twitch tension declines and r.m.s. fluctuation amplitude increases. The results were taken from a single muscle (0·9 mm diameter) and are typical of those obtained in nine other muscles.

The inverse relationship between increases in the amplitude of tension noise and the decline in twitch tension supports the view that the two phenomena may be causally related and that $Ca^{2+}$ oscillations may in some way interfere with twitch development (Allen *et al.* 1985). The tension fluctuations reported here are presumably due to oscillations of cytoplasmic $Ca^{2+}$ as measured by Allen *et al.* (1984, 1985) and Eisner & Valdeolmillos (1986) and presumably underlie the scattered light intensity fluctuations described by Kort & Lakatta (1984). In the next section we will consider some mechanisms that may account for this interference.

*Model of $Ca^{2+}$ oscillations and resting tension*

To consider the influence of such $Ca^{2+}$ oscillations on tension, we have developed a simple mathematical model (see Appendix for details). We considered the

behaviour of a section of muscle 0·9 mm in diameter (to simulate the muscle illustrated by Fig. 6) and the length of one muscle cell. Each cell was assumed to be 20 $\mu$m in diameter and to oscillate independently. Thus, we assumed that the cells were oscillating in parallel with random phase angle, frequency (2–4 Hz) and amplitude (maximum = 2–70% SR $Ca^{2+}$ content). Similar assumptions have been stated elsewhere in an attempt to model the oscillatory behaviour of heart muscle (Stern, Kort, Bhatnagar & Lakatta, 1983; Cannell, Vaughan-Jones & Lederer, 1985). Cytosolic $Ca^{2+}$ oscillations were transformed to tension using estimates of cellular $Ca^{2+}$ buffering reported by Fabiato (1983).

For this muscle we first calculated the relationship between background (diastolic free + bound $[Ca^{2+}]$) and r.m.s. tension for different maximum fractions of spontaneous SR $Ca^{2+}$ release (curves with positive slopes in Fig. 8). We then determined the points along these curves which corresponded to the experimentally observed resting tension from Fig. 6 (3·5 mN). This resulted in the curve with negative slope in Fig. 8. Point A in Fig. 8 is the unique point where the experimentally observed values for r.m.s. noise (7·3 $\mu$N) and resting force coincide (for the muscle in Fig. 6). This corresponds to a rather small SR $Ca^{2+}$ release (0–5%) and a high background total $Ca^{2+}$ (49 $\mu$mol/l cell water or about 6% of maximum force). This would imply a decline in the time-averaged SR $Ca^{2+}$ content of 2·5% which alone is unlikely to reduce twitches substantially. However, it has been estimated that $Ca^{2+}$ oscillations are quite large, reaching 4–40 $\mu$M (Orchard, Eisner & Allen, 1983; Weir, Kort, Stern, Lakatta & Marban, 1983; Eisner & Valeolmillos, 1986). This conclusion is further supported by the observation of spontaneous oscillations in $Ca^{2+}$-tolerant myocytes which indicates that free $[Ca^{2+}]_i$ reaches micromolar levels (Capogrossi & Lakatta, 1985). On this basis the fractional SR $Ca^{2+}$ release at point A seems unrealistically low.

The same observed r.m.s. value (7·3 $\mu$N) can be obtained with the model if the fractional SR $Ca^{2+}$ release is increased and the background $Ca^{2+}$ is decreased. Point B in Fig. 8 indicates such a situation, but is unlikely to explain our result since the predicted resting force is considerably lower than actually observed. Nevertheless, this calculation illustrates that $Ca^{2+}$ oscillations large enough to significantly reduce time-averaged SR $Ca^{2+}$ content can sum in this parallel array to produce very small tension fluctuations. If instead we increase the fractional SR $Ca^{2+}$ release and hold the resting tension at the observed level, the predicted r.m.s. values would fall on the curve in Fig. 8 from point A towards point C. On this curve the predicted magnitude of the r.m.s. tension fluctuations exceeds those observed experimentally. This is reasonable because the series compliance of some of the cells would damp the amplitude of these oscillations. Thus, while large $Ca^{2+}$ oscillations may occur and reduce time-averaged SR $Ca^{2+}$ content in a single unit (e.g. single cell) the tension fluctuations recorded at the end of the muscle would be smaller than those expected if no compliance existed. We suggest that a correct description of the muscle behaviour lies somewhere on the curve connecting points A and C in Fig. 8. It is also possible that the resting tension is underestimated due to the same series compliance effect. In this case a correct description of muscle behaviour would lie above and to the right of the curve connecting points A and C in Fig. 8. Our model suggests that series compliance may be responsible for decreasing the amplitude of tension

