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THE EFFECTS OF HYPERTONICITY ON TENSION AND INTRACELLULAR CALCIUM CONCENTRATION IN FERRET VENTRICULAR MUSCLE

BY D. G. ALLEN AND G. L. SMITH

From the Department of Physiology, University College London, Gower Street, London WC1E 6BT

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SUMMARY

1. Tension and intracellular calcium concentration ($[Ca^{2+}]_i$) were measured in isolated ferret papillary muscles exposed to hypertonic solutions. $[Ca^{2+}]_i$ was measured with aequorin which was microinjected into surface cells of the preparation. Correction was made for the effects of ionic strength on aequorin sensitivity to Ca^{2+}.

2. Application of 100 mM-mannitol increased both developed tension and the intracellular Ca^{2+} signals on contraction (the Ca^{2+} transients). 300 mM-mannitol increased the Ca^{2+} transients further but led to a decrease in developed tension.

3. Mannitol caused a concentration-dependent slowing in the time course of a stimulated contraction but had no effect on that of the Ca^{2+} transient.

4. As the mannitol concentration was increased, the muscles exhibited increased viscosity which was demonstrated by measuring the tension response to a sudden stretch during diastole. This is probably a consequence of cell shrinkage and may cause the slower time course of the contraction.

5. In the presence of 300 mM-mannitol, oscillations of diastolic $[Ca^{2+}]_i$ were detectable in both stimulated and quiescent preparations. However, in stimulated preparations the oscillations in mannitol were smaller than when a Ca^{2+} transient of similar amplitude was achieved by other means.

6. Immediately after the application or removal of mannitol large spontaneous Ca^{2+} signals were often observed. These signals were even larger in Na^+-free solutions, suggesting that they cannot be attributed to Na^+–Ca^{2+} exchange.

7. The increase in developed tension in 100 mM-mannitol can be accounted for by the increased Ca^{2+} transients in combination with the inhibitory effects of ionic strength on myofibrillar tension production (Kentish, 1984). The decrease in developed tension at 300 mM-mannitol is dominated by the inhibitory effect of increased ionic strength on maximum Ca^{2+}-activated tension.

INTRODUCTION

Koch-Weser (1963) showed that small increases in the tonicity of the perfusate of isolated cardiac preparations increased the developed tension whereas higher tonicities decreased developed tension and slowed the time course of the twitch. When

extracellular tonicity is raised water rapidly leaves the cells to equalize extracellular and intracellular osmolarity (Dick, 1966) and consequently both intracellular osmolarity and ionic strength are increased. Experiments on skinned muscle preparations have demonstrated that increases in the ionic strength of the activating solutions lead to a monotonic decline in the Ca^{2+}-activated tension produced by the contractile proteins (Gordon, Godt, Donaldson & Harris, 1973; Kentish, 1984). The observation that hypertonicity can increase developed tension under conditions in which the Ca^{2+}-activated tension of the contractile proteins is depressed has led to the suggestion that hypertonicity increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Koch-Weser, 1963; Wildenthal, Adcock, Crie, Templeton & Willerson, 1975; Chapman, 1978).

Recently Lado, Sheu & Fozzard (1984), using ion-sensitive electrodes, demonstrated that the resting $[Ca^{2+}]_i$ in quiescent cardiac preparations is increased by hypertonicity. In the present study we have used the Ca^{2+}-sensitive photoprotein aequorin to re-examine the effects of hypertonicity on resting $[Ca^{2+}]_i$. In addition, we have measured both the rapid rise in $[Ca^{2+}]_i$ which triggers contraction (the Ca^{2+} transient) and the developed tension. The aim of our experiments was to identify the mechanisms by which hypertonicity causes changes in the magnitude and time course of the developed tension.

A preliminary report of some of these findings has already appeared (Allen & Smith, 1985).

METHODS

The experiments were performed on papillary muscles dissected from the right ventricles of ferrets. The methods used were similar to those described by Allen, Eisner, Lab & Orchard (1983). $[Ca^{2+}]_i$ was measured with aequorin, which was pressure-injected from conventional glass microelectrodes into 50–100 cells on the surface of the preparations. Light emission from aequorin, which is a non-linear indicator of $[Ca^{2+}]_i$, was measured with a photomultiplier tube whose output current was proportional to light intensity. When measuring the very low levels of light from quiescent preparations, light emitted from the preparation was distinguished from the dark current of the photomultiplier tube (0·1 nA) by closing a shutter between the preparation and the photomultiplier tube.

All the experiments were at 30 °C. The muscles were stretched to the length at which developed tension was maximal. Stimulation was generally at 0·33 Hz; resting $[Ca^{2+}]_i$ was measured in quiescent preparations. Length change experiments were carried out using the lever system described by Allen & Kurihara (1982). The diameter of the muscle was measured under control conditions with a binocular microscope and used to calculate the cross-sectional area, assuming the muscle had a circular cross-section.

Fourier transforms were performed on unfiltered light records using methods described by Orchard, Eisner & Allen (1983).

Solutions. Under control conditions the muscles were bathed in a Tyrode solution with the following composition (mM): Na^+, 135; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 2; Cl^-, 104; HCO_3^-, 20; HPO_4^{2-}, 1; acetate, 20; glucose, 10; insulin, 4×10^{-5}; equilibrated with 95 % O_2/5 % CO_2 to give a final pH of 7·4. Na^+-free solutions were made by replacing all sodium salts by the appropriate potassium salt. Tonicity was increased by adding solid chemicals to the Tyrode solution. The osmolarity of the Tyrode solution was measured with a freezing point depression osmometer and was found to be 285 mosmol/l (sum of particles = 299 mosmol/l). The addition of 100, 200 and 300 mmol mannitol/l increased osmolarity to 385, 500 and 605 mosmol/l respectively. These figures correspond reasonably well to calculated osmolarities using published osmotic coefficients.

