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# EFFECTS OF HYPERTONIC SOLUTIONS ON CALCIUM TRANSIENTS IN FROG TWITCH MUSCLE FIBRES

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## SUMMARY

1. The effects of hypertonic solutions on excitation–contraction (e.–c.) coupling in frog skeletal muscle fibres were investigated using Arsenazo III to monitor intracellular calcium transients in voltage-clamped fibres.

2. In solutions made hypertonic with sucrose or sodium chloride, the size of the Arsenazo signal evoked by a 5 ms depolarization to 0 mV was little altered by increases in tonicity up to about twice normal, but declined in higher tonicities, and was almost completely suppressed at 4 times normal tonicity.

3. The latency to onset of the Arsenazo signal was increased in hypertonic solutions (2·3 and 3·1 times normal tonicity), but the decay time constant of the signal was little changed with tonicities up to 2·3 times normal.

4. The rheobase potential for a just-detectable Arsenazo signal was shifted about 4 mV more negative by increases in tonicity up to 2·3 times normal, but further increases reversed the direction of the shift, and in 3·95 times normal tonicity the rheobase was 10 mV more positive than in normal Ringer solution.

5. With short ($< 10$ ms) pulse durations the depolarization needed to elicit a threshold Arsenazo signal increased steeply with increasing tonicity. Changes in the strength–duration curve could be accounted for by an increase in the time constant for build-up of a hypothetical coupler in the e.–c. coupling process.

6. Solutions of about twice normal tonicity are commonly used to suppress muscle contraction. Since the size of the Arsenazo signal was only slightly reduced by this tonicity, the main effect is presumably on the contractile proteins.

## INTRODUCTION

Hodgkin & Horowicz (1957) first showed that hypertonic solutions reduce the twitch tension developed by muscle fibres, without affecting the size or time course of the action potential. Since then, the use of bathing solutions made hypertonic by addition of sucrose or sodium chloride has become a common technique to reduce contraction artifacts in experiments where intracellular recordings are made from stimulated muscle fibres.

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Several lines of evidence indicate that the main effect of increasing tonicity to around twice normal is on the contractile mechanism, and that any actions on the excitation–contraction (e.–c.) coupling process are of less importance (Howarth, 1958; Gordon & Godt, 1970; Rapoport, Nassar-Gentina & Passonneau, 1982; for review see Caputo, 1983). The most direct observation supporting this view is that of Taylor, Rudel & Blinks (1975), who used aequorin to monitor intracellular calcium, and found that although twitch tension was practically abolished in a solution made 2·3 times hypertonic with sucrose, the size of the aequorin signal was almost unchanged. However, there are several indications that hypertonicity may affect the e.–c. coupling process in addition to its actions on tension development. For example, hypertonic solutions were found to depress depolarization-induced tension development to a greater extent than caffeine-induced tension (Caputo, 1966; Homsher, Briggs & Wise, 1974), an effect which suggests an action on e.–c. coupling, since caffeine is presumed to bypass this process and directly release calcium from the sarcoplasmic reticulum. Furthermore, depression of e.–c. coupling by hypertonic solutions has been demonstrated by using heat measurements (Homsher *et al.* 1974), or turnover of high-energy phosphate compounds (Rapoport *et al.* 1982), to monitor the activation process. Also, Shlevin & Taylor (1979) noted that hypertonicity reduced the aequorin calcium transient during tetanic stimulation.

A more detailed knowledge of the possible effects of hypertonicity on e.–c. coupling would be important, both in its own right, and because hypertonic solutions are frequently used in the study of membrane charge movements which are presumed to reflect an initial stage in the e.–c. coupling process (Schneider, 1981). We therefore made a study of the actions of hypertonic solutions on the calcium transient evoked by depolarization of frog twitch muscle fibres, using Arsenazo III as an intracellular calcium monitor (Miledi, Parker & Zhu, 1982, 1983*b*).

## METHODS

### *Preparation and Arsenazo recording*

Experiments were performed on the isolated cutaneous pectoris muscle of *Rana temporaria*. Details of the preparation, and techniques for voltage clamping and recording of intracellular calcium transients were as described previously (Miledi *et al.* 1982, 1983*b*). All bathing solutions included tetrodotoxin ($10^{-6}$ g $ml^{-1}$) and tetraethylammonium bromide (20 mM) to reduce voltage-activated sodium and potassium conductances, and hence improve voltage-clamp control. Optical measurements of calcium-dependent changes in Arsenazo III absorbance were made using a rectangle of light (*ca.* 100 $\mu$m long), positioned between the recording and current-passing electrodes, and adjusted to match the fibre diameter. Thus, the calcium signals were obtained from a restricted region of the fibre which was under good voltage-clamp control, despite the use of a two-electrode clamp. Muscles were stretched to a striation spacing of about 3·7 $\mu$m. Fibres were clamped at a holding potential of −75 mV, and examined at a temperature of 9–11 °C.