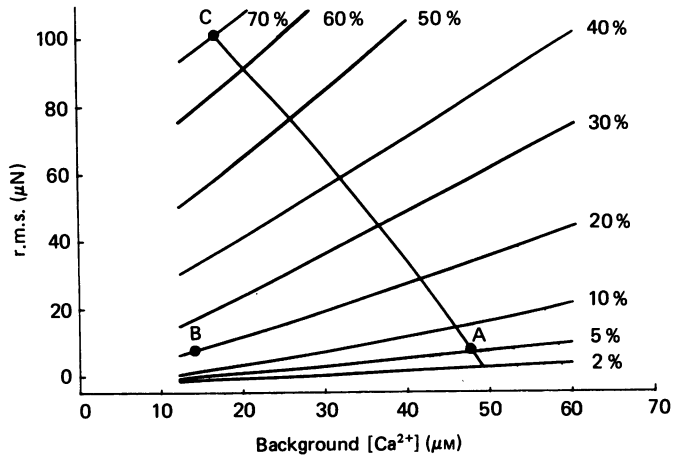


Fig. 8. Calculated variation of r.m.s. tension fluctuation against background $Ca^{2+}$ levels for 2025 cells (diameter 0·9 mm) oscillating sinusoidally in parallel. Each curve is plotted for a different maximum amplitude of $Ca^{2+}$ oscillation ranging from 2 to 70% of the assumed SR content. Various r.m.s. required to produce an observed rest tension (3·5 mN in a 0·9 mm diameter muscle) were calculated. These are displayed as the curve A–C. As the r.m.s. amplitude increases, less background $Ca^{2+}$ is required to achieve the observed rest tension.

oscillations in our resting multicellular preparations. The contribution of these $Ca^{2+}$ oscillations to the decline in developed twitches will be addressed in the Discussion.

## DISCUSSION

We find that in the concentration range 1–4 μM ACS produces positive inotropic effects which are generally accompanied by increases in RCC amplitude. Between 6 and 10 μM twitch tension declines, but RCCs rise to a plateau level. Rest tension and total tension also decline and the twitches begin to exhibit after-contractions.

### *The positive inotropic effect of acetylstrophanthidin*

Our results may conveniently be interpreted by supposing that cooling contractures provide a relative index of the availability of SR $Ca^{2+}$ for contraction (Kurihara & Sakai, 1985; Bridge, 1986). A simple explanation for glycoside inotropy is that these drugs produce an imbalance of $Ca^{2+}$ fluxes during the contraction–relaxation cycle. Specifically, small increases in intracellular $Na^+$ produced by partial inhibition of the $Na^+$ pump might lead to a slowing of $Ca^{2+}$ efflux via $Na^+$–$Ca^{2+}$ exchange. If $Ca^{2+}$ influx per complete cycle of contraction and relaxation is either increased or remains unchanged, then there must be a net gain of $Ca^{2+}$. This gain of $Ca^{2+}$ could produce the observed inotropy. The increase in cooling contracture is consistent with the view that there was a net gain in $Ca^{2+}$ which enlarged SR $Ca^{2+}$ stores. It seems likely to us that this simple hypothesis will at least provide a partial explanation of positive inotropy. However, additional observations must be considered.