The effect of ionic strength on aequorin. Increases in ionic strength are known to reduce the sensitivity of aequorin to $[Ca^{2+}]$ (Moisescu & Ashley, 1977; Allen & Blinks, 1979). Fig. 1 shows the aequorin light emission as a function of $[Ca^{2+}]$ at five different KCl concentrations ranging from

3 to 507 mM. Details of the methods used are given in Allen, Blinks & Prendergast, 1977. Note that these results should not be used to calibrate the intracellular aequorin signals as, for simplicity, they were performed in the absence of Mg^{2+}.

The results in Fig. 1 show that both at the resting $[Ca^{2+}]_i$ (0·1–0·2 μM) and at the peak of the Ca^{2+} transient (1–5 μM) the increase in $[K^+]_i$ produced by external hypertonicity (roughly equivalent to changing from 150 to 300 mM-KCl) will reduce the aequorin light emission at a given $[Ca^{2+}]$. Thus the increase in light emission observed under these conditions will tend to underestimate the true changes in $[Ca^{2+}]_i$.

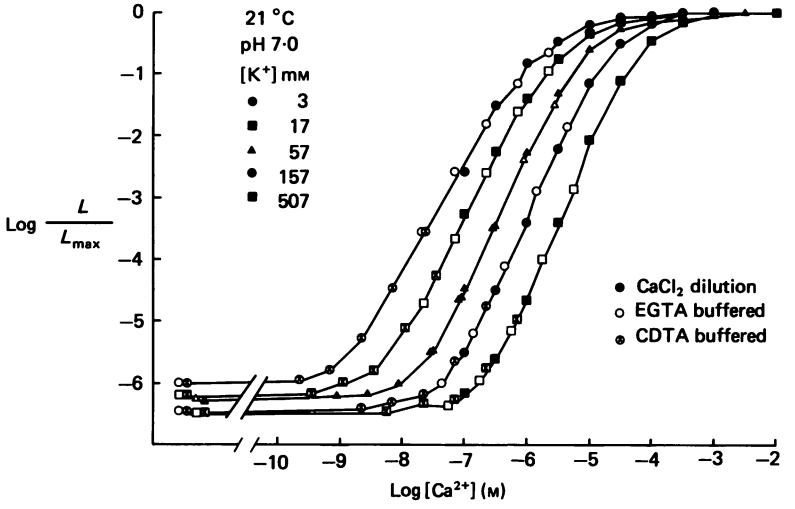

Fig. 1. The effect of [KCl] on the relationship between aequorin light emission and free $[Ca^{2+}]$. Ordinate: $\log_{10}$ (aequorin light (L) at given $[Ca^{2+}]$/maximum aequorin light (L_{max}) in a saturating $[Ca^{2+}]$). Abscissa: $\log_{10}[Ca^{2+}]$. All measurements were performed at 21 °C, pH 7·0, buffered with 5 mM-PIPES (piperazine-*N*,*N*′-bis[2-ethanesulphonic acid]) except the curve labelled 3 mM for which the PIPES was lowered to 2 mM. The concentrations of KCl varied from 3 to 507 mM as indicated on the Figure. The free $[Ca^{2+}]$ was determined either by dilution of 1 M-$CaCl_2$ or by the use of 1 mM-EGTA or CDTA (*trans*-1,2,-cyclohexylenedinitrilo)tetraacetic acid buffers as indicated by the symbols. The leftmost points (unlabelled abscissa) had Ca^{2+} buffer with no added Ca^{2+}; the free $[Ca^{2+}]$ is unknown but $< 10^{-10}$ M. Details of the experimental methods are given by Allen *et al.* 1977.

In some experiments the aequorin light was calibrated using the method described by Allen & Blinks (1979). This involved discharging all the aequorin remaining at the end of the experiment so that the fractional luminescence of the light levels recorded during the experiment could be determined. Fractional luminesence is the ratio of light emission at a given $[Ca^{2+}]$ to the maximum light emission in a saturating $[Ca^{2+}]$; for details see Allen *et al.* (1983). Under control conditions fractional luminescence was converted to $[Ca^{2+}]$ using a calibration curve obtained in 157 mM-K^+, 2 mM-Mg^{2+}, pH 7·0, 30 °C. To allow for the effects of ionic strength on the aequorin light signals the following assumptions were made. (i) The effects of ionic strength on aequorin sensitivity shown in Fig. 1 were equivalent in solutions containing 2 mM-Mg^{2+}. (ii) $[Mg^{2+}]_i$ was unaffected by changes in ionic strength because it can be effectively buffered by mitochondria (Flatman, 1984). If $[Mg^{2+}]$ does increase when the cell is shrunk then the peak $[Ca^{2+}]_i$ in Table 1 will underestimate the true changes. (iii) Increases in external osmolarity increased intracellular ionic strength in proportion to the fractional increase in external osmolarity. This is equivalent to assuming that the muscle acts as a perfect osmometer, which has been demonstrated experimentally for skeletal muscle by Blinks (1965) and for cardiac muscle by Page & Storm (1966).