### *Solutions*

Normal Ringer solution contained (in mM): sodium chloride, 120; potassium chloride, 2; calcium chloride, 1·8; HEPES, 5, at pH 7·2. The osmolality of this solution would have been about 230 mosmol/l (cf. Dydynska & Wilkie, 1963). We refer to this solution as isotonic (i.e. tonicity = 1 ×), and the tonicities of other solutions are expressed relative to this. Hypertonic solutions were made by addition of either sucrose or sodium chloride to the normal Ringer solution. The tonicities of these solutions were calculated from the tables of freezing-point depressions given

in Weast & Selby (1962) for sucrose and sodium chloride solutions (osmolality of solute = freezing-point depression in °C/1·86). Table 1 gives the various concentrations of sucrose and sodium chloride used and the corresponding tonicities of the bathing solutions.

TABLE 1. Concentrations of sucrose and sodium chloride added to Ringer solution to increase tonicity. Osmolalities and tonicities were derived as described in the text

| Concentration (mM) | Osmolality of solute (mosmol/l) | Tonicity of final solution (× normal) |
|---|---|---|
| | Sucrose | |
| 140 | 145 | 1·63 |
| 280 | 310 | 2·34 |
| 420 | 485 | 3·11 |
| 560 | 680 | 3·96 |
| | Sodium chloride | |
| 70 | 140 | 1·61 |
| 140 | 260 | 2·13 |
| 280 | 515 | 3·23 |

## RESULTS

### *Effects of tonicity on size of Arsenazo signals*

Changes in tonicity had little effect on the resting potentials of muscle fibres, except for a fall at very high (4 ×) tonicity. In solutions made hypertonic with sucrose the following mean values were recorded (all errors are given as ±S.E. of mean, with number of fibres in brackets): normal Ringer solution, $-84{\cdot}2 \pm 0{\cdot}9$ mV ($n = 24$); 2·3 × hypertonic solution, $-88{\cdot}6 \pm 1{\cdot}2$ mV ($n = 25$); 3·1 × hypertonic solution, $-81{\cdot}6 \pm 2{\cdot}2$ mV ($n = 12$); 4 × hypertonic solution, $-71{\cdot}0 \pm 2{\cdot}1$ mV ($n = 3$).

Arsenazo signals were recorded in response to a standardized voltage-clamped depolarizing pulse of 5 ms duration to 0 mV, from a holding potential of −75 mV. At the temperature used (9–11 °C) and in normal Ringer solution, this pulse gives a response size similar to that elicited by a single action potential (Miledi, Parker & Zhu, 1984, 1985). However, the use of voltage-clamped pulses obviates any complications from possible changes in action potential parameters. Fig. 1 shows sample records of Arsenazo signals, recorded from different fibres in the same muscle at different tonicities. It was not possible to retain the micro-electrodes in a fibre while changing solutions, so measurements were made from several fibres in normal Ringer solution, and then from other fibres beginning about 30 min after changing to hypertonic solution. In the solution made 2·3 × hypertonic with sucrose the size of the Arsenazo signal was reduced compared to that in normal Ringer solution, but the time course of the calcium transient appeared little changed (Fig. 1*a*, *b*). A further increase in tonicity to 4 × normal caused the signal to be practically abolished (Fig. 1*c*).

The sizes of Arsenazo signals like those in Fig. 1 cannot be directly compared, because of differences in fibre diameters and amounts of dye injected. To standardize the measurements, we expressed the calcium-dependent change in dye absorption at 650–700 nm by dividing by the resting dye absorbance at 570 nm ($\Delta A/A_{570}$); see

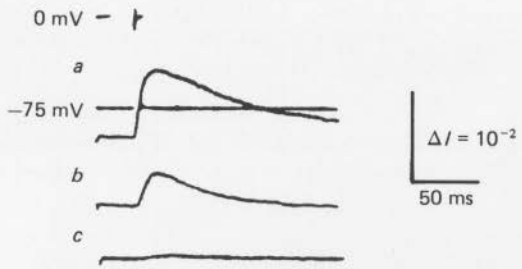


Fig. 1. Arsenazo–calcium signals recorded from voltage-clamped muscle fibres in solutions of different tonicities. Upper trace shows membrane potential, which was held at −75 mV and stepped to 0 mV during a 5 ms duration pulse, in all records. Lower traces (*a*, *b* and *c*) show light absorption changes recorded at the differential wave-length pair 650–700 nm. Calibration bar indicates the fractional change in light transmission ($\Delta I$). *a*, fibre in normal Ringer solution. *b*, after addition of 280 mM-sucrose to increase the tonicity to 2·3 × normal. *c*, after addition of 560 mM-sucrose (4 × normal tonicity). Records from three fibres in one muscle.