The dependence of twitches and cooling contracture amplitude on ACS concentration (up to 4 $\mu$M) are similar (Fig. 2). Twitches increase at lower ACS concentration than that required to increase cooling contracture amplitude. Although this effect is small it was consistently observed in all muscles (and is most apparent in Fig. 4). This means that twitch tension can increase before there has been measurable enlargement of SR $Ca^{2+}$ stores. It is conceivable that if $Ca^{2+}$ efflux is slowed a modest amount, then the $Ca^{2+}$ transient might be prolonged without any change in the integrated net flux for one cycle of contraction and relaxation. If the $Ca^{2+}$ transient did slow sufficiently to allow the contractile elements to come closer to equilibrium, then strengthening of contraction might occur. If in addition, $Ca^{2+}$ influx during the cardiac cycle is enhanced by ACS (Bers, 1987), this could increase the developed twitch either directly or by increasing the fraction of SR $Ca^{2+}$ released since $Ca^{2+}$-induced $Ca^{2+}$ release is known to be graded (Fabiato, 1985). ACS-induced $Na^+$ accumulations might also lead to an alteration in mitochondrial $Ca^{2+}$ fluxes (Crompton, Capano & Carafoli, 1976) which our approach cannot evaluate.

The present results with caffeine (and those of Bers, 1987) indicate that while the SR can play a role in producing glycoside inotropy it is not essential. Muscles treated with 10·0 mM-caffeine do not exhibit RCCs (presumably because the SR is empty) (Weber & Herz, 1968; Blaney *et al.* 1978; Su & Hasselbach, 1984; Bridge, 1986) but still exhibit positive inotropy in response to moderate doses of ACS. The increase $[Na^+]_i$ may increase $Ca^{2+}$ influx and/or slow $Ca^{2+}$ efflux thereby prolonging the cytoplasmic $Ca^{2+}$ transient (as discussed above). This $Na^+$–$Ca^{2+}$ exchange imbalance may also produce a small increase in the resting or free $[Ca^{2+}]$. Small increases in free $[Ca^{2+}]$ about the foot of the $[Ca^{2+}]_i$ *vs.* tension relationship may not have observable effects on resting tension. However, after an aliquot of $Ca^{2+}$ has been delivered to the cytosol, small differences in background $Ca^{2+}$ will in effect be amplified on the steep portion of the tension–pCa relationship (e.g. see Fig. 10 of Bers, 1987). In either case the positive inotropy results from $Ca^{2+}$ flux imbalances (and their consequences).

*The negative inotropic effect of ACS*

The results suggest a striking inverse relationship between the increase in r.m.s. amplitude of the microscopic tension fluctuations and the decline in developed twitches. This supports the suggestion by Allen *et al.* (1985) that the presence of microscopic tension fluctuations and their underlying $Ca^{2+}$ oscillations might somehow interfere with developed twitch tension. Some authors (Allen *et al.* 1985; Canell *et al.* 1985) suggest that the presence of series elasticity might, in the presence of $Ca^{2+}$ oscillations, introduce a compliance that reduces tension. Thus, cells that have just released their SR $Ca^{2+}$ will provide a compliance against which loaded cells will contract when the muscle is activated. It seems clear that this mechanism will necessarily be involved in the observed decline in twitch force.

Time-average SR $Ca^{2+}$ content may also be reduced, as suggested by the results of Orchard *et al.* (1983), Wier *et al.* (1983), Eisner & Valdeolmillos (1986) and also suggested by our model. However, oscillatory losses of SR $Ca^{2+}$ will presumably be accompanied by complementary increases in cytosolic $Ca^{2+}$. This might explain why the RCC magnitude does not decline appreciably even at high ACS concentration (see Fig. 2). It is conceivable that the magnitude of an RCC reflects the sum of SR $Ca^{2+}$