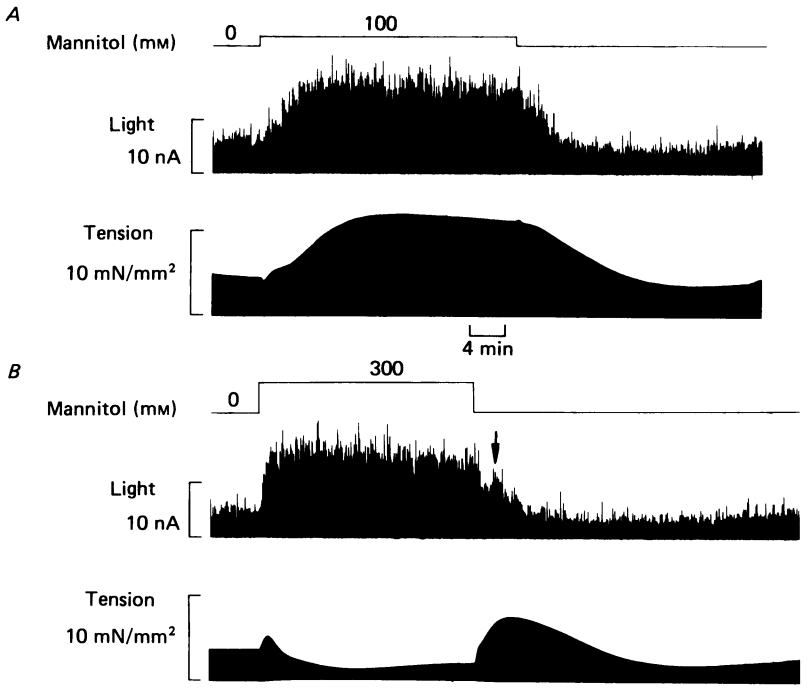

Fig. 2 The time course of the effects of raising external osmolarity with mannitol on aequorin light and tension. *A*, the effect of the application of 100 mM-mannitol. *B*, the effect of the application of 300 mM-mannitol. In both panels the traces show: top, light; bottom, tension. The solution applications are illustrated above the record. The slow chart speed used means that light and tension signals are fused into a continuous envelope. The arrow in *B* indicates the peak of an irregular burst in the aequorin light signal (see text).

RESULTS

Effects of hypertonicity on developed tension and Ca^{2+} transients

Fig. 2 shows continuous records of aequorin light and tension from an aequorin-injected papillary muscle when 100 and 300 mM-mannitol were added to the perfusing Tyrode solution. Addition of 100 mM-mannitol led to a 2·5-fold increase in the aequorin light transient over about 5 min. Tension also increased by about 2·5-fold but the increase was slower than that of light. When mannitol was removed both light and tension returned to the control values after a slight undershoot. The faster time course of the changes of light compared with tension probably reflects the fact that aequorin-injected cells are on the surface of the preparation whereas tension depends on the whole cross-section of the muscle.

The increase in developed tension in 100 mM-mannitol was quite variable; most preparations (e.g. those in Figs. 2 and 3) show a substantial increase while a few (e.g. those in Fig. 4*B*) show a small decrease. The average of five preparations was an increase of 50% (see Table 1). In one preparation we confirmed the finding (Wildenthal *et al.* 1975) that when developed tension was low, 100 mM-mannitol

increased tension, whereas after tension had been increased, by raising $[Ca^{2+}]_o$, 100 mM-mannitol then decreased tension. Thus the variability in the effects of mannitol probably reflects intrinsic variations in the degree of activation of different preparations.

When 300 mM-mannitol was applied to the muscle (Fig. 2*B*) the aequorin light transients rose over 2 min to a level of about 3·5 times the control. Developed tension showed a rapid rise followed by a fall, so that in the steady state developed tension was about one-third of the control value. When mannitol was removed light declined but there were often irregular bursts of light (arrowed on Fig. 2*B*) in both stimulated and quiescent preparations. When mannitol was removed tension showed a rapid increase to a level which exceeded the control, followed by a slower fall towards the control level.

In three out of six preparations, application of 300 mM-mannitol produced a transient effect in addition to the responses shown in Fig. 2*B*. Within 20–30 s of its application, the muscle stopped responding to stimuli, and there was a very large increase in light emission and a small increase in resting tension. During this period of increased light there were pronounced oscillations of light, similar to those described by Orchard *et al.* (1983) when Na^+-free solutions were applied to a muscle. After 1–2 min these rapid effects recovered and the response of the muscle was then similar to that shown in Fig. 2. This kind of response was quite variable; the same preparation on repeated application of identical solutions would sometimes show this behaviour and sometimes not.

The effects of hypertonicity were similar when sucrose was used instead of mannitol. However, addition of the membrane-permeable solute urea to the perfusing solutions produced only transient changes in tension and light which returned to control levels within 3–4 min (cf. Koch-Weser, 1963). In the steady state urea will raise internal osmotic strength but not ionic strength, so that this observation suggests that increased osmotic strength is not an important factor in determining the effects of hypertonicity on $[Ca^{2+}]_i$ and tension.

The time course of tension and Ca^{2+} transients in hypertonic solutions

A characteristic feature of hypertonicity is a slowing of the time course of contraction (Koch-Weser, 1963). Fig. 3*A* shows the effects of various concentrations of mannitol on aequorin light and tension. Developed tension was greatly depressed in 300 mM-mannitol and there was an obvious slowing of both the rate of rise and the rate of decline of tension. This is clearer in Fig. 3*B* in which the light and tension responses have been normalized and superimposed. The addition of mannitol had no discernible effect on the time course of the aequorin light transients, but it caused a progressive slowing in the rate of rise of tension and an even more pronounced effect on the rate of relaxation.

Changes in the mechanical properties of cardiac muscle in hypertonic solution

Since hypertonicity has no effect on the time course of the Ca^{2+} transients but slows the development of tension, there may be changes in the mechanical properties of the preparation. Studies in both cardiac muscle (Wildenthal, Skelton & Coleman, 1969) and skeletal muscle (Gulati & Babu, 1982) have shown that the maximal