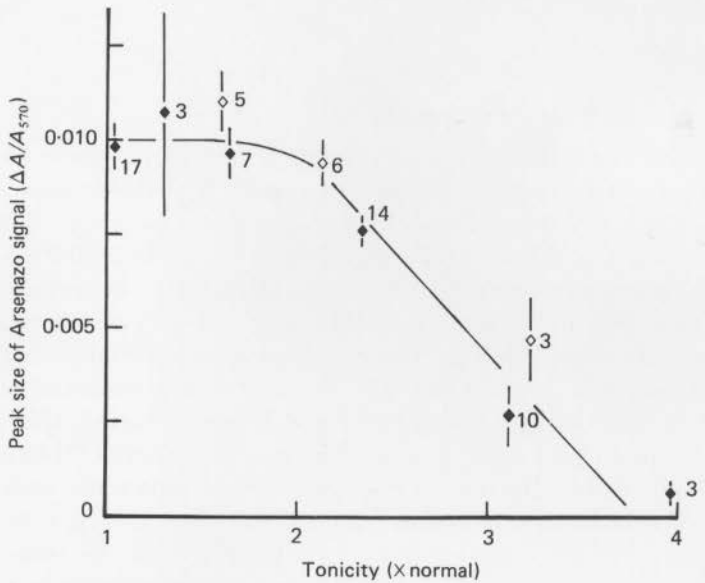


Fig. 2. Peak size of Arsenazo signals elicited by the standard depolarizing pulse (0 mV, 5 ms, from a holding potential of −75 mV) in solutions of different tonicities. Bathing solution was normal Ringer solution, made hypertonic with sucrose (filled symbols) or sodium chloride (open symbols). Error bars indicate ± S.E. of mean. Numbers of fibres examined are indicated next to the data points.

Miledi *et al.* (1982) for more details. Mean values of the standardized absorbance change elicited by the 5 ms depolarizing pulse to 0 mV are plotted in Fig. 2 as a function of tonicity. There were no clear differences in the effects of solutions made hypertonic with sucrose or with sodium chloride; in both cases the mean size of the Arsenazo signal remained about constant with increasing tonicity up to twice normal, but then declined at higher tonicities. The response was reduced to roughly one-half

of the normal value at a tonicity of three times normal, and was almost abolished at four times normal tonicity.

The effect of very high tonicities on the Arsenazo signal was only partly reversible on returning to normal Ringer solution. In one experiment, mean values of the Arsenazo signals ($\Delta A/A_{570}$) were 0·13 in normal Ringer solution, 0·006 in 4×

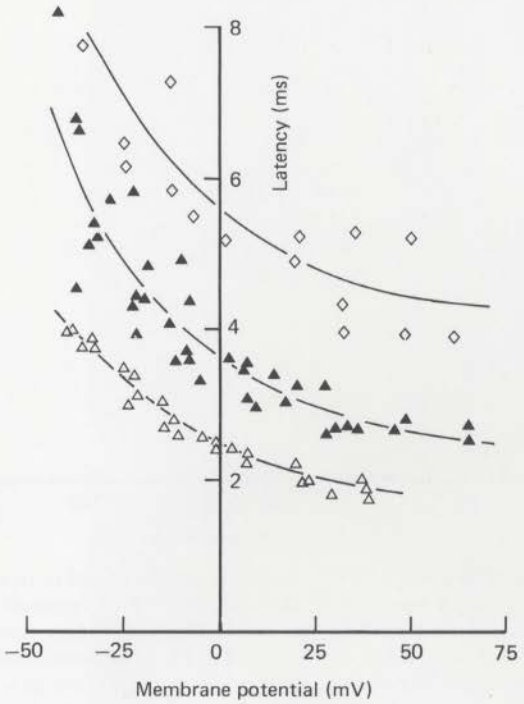


Fig. 3. Voltage dependence of latency to onset of Arsenazo signal in normal Ringer solution (△) and solutions made 2·3× (▲) and 3·1× (◇) hypertonic with sucrose. Abscissa is membrane potential during the test pulse, and ordinate is the latency from the onset of the pulse to the time at which the tangent to the main rising phase of the Arsenazo signal crossed the base line (cf. Miledi *et al.* 1983*b*). Temperature, 10 °C. Data from two muscles; four fibres in normal Ringer solution, six in 2·3× and three in 3·1× hypertonic solution. Each point represents a single measurement. Curves drawn by eye.

hypertonic sucrose Ringer solution, and 0·064 after returning to normal Ringer solution (three fibres examined in each solution).