plus the cytoplasmic $Ca^{2+}$ to which it is added. If $Ca^{2+}$ oscillation takes place solely between the SR and cytosol this sum and hence the magnitude of RCC may tend to a maximum as $Ca^{2+}$ oscillations occur. Taken at face value the results with RCCs suggest that the decrease in the time-averaged SR $Ca^{2+}$ content of the muscle *is* associated with a complementary increase in cytoplasmic $Ca^{2+}$. In this case one could only explain a decrease in developed tension which is associated with an increase in resting tension of similar magnitude (i.e. total tension, or rest plus developed tension would be constant). This is not our typical observation in Figs 1 and 6 where total tension is substantially reduced. Therefore, it appears that factors other than the decrease in time-averaged SR $Ca^{2+}$ content are likely to contribute significantly to the negatively inotropic effect or a decreased $Ca_i^{2+}$ transient at high ACS concentration.

The presence of $Ca^{2+}$ oscillations may lead to a decrease in the fraction of SR $Ca^{2+}$ release in response to an action potential. After an oscillation the SR $Ca^{2+}$ release mechanism may be somewhat refractory (even if the cytoplasmic $Ca^{2+}$ has been resequestered). This refractoriness might have several explanations, e.g. time may be required for $Ca^{2+}$ to move from SR uptake to SR release sites. Alternatively, the SR $Ca^{2+}$ release channel may remain in a refractory state (e.g. due to $Ca_i^{2+}$-dependent inactivation as suggested by Fabiato (1985).

If as some authors suggest, the slow inward current is responsible for inducing $Ca^{2+}$ release from the SR (Fabiato, 1985; London & Krueger, 1986), it is also possible that $Ca^{2+}$ oscillations may interfere with and reduce the extent of that current. $Ca^{2+}$ current could be reduced if $Ca^{2+}$ oscillations bring $Ca^{2+}$ into the micromolar range by reducing the driving force and by directly inactivating $Ca^{2+}$ current (Lee, Marban & Tsien, 1985).

Any inference about contractile $Ca^{2+}$ that is made from tension measurement is extremely difficult in the presence of $Ca^{2+}$ oscillations. However, microscopic tension fluctuations (and presumably $Ca^{2+}$ oscillations) are abolished by cooling (Bridge, 1986). Thus, while $Ca^{2+}$ oscillations may suppress contraction at 30 °C, cooling contractures will not be so affected.

Our results with RCCs suggest that increases in SR $Ca^{2+}$ content may partly, but not completely, explain the positive inotropic effects of ACS. Specifically, some increase in force occurs in the absence of increased SR $Ca^{2+}$ content. The negative inotropic effects of ACS were not associated with a decline in average SR $Ca^{2+}$ content assessed by RCCs, but were associated with microscopic tension fluctuations, presumably due to intracellular $Ca^{2+}$ oscillations. Such $Ca^{2+}$ oscillations may contribute to the negative inotropic effects of ACS in several ways. These include a significant effect of series compliance and possibly of reduced SR $Ca^{2+}$ release. Our results suggest that a decline in average SR $Ca^{2+}$ content is less likely to contribute *per se* to the decline in contractile force at higher ACS concentrations.

## APPENDIX

### *Mathematical model of $Ca^{2+}$ oscillations*

Our underlying assumption is that $Ca^{2+}$ oscillations between the SR and cytosol do underlie microscopic tension fluctuations (Allen *et al.* 1985). For simplicity we assume

that the oscillations are sinusoidal, are of frequency $f$ and amplitude $A$. We let each cell oscillate as a unit (although this may not be true, Eisner & Valdeolmillos, 1986) with these characteristics but that different cells oscillate out of phase (phase angle = $\phi$ rad). If the SR $Ca^{2+}$ content oscillates sinusoidally then there is a reciprocal and sinusoidal gain and loss of cytosolic $Ca^{2+}$ given by the following equation:

$$C_o(t) = A/2\,(1-\sin(2\pi ft+\phi)), \tag{1}$$

when $C_o(t)$ = cytosolic $Ca^{2+}$ content at time $t$.