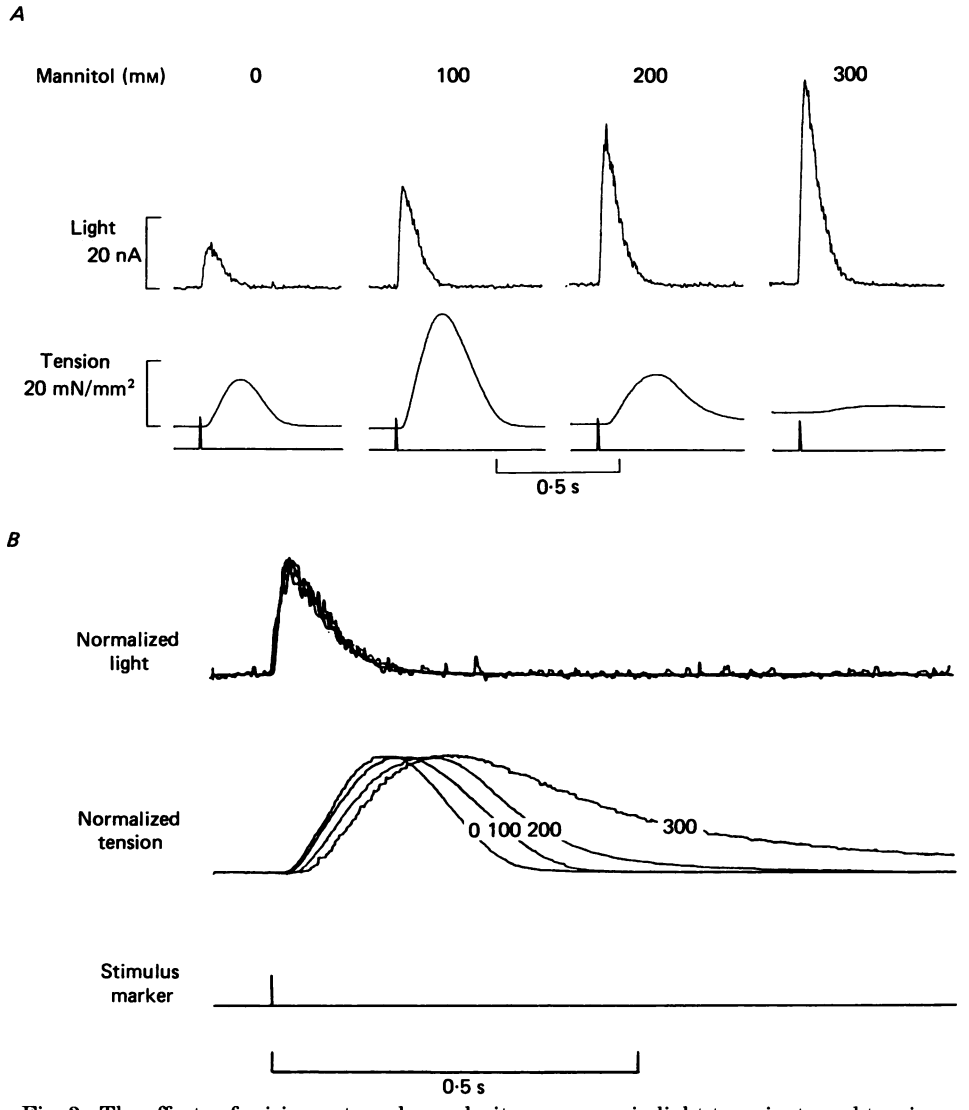

Fig. 3. The effects of raising external osmolarity on aequorin light transients and tension. *A*, averaged records ($n = 16$) of aequorin light and tension for contractions at various mannitol concentrations. Each panel shows: top, light; bottom, tension. The stimulus markers are shown below. The external mannitol concentrations are indicated above each record. *B*, the individual light transients and twitches shown in *A* are normalized and superimposed to indicate any changes in their time courses. The external mannitol concentration (mM) is indicated on the appropriate tension record.

velocity of shortening is considerably reduced by hypertonicity. Gulati & Babu (1982) showed that this mechanical effect is not due to increased intracellular ionic strength but is a consequence of the shrinkage of the cell and, presumably, the associated compression of the myofibrillar lattice. To study the mechanical properties of papillary muscles we imposed stretches and releases on the relaxed muscle, as

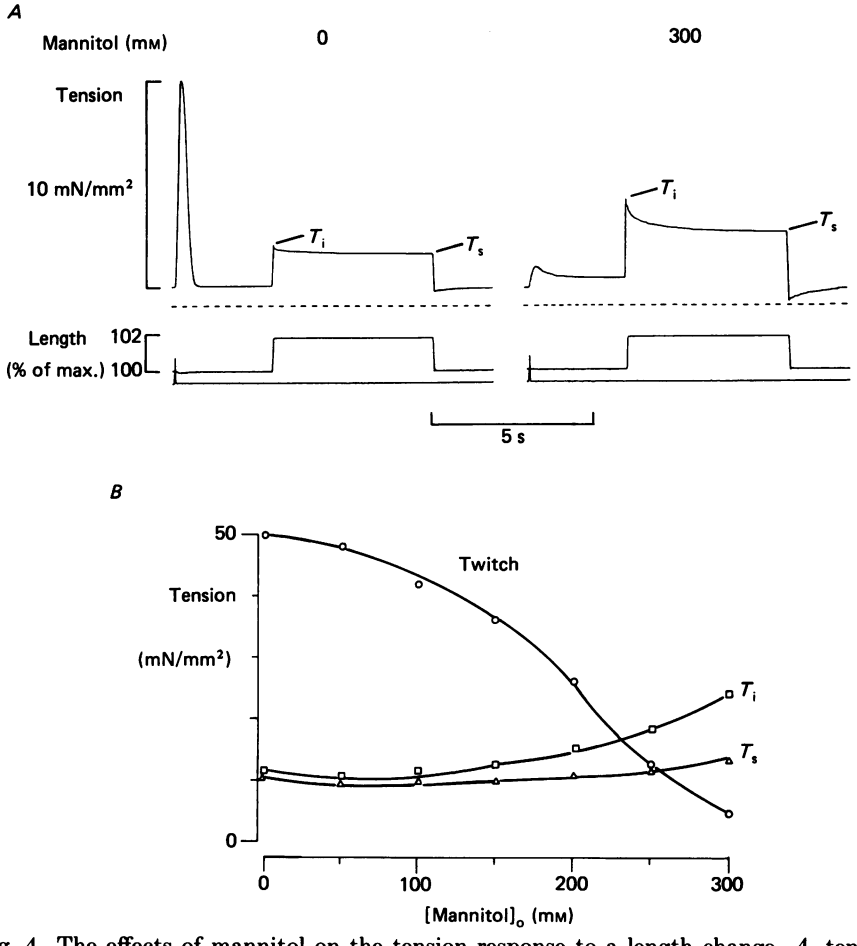

Fig. 4. The effects of mannitol on the tension response to a length change. *A*, tension responses to a rapid (5 ms) length change during diastole. Left panel, control conditions: right panel, plus 300 mM-mannitol. The traces show signal-averaged records ($n = 8$) of: top, tension; middle, muscle length (as % of maximum); bottom, stimulus marker. Dashed line below tension record indicates the zero tension level. T_i indicates instantaneous tension, T_s indicates the steady tension following the stretch. Stimulation rate 0·1 Hz. *B*, the effects of mannitol on the developed tension during a twitch and on T_i and T_s produced by stretch during diastole. The results shown in *B* were taken from a different preparation to those shown in *A*.

shown in Fig. 4*A*. A stretch of 2% of muscle length was applied over 5 ms and maintained for about 5 s. The stretch led to an instantaneous increase in tension (T_i), which declined with a half-time of 20–40 ms to a steady level (T_s). The difference in tension between the instantaneous level and the steady level can be ascribed to the viscous properties of the preparation. In 300 mM-mannitol the instantaneous component of tension increased substantially while the steady component increased by a smaller amount. Furthermore, the half-time of the change from instantaneous to steady tension increased dramatically to 200 ms. Both of these changes suggest that hypertonicity increases the viscosity of the preparation.

The relationship between solution tonicity and the tension following a stretch is shown in Fig. 4*B* for a different preparation. The steady tension following a stretch fell slightly in the presence of 50–200 mM-mannitol (cf. Chapman, 1978) before rising above control levels in 300 mM-mannitol. The difference between instantaneous and steady tension increased at 150 mM-mannitol and above, suggesting that the effects of increased viscosity occur mainly at the higher concentrations of mannitol.

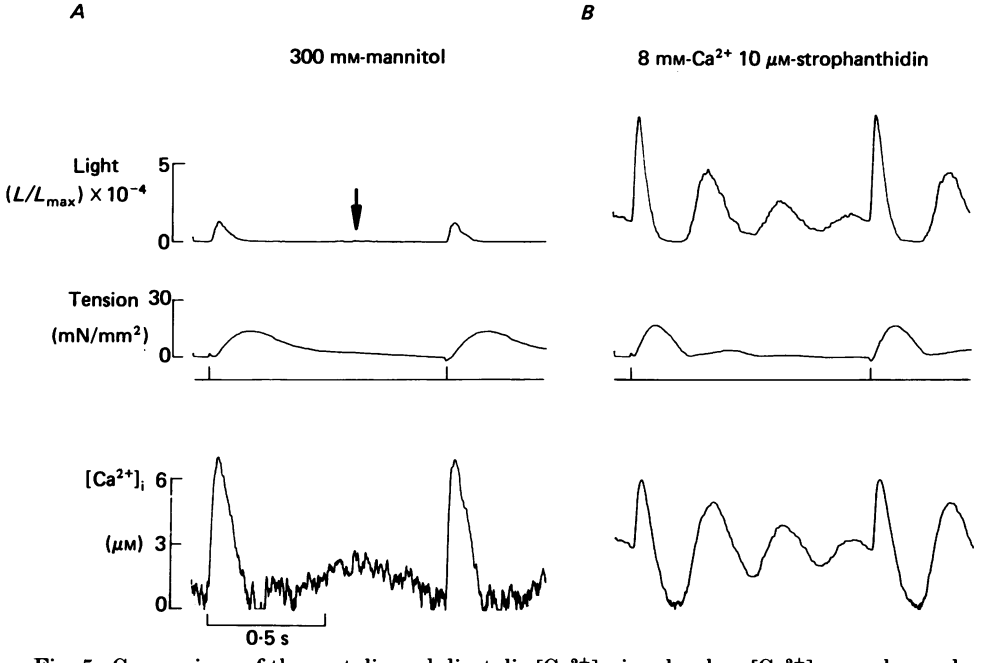

Fig. 5. Comparison of the systolic and diastolic $[Ca^{2+}]_i$ signals when $[Ca^{2+}]_i$ was changed by two different methods. *A*, external osmolarity was raised with 300 mM-mannitol. *B*, $[Ca^{2+}]_o$ was raised to 8 mM and 10 μM-strophanthidin applied. All traces are averaged records ($n = 8$) of: top, light (L, as a fraction of maximum light (L_{max})); middle, tension; bottom, $[Ca^{2+}]_i$ calculated from the light record. The stimulus marker is shown below the tension record. The arrow in *A* indicates the peak of a small rise in diastolic light (see text).

Oscillations of $[Ca^{2+}]_i$ in hypertonic solutions

A variety of manoeuvres which increase $[Ca^{2+}]_i$ have been shown to cause spontaneous oscillations of $[Ca^{2+}]_i$ (Orchard *et al.* 1983; Wier, Kort, Stern, Lakatta & Marban, 1983). These oscillations result from spontaneous release of Ca^{2+} from overloaded sarcoplasmic reticulum. It might therefore be expected that the application of hypertonic solutions, which also elevate $[Ca^{2+}]_i$, would lead to oscillations. Fig. 5*A* shows the light signal from a preparation in 300 mM-mannitol; a small rise in diastolic light is barely visible (arrow). This record was taken when the response to mannitol was stable; as noted earlier, immediately after exposure to mannitol oscillations are pronounced but in the steady state they are relatively small. This light signal has been converted to $[Ca^{2+}]_i$ in the bottom panel of Fig. 5*A* after correction for the effects of ionic strength on aequorin. A moderate increase in diastolic $[Ca^{2+}]_i$ is now apparent at 0·7 s. For comparison $[Ca^{2+}]_i$ was then increased in the same

muscle by applying 10 μM-strophanthidin and elevating $[Ca^{2+}]_o$ to 8 mM (Fig. 5*B*). We chose these conditions because, after conversion to $[Ca^{2+}]_i$, the amplitudes of the Ca^{2+} transients were similar in Fig. 5 *A* and *B*. This comparison makes it clear that, even after appropriate correction for the effects of ionic strength on aequorin, the oscillations of diastolic $[Ca^{2+}]_i$ are smaller and lower in frequency in the presence of mannitol than they are for an equivalent Ca^{2+} transient produced by strophanthidin and raised $[Ca^{2+}]_o$.