If this time-dependent cytosolic $Ca^{2+}$ can be transformed to tension then we obtain an expression for the sinusoidal variation of tension ($P$) of a cell

$$P = T(C_o(t, \phi, A)) = T(A/2(1-\sin(2\pi ft+\phi))), \tag{2}$$

where time = $t$, and $T$ is the function that transforms cytosolic $Ca^{2+}$ to tension $P$. If $n$ cells oscillate in parallel then the total tension $P$ will be given by

$$P_n = \sum_{i-1}^{n} T(C_o(t_i, \phi_i, A_i)). \tag{3}$$

Fabiato (1983) has calculated the relationship between the total cell $Ca^{2+}$ (in $\mu$moles per litre of accessible cell water) and relative steady-state tension. This relationship was fitted by a sigmoid curve described by the following equation

$$P = P_o/\{1+(1/(B[Ca^{2+}]^{3\cdot2}))\}, \tag{4}$$

where $B = 2{\cdot}11 \times 10^{-7}$. The maximum tension that a single cell produces was assumed to be 26 $\mu$N (Fabiato, 1981*b*). The relationship described by eqn (4) was assumed for the sake of the illustration to hold for twitches as well as steady state.

We used the foregoing equations to calculate the tension produced by up to 2500 cells oscillating asynchronously in parallel. The calculations were repeated at 40 ms (corresponding to the experimental sampling frequency) intervals for 10 s real time. The amplitude frequency and phase angle for each cell were generated by a random number generator. Frequencies ranged from 2 to 4 Hz, phase angles from 0 to $2\pi$ rad and amplitudes were varied from zero to various maxima (0·1–0·7 of SR $Ca^{2+}$ content). The maximum SR $Ca^{2+}$ content was assumed to be 142 $\mu$mol/l cell water. The r.m.s. values of the tension oscillations were calculated from the computed tension values.

We would like to thank Dr David A. Eisner for critically reading an earlier version of the manuscript and providing useful suggestions and Dr Bruce Steinhaus for providing valuable discussion and assistance with the Fourier analysis and computer modelling. The authors also thank Mrs R. K. Richards and Mr R. D. Takeda for technical assistance and Ms Doris Land for typing the manuscript. This work was supported by grants from the U.S. Public Health Service: HL30077 and HL13340; National Institutes of Health New Investigator Research Award HL31140 (J.H.B.B.) and Research Career Development Award HL01526 (D.M.B.); an award from the American Heart Association, Utah affiliate; and awards from the Nora Eccles Treadwell Foundation and the Richard A. and Nora Eccles Harrison Fund for Cardiovascular Research.

REFERENCES

ALLEN, D. G., EISNER, D. A. & ORCHARD, C. H. (1984). Characterization of oscillations of intracellular calcium concentration in ferret ventricular muscle. *Journal of Physiology* **352**, 113–128.

ALLEN, D. G., EISNER, D. A., PIROLO, J. S. & SMITH, G. L. (1985). The relationship between intracellular calcium and contraction in calcium-overloaded ferret papillary muscles. *Journal of Physiology* **364**, 169–182.

ALLEN, D. G., JEWELL, B. R. & WOOD, E. H. (1976). Studies of the contractility of mammalian myocardium at low rates of stimulation. *Journal of Physiology* **254**, 1–17.

AXELSON, P. H. & BRIDGE, J. H. B. (1985). Electrochemical ion gradients and the Na/Ca exchange stoichiometry. Measurements of these gradients are thermodynamically consistent with a stoichiometric coefficient. *Journal of General Physiology* **85**, 471–475.

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1969). The influence of calcium on sodium efflux in squid axons. *Journal of Physiology* **200**, 431–458.

BERS, D. M. (1987). Mechanism contributing to the cardiac inotropic effect of Na-pump inhibition and reduction of extracellular Na. *Journal of General Physiology* **90**, 479–504.