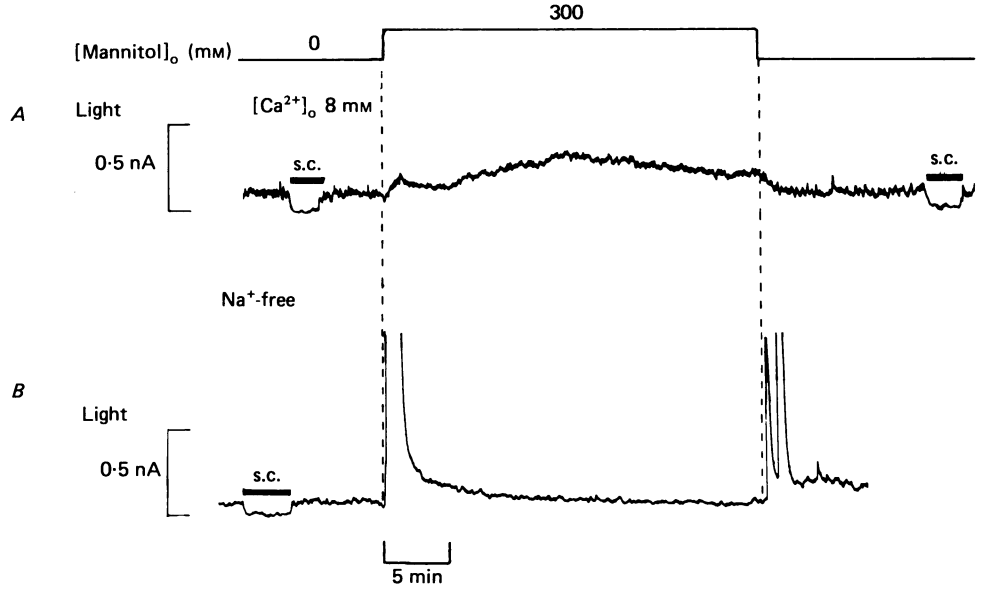

Fig. 6. The effects of raising the external osmolarity with 300 mM-mannitol on the resting aequorin light signal (12 s filter) under the following conditions: *A*, Tyrode solution with $[Ca^{2+}]_o$ of 8 mM; *B*, Na^+-free (replaced by K^+) Tyrode solution with $[Ca^{2+}]_o$ of 2 mM. The period during which the photomultiplier shutter was closed is indicated by s.c.

The effects of hypertonicity on resting light

In quiescent preparations under control conditions (in 2 mM-Ca^{2+}), light emission was only just detectable and addition of 300 mM-mannitol produced no significant effect on this light. Because the associated increase in intracellular ionic strength reduces aequorin light emission, this result is compatible with a rise in $[Ca^{2+}]_i$, as reported by Lado *et al.* (1984). However, by elevating $[Ca^{2+}]_i$ it became possible to observe substantial increases in aequorin light on exposure to mannitol and some of the properties of this increase are shown in Fig. 6.

In the preparation shown in Fig. 6*A*, $[Ca^{2+}]_i$ has been elevated by increasing $[Ca^{2+}]_o$ to 8 mM. This led to an increase in resting light emission indicated by the difference in light signal with the shutter open and closed. When 300 mM-mannitol was applied the following features were observed. (i) There was an early increase in light which reached a peak within 1–2 min and then declined; the amplitude of this phase increased with increased Ca^{2+} loading of the preparation. (ii) There was a slow increase in light which reached a maximum after 10–20 min and then showed a decline to an intermediate level. Oscillations of light at relatively low frequencies (0·25 Hz) were

detectable in Fourier transforms during both phases (i) and (ii), indicating spontaneous Ca^{2+} release from the sarcoplasmic reticulum (s.r.) (Orchard *et al.* 1983; Wier *et al.* 1983). (iii) On return to Tyrode solution the light signal fell towards the preceding control level but often remained noisier and elevated for many minutes. Fourier transforms during this period did not reveal any significant oscillatory components above 0·1 Hz.

The drug ryanodine, which inhibits the release of Ca^{2+} from the sarcoplasmic reticulum (Sutko & Kenyon, 1983), was used to investigate the contribution the s.r. may make to the responses seen in quiescent preparations. Ryanodine (1 μM for 30 min) eliminated all three phases of light increase associated with mannitol (data not shown).

Fig. 6*B* shows the effects of 300 mM-mannitol on the same preparation as in Fig. 6*A* but after replacement of the Na^+ in the perfusate with K^+. This substitution (not shown) occurred 10 min before the start of Fig. 6*B* and caused a large (peak 120 nA) transient increase in light (cf. Allen *et al.* 1983). Measurements of $[Na^+]_i$ in preparations in Na^+-free solutions show that $[Na^+]_i$ falls with a half-time of about 1 min (Chapman, Coray & McGuigan, 1983) so that the preparation in Fig. 6*B* should have a very low $[Na^+]_i$. On exposure to 300 mM-mannitol there was a very large burst of light (peak 1500 nA) which reached a maximum after 20 s and then declined to about the control level in the absence of mannitol. Oscillations of light (peak frequency 3 Hz) were detectable during the large burst. Comparison with Fig. 6*A* suggests that in Na^+-free conditions, when Na^+–Ca^{2+} exchange cannot operate, hypertonicity produces a greatly increased early phase of increased light but no slow phase. On removal of mannitol there were large transient increases in light (peak 1000 nA) which also demonstrated oscillations at around 3 Hz. This transient increase in Ca^{2+} probably underlies the contracture seen by Chapman (1978) under similar conditions in frog atrial trabeculae.

DISCUSSION

Mechanism of the rise in resting $[Ca^{2+}]_i$ during hypertonicity

It has been established that when muscle cells are subjected to increases in external osmolarity, their volume rapidly falls due to water loss (Blinks, 1965; Dick, 1966). Under these circumstances the concentrations of the major intracellular ions should increase in proportion to cell shrinkage. The addition of 300 mM-mannitol approximately doubles the extracellular osmolarity so that the concentration of the major intracellular ions, e.g. K^+, should double. In fact Fozzard & Lee (1976) found that intracellular K^+ activity rose somewhat less than expected but suggested that this might have been because the activity coefficient had declined. For Na^+ one would expect that, following an initial doubling of concentration, the Na^+–K^+ pump should then reduce the $[Na^+]_i$ to the original level with a half-time of 1–2 min (Eisner, Lederer & Vaughan-Jones, 1981). In fact Lado *et al.* (1984) found that $[Na^+]_i$ increased by a factor of about 1·5 and was still increasing slowly at the end of a 5 min exposure. They suggested that the concomitant rise in $[K^+]$ inhibited the Na^+ pump, as has been observed in red blood cells (Garay & Garrahan, 1973).

For an intracellular ion such as Ca^{2+}, whose level is controlled by a variety of

mechanisms (Chapman, 1979), it is less easy to predict its behaviour in the face of external hypertonicity. The expected initial doubling of concentration may be reduced both by protein buffers such as troponin and calsequestrin and/or by pumping Ca^{2+} out of the cell or into intracellular organelles. Alternatively, control of $[Ca^{2+}]_i$ might depend on a pump or exchanger whose activity is very sensitive to the concentration of one of the other intracellular ions. Lado *et al.* (1984) found that resting $[Ca^{2+}]_i$ increased almost 4-fold and this was consistent with $[Ca^{2+}]_i$ being controlled by a Na^+–Ca^{2+} exchanger which was at equilibrium and exhibited an apparent stoicheiometry of 2·6. A number of recent studies suggest that the true stoicheiometry is 3 Na^+:1 Ca^{2+} (for review see Eisner & Lederer, 1985) so that the exchanger is probably not at equilibrium but utilizes the Na^+ gradient to generate a net influx of Na^+ and efflux of Ca^{2+}.

In quiescent preparations in 2 mM-external Ca^{2+} we could not detect an increase in aequorin light on the addition of 300 mM-mannitol. The relationship between aequorin light and $[Ca^{2+}]$ at the resting $[Ca^{2+}]$ (0·1–0·2 μM) is such that an increase in $[K^+]$ from 150 to 300 mM would reduce light by about a factor of 2·0. Under these conditions an increase in $[Ca^{2+}]_i$ by a factor of 2·9 would be required to maintain light at the original level. Thus our experimental result suggests that $[Ca^{2+}]_i$ may have increased by a factor of about 3, reasonably consistent with the 4-fold increase in $[Ca^{2+}]_i$ observed by Lado *et al.* (1984). This increase in $[Ca^{2+}]_i$ was attributed by Lado *et al.* (1984) to Na^+–Ca^{2+} exchange on the grounds that it could be explained by the accompanying rise in $[Na^+]_i$. Our results show that, in the presence of raised $[Ca^{2+}]_o$, hypertonic solutions caused an increase in resting light signal with a complicated time course. An early rise in $[Ca^{2+}]_i$ was followed by a slower rise. These phases were also detectable in the measurements of Lado *et al.* (1984) for both $[Ca^{2+}]_i$ and $[Na^+]_i$. In the present study the $[Ca]_i$ fell after more than 10 min in hypertonic solutions. The shorter exposures used by Lado *et al.* (1984) may explain why this phase was absent from their records.

Our results show that processes other than Na^+–Ca^{2+} exchange must also contribute to the changes in $[Ca^{2+}]_i$ in hypertonic solutions, as follows.

(i) Oscillations of $[Ca^{2+}]_i$ are apparent in both stimulated and quiescent preparations. These might not be detected by ion-sensitive electrodes because of their limited frequency response. These oscillations are attributed to spontaneous release of Ca^{2+} from overloaded s.r. and this interpretation is supported by our observation that they were inhibited by ryanodine. As shown in Fig. 5, the oscillations of $[Ca^{2+}]_i$ in hypertonic solutions were less pronounced than those observed when $[Ca^{2+}]_i$ was raised by other means. This could be due to the raised intracellular ionic strength inhibiting Ca^{2+} binding to a site involved in triggering of release. Alternatively the changes in s.r. volume in hypertonic solutions noted by Page & Upshaw-Early (1977) might be involved.

(ii) The large increase in light emission seen when mannitol was applied to a muscle in Na^+-free conditions cannot be caused by Na^+–Ca^{2+} exchange and probably represents release of Ca^{2+} from the s.r. The s.r. is already loaded with Ca^{2+} in Na^+-free conditions and the extra loading precipitated by cell shrinkage may cause Ca^{2+} release. This Ca^{2+} release is probably the cause of the contractures observed by Chapman (1978) under similar conditions.

(iii) Removal of mannitol also sometimes promotes irregular Ca^{2+} signals (see Figs. 2*B* and 6*B*) and contractures (Chapman, 1978; Ohba, 1984). Ohba (1984) has shown that this kind of Ca^{2+} release can be triggered in skinned muscles with intact s.r. at constant $[Ca^{2+}]$ if external ionic strength is lowered. Thus this component of Ca^{2+} release seems to be a direct effect of intracellular ionic strength on the s.r.

TABLE 1. Experimental and calculated data concerning the effects of mannitol on Ca^{2+} transients and developed tension. Fractional luminescences were determined from the peak of the aequorin light transients as described in the Methods. Peak developed tension was measured under the various conditions and normalized to the calculated cross-sectional area of the papillary muscle under control (mannitol-free) conditions. Peak $[Ca^{2+}]_i$ was determined from the fractional luminescence using an aequorin calibration curve after correction for the effects of increase of $[K^+]$ when mannitol was increased. The final column shows the calculated tension determined on the basis of the peak $[Ca^{2+}]_i$ and the effects of ionic strength (i.s.) and $[K^+]$ on the contractile proteins (see Discussion)

		Experimental data		Calculated data	
Mannitol (mM)	No. of experiments	Fractional luminescence ($\times 10^{-4}$)	Peak tension (mN/mm²)	Peak $[Ca^{2+}]_i$ (μM)	Ca^{2+}- and i.s.-dependent tension (mN/mm²)
0	7	1·8±0·2	19±5	2·2	19
100	5	2·1±0·4	28±6	3·7	34
200	4	4·6±0·3	14±3	7·9	36
300	7	4·3±0·4	5±1	8·9	0