BERS, D. M., BRIDGE, J. H. B. & MACLEOD, K. T. (1987). The mechanism of ryanodine action in cardiac muscle assessed with Ca selective microelectrodes and rapid cooling contractures. *Canadian Journal of Physiology and Pharmacology* **65**, 610–618.

BLANEY, L., THOMAS, H., MUIR, J. & HENDERSON, A. (1978). Action of caffeine on calcium transport by isolated fractions of myofibrils, mitochondria and sarcoplasmic reticulum from rabbit heart. *Circulation Research* **43**, 520–526.

BRIDGE, J. H. B. (1986). Relationships between the sarcoplasmic reticulum and transarcolemmal Ca transport revealed by rapidly cooling rabbit ventricular muscle. *Journal of General Physiology* **88**, 437–473.

CANNELL, M. B., VAUGHAN-JONES, R. D. & LEDERER, W. J. (1985). Ryanodine block of calcium oscillations in heart muscle and the sodium–tension relationship. *Federation Proceedings* **44**, 2964–2969.

CAPOGROSSI, M. C. & LAKATTA, E. G. (1985). Frequency modulation and synchronization of spontaneous oscillations in cardiac cells. *American Journal of Physiology* **248**, H412–418.

CHAPMAN, R. A. & ELLIS, E. (1974). Synergistic effects of cooling and caffeine on the contraction of the frog's heart. *Journal of Physiology* **232**, 101–102*P*.

CROMPTON, M., CAPANO, M. & CARAFOLI, E. (1976). The sodium-induced efflux of calcium from heart mitochondria: A possible mechanism for the regulation of mitochondrial calcium. *European Journal of Biochemistry* **69**, 453–462.

EISNER, D., LEDERER, W. J. & VAUGHAN-JONES, R. (1983). The control of tonic tension by membrane potential and intracellular Na activity in the sheep cardiac Purkinje fibre. *Journal of Physiology* **335**, 723–743.

EISNER, D. A. & VALDEOLMILLOS, M. (1986). A study of intracellular calcium oscillations in sheep cardiac Purkinje fibres measured at the single cell level. *Journal of Physiology* **372**, 539–556.

ELLIS, D. (1977). The effects of external cations and ouabain on the intracellular sodium activity of sheep heart Purkinje fibres. *Journal of Physiology* **273**, 211–240.

FABIATO, A. (1981*a*). Effects of cyclic AMP and phosphodiesterase inhibitors on the contractile activation and the $Ca^{2+}$ transient detected with aequorin in skinned cardiac cells from rat and rabbit ventricles. *Journal of General Physiology* **78**, 15–16 (abstract).

FABIATO, A. (1981*b*). Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. *Journal of General Physiology* **78**, 457–497.

FABIATO, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology* **245**, C1–14.

FABIATO, A. (1985). Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* **85**, 291–320.

FABIATO, A. & FABIATO, F. (1973). Activation of skinned cardiac cells. Subcellular effects of cardioactive drugs. *European Journal of Cardiology* **1**, 143–155.

GLYNN, I. M. (1964). The action of cardiac glycosides on ion movements. *Pharmacological Reviews* **16**, 381–407.

KORT, A. A. & LAKATTA, E. G. (1984). Calcium-dependent mechanical oscillations occur spontaneously in unstimulated mammalian cardiac tissues. *Circulation Research* **54**, 396–404.

KURIHARA, S. & SAKAI, T. (1985). Effects of rapid cooling on mechanical and electrical responses in ventricular muscle of guinea-pig. *Journal of Physiology* **361**, 361–378.

LANGER, G. A. (1965). Calcium exchange in dog ventricular muscle. Relation to frequency of contraction and maintenance of contractility. *Circulation Research* **17**, 78–90.

LANGER, G. A. & SERENA, S. D. (1970). Effects of strophanthidin upon contraction and ionic exchange in rabbit ventricular myocardium, relative to control of active state. *Journal of Molecular and Cellular Cardiology* **1**, 65–90.

LEE, C. O. & DAGOSTINO, M. (1982). Effect of strophanthidin on intracellular Na ion activity and twitch tension of constantly driven canine Purkinje fibers. *Biophysical Journal* **40**, 185–198.

LEE, C. O., KANG, D. H., SOKOL, J. H. & LEE, K. S. (1980). Relation between intracellular Na ion activity and tension in sheep cardiac purkinje fibers exposed to dihydro-ouabain. *Biophysical Journal* **29**, 315–330.

LEE, K. S., MARBAN, E. & TSIEN, R. W. (1985). Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *Journal of Physiology* **364**, 395–411.

LONDON, B. & KRUEGER, J. W. (1986). Contraction in voltage-clamped, internally perfused single heart cells. *Journal of General Physiology* **88**, 475–505.

MULLINS, L. J. (1979). The generation of electric currents in cardiac fibers by Na/Ca exchange. *American Journal of Physiology* **236**, C103–110.

NAYLER, W. G. (1973). Effect of inotropic agents on canine trabecular muscle rendered highly permeable to calcium. *American Journal of Physiology* **225**, 918–924.

NIEMAN, C. J. & EISNER, D. A. (1985). Effects of caffeine, tetracaine, and ryanodine on calcium-dependent oscillations in sheep cardiac Purkinje fibers. *Journal of General Physiology* **86**, 877–889.

ORCHARD, C. H., EISNER, D. A. & ALLEN, D. G. (1983). Oscillations of intracellular $Ca^{2+}$ in mammalian cardiac muscle. *Nature* **304**, 735–738.

REPKE, K. (1964). Uber den biochemischen Wirkingsmodus von digitalis. *Klinische Wochenschrift* **41**, 157–165.

SAKAI, T. (1965). The effects of temperature and caffeine on action of the contractile mechanism in the striated muscle fibres. *Jikeikai Medical Journal* **12**, 88–102.

SHEU, S.-S. & FOZZARD, H. A. (1982). Transmembrane Na and Ca electrochemical gradients in cardiac muscle and their relation to force development. *Journal of General Physiology* **80**, 325–351.

STERN, M. D., KORT, A. A., BHATNAGAR, G. M. & LAKATTA, E. G. (1983). Scattered-light intensity fluctuations in diastolic rat cardiac muscle caused by spontaneous $Ca^{2+}$-dependent cellular mechanical oscillations. *Journal of General Physiology* **82**, 119–153.

SU, J. Y. & HASSELBACH, W. (1984). Caffeine-induced calcium release from isolated sarcoplasmic reticulum of rabbit skeletal muscle. *Pflügers Archiv* **400**, 14–21.

SUTKO, J. L., BERS, D. M. & REEVES, J. P. (1986). Post rest inotropy in rabbit ventricle: $Na^+$–$Ca^{2+}$ exchange determines sarcoplasmic reticulum $Ca^{2+}$ content. *American Journal of Physiology* **250**, H654–661.

WEBER, A. & HERZ, R. (1968). The relationship between caffeine contracture of intact muscle and the effect of caffeine on reticulum. *Journal of General Physiology* **52**, 750–759.

WENDT, I. R. & STEPHENSON, D. G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibres of the rat. *Pflügers Archiv* **398**, 210–216.

WIER, W. G. & HESS, P. (1984). Excitation–contraction coupling in cardiac Purkinje fibers. Effects of cardiotonic steroids on the intracellular $[Ca^{2+}]$ transient, membrane potential, and contraction. *Journal of General Physiology* **83**, 395–415.

WIER, W. G., KORT, A. A., STERN, M. D., LAKATTA, E. G. & MARBAN, E. (1983). Cellular calcium fluctuations in mammalian heart: Direct evidence from noise analysis of aequorin signals in Purkinje fibers. *Proceedings of the National Academy of Sciences of the U.S.A.* **80**, 7367–7371.