Mechanism of the increased Ca^{2+} transients in hypertonic solutions

Two possible mechanisms for the increased Ca^{2+} transients seen in hypertonic solutions are (i) an increase in the influx of Ca^{2+} during each action potential and (ii) an increase in Ca^{2+} release from the s.r. Evidence against (i) comes from a recent study of cardiac action potentials and tension on exposure to hypertonic solutions (Beyer, Jepsen, Lüllman & Ravens, 1985). The action potential was shown to shorten significantly in hypertonic solutions; this and other indirect evidence suggested that changes in the slow inward Ca^{2+} current were not important in positive inotropic effect of hypertonic solutions. In support of (ii) is the observation that resting $[Ca^{2+}]_i$ increases. This increase may lead to increased uptake of Ca^{2+} by the s.r., leading to an increased Ca^{2+} release and to larger Ca^{2+} transients. Quantitative arguments also support this view. According to Lado *et al.* (1984) the resting $[Ca^{2+}]_i$ is about 0·2 μM (we have converted their activities to concentration) and this rises to 0·8 μM after exposure to 300 mM-mannitol. Since the s.r. Ca^{2+} pump has a K_m of about 5 μM (Shigekawa, Finegan & Katz, 1976) an increase in myoplasmic $[Ca^{2+}]$ over the above range will lead to an approximately proportional increase in the rate of Ca^{2+} uptake. If the amount of Ca^{2+} release from the s.r. is a linear function of loading and the amplitude of the Ca^{2+} transient is a linear function of release, then the amplitude of the Ca^{2+} transient should be about 4 times larger in 300 mM-mannitol. After calibration of the light signals, this is approximately what we found (Table 1).

It is interesting to note that a study on frog skeletal muscle (Taylor, Rüdel & Blinks, 1975) showed that the amplitude of the Ca^{2+} transient did not change in hypertonic solutions, despite the complete suppression of twitch force. The difference

between the result in cardiac and skeletal muscle may reflect the absence of a Na^+–Ca^{2+} exchanger in the surface membrane of skeletal muscle.

Mechanism of the changes in developed tension

The increased Ca^{2+} transients which we have observed would be expected to lead to increased developed tension. On the other hand, the reduction in maximum Ca^{2+}-activated tension associated with increased ionic strength (Kentish, 1984) and the reduction in Ca^{2+} sensitivity associated with increased $[K^+]_i$ (Kentish, 1984) would be expected to reduce force. To determine whether our results can account for the increased tension at 100 mM-mannitol and decreased tension in 300 mM-mannitol we have calculated the expected tension production on the following basis.

First, the tension expected on the basis of our calculated peak $[Ca^{2+}]_i$ (Table 1) was determined using the pCa–tension curve of Kentish, ter Keurs, Ricciardi, Bucx & Noble (1986). The peak $[Ca^{2+}]_i$ under control conditions (2·2 μM) was assumed to produce the tension observed under control conditions (19 mN/mm^2); on this basis the expected tensions at the other peak $[Ca^{2+}]_i$ were determined. Secondly, the expected tension was corrected for the reduction in Ca^{2+} sensitivity due to change in $[K^+]$, using the data of Kentish (1984). This required extrapolating his data over a different range, but the results of Fink, Stephenson & Williams (1986) suggest that this assumption is reasonable. Thirdly, the tension was corrected for the decrease in maximum Ca^{2+}-activated tension with ionic strength (Kentish, 1984). The results of this exercise are given in the final column of Table 1. The main points are that the predicted tension is increased at intermediate mannitol concentrations but decreased at the high mannitol concentrations. Thus in general terms the calculated tension matches our experimental results. However, there are many assumptions involved. One critical assumption is the steepness of the pCa–tension curve at Ca^{2+} concentrations between 2·2 and 3·7 μM; clearly a steep curve is required if the increased tension due to the rise in $[Ca^{2+}]_i$ is to exceed the decrease associated with increased ionic strength and $[K^+]$. The steepness *in vivo* is not known with certainty, but recent evidence (Yue, Marban & Wier, 1986) suggests that it is extremely steep, certainly much steeper than skinned fibre results suggest. A second critical assumption is the intracellular ionic strength under control conditions. We used the value of 200 mM (Kentish, 1984) which can be obtained by straightforward addition of the known intracellular constituents. Since the addition of 300 mM-mannitol increases external osmotic strength by 2·13, we assumed intracellular ionic strength to increase by the same factor. On this basis the maximum Ca^{2+}-activated tension at the highest mannitol concentration would be zero (Kentish, 1984). However, if we had assumed that the intracellular ionic strength was lower (e.g. according to Fabiato, 1981) then the calculated developed tension in 300 mM-mannitol would be much higher. Clearly no detailed significance should be attached to the results of this exercise; it simply shows that, with reasonable assumptions taken from the literature, the main features of our results can be explained.

The above calculation predicts the effects of the altered Ca^{2+} transients and myofibrillar properties on tension development. In addition, the following factors may contribute to the changes in developed tension. (i) Under certain conditions oscillations of $[Ca^{2+}]_i$ during diastole are associated with a fall of tension in

'Ca^{2+}-overloaded' preparations (Allen, Eisner, Pirolo & Smith, 1985). However, in hypertonic solutions the diastolic oscillations are small and, as noted in the Results, tend to decline during a period of exposure while the developed tension remains depressed. On this basis we suspect that their contribution to the fall of tension in the present experiments is small. (ii) The increased viscosity of the preparation will both slow the time course of contraction and reduce the peak developed tension. This increase in viscosity is probably due to the shrinking of the myofilament lattice (Gulati & Babu, 1982). (iii) There may be an intracellular acidosis during hypertonicity (Ellis & Thomas, 1976) and this would also contribute to a reduced tension at all mannitol concentrations.

In conclusion, our results show the hypertonicity increases the Ca^{2+} transients in cardiac muscle and that this can account for the increased developed tension in moderately hypertonic solutions.

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