*Effect of tonicity on latency of Arsenazo signals*

We have previously shown that there is a latency of a few milliseconds between depolarization of a muscle fibre and the beginning of the Arsenazo signal, which decreases with increasing depolarization (Miledi *et al.* 1983*b*). Fig. 3 shows the voltage dependence of this latency, derived from experimental records like those in Fig. 6 of Miledi *et al.* (1983*b*). In normal Ringer solution the delay to onset of the signal was about 4 ms for depolarization to −40 mV, decreasing to about 2 ms at +30 mV (open triangles, Fig. 3). After addition of sucrose to bring the tonicity to 2·3× normal

the latency showed a similar voltage dependence, but at each potential was about 1 ms longer than in normal Ringer solution. This increase in latency could not be attributed to a decreased size of the Arsenazo response, since the signals were of similar size to that in normal Ringer solution. In 3·1× hypertonic solution the

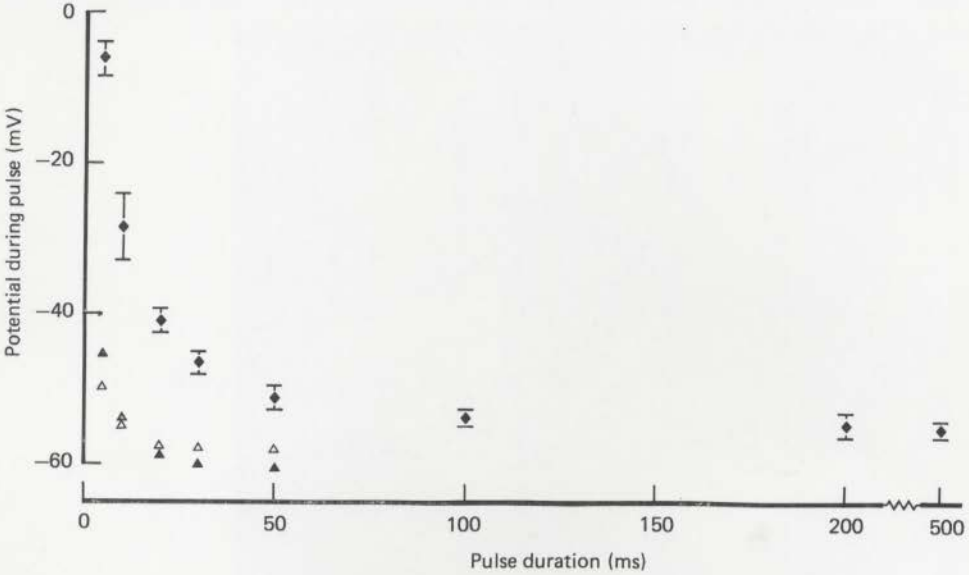


Fig. 4. Strength–duration curves for threshold Arsenazo signal in normal Ringer solution (△), and in Ringer solution made 2·3× (▲) and 3·1× (◆) hypertonic with sucrose. Data points are mean values from two fibres in normal Ringer solution, and three fibres in each of the hypertonic solutions. Error bars indicate ±S.E. of mean; bars are smaller than symbol width for the 2·3× hypertonic solution. Holding potential was −75 mV.

increase in latency was more pronounced, being about 8 ms at −40 mV and failing to reduce below 4 ms even with depolarization to +50 mV.

*Decay time course of Arsenazo signal*

The time course of decay of the Arsenazo signal following a depolarizing pulse was measured by plotting out records on semilogarithmic graph paper. In normal and hypertonic solutions the initial part of the decay (down to one-third or less of the peak size) followed closely to a single exponential decline, and the time constants were measured from the slope of the line on semilogarithmic coordinates. Mean values measured in this way from fibres in one muscle exposed to solutions made hypertonic with sucrose were: normal Ringer solution, 64±9 ms ($n = 5$); 1·3× hypertonic solution, 59 ms ($n = 2$); 1·6× hypertonic solution, 69±2·5 ms ($n = 4$); 2·3× hypertonic solution, 51 ms ($n = 2$). Additional data were obtained from three other muscles, by measuring the half-decay time of the Arsenazo signal, beginning about 10 ms after the peak. Mean values of the half-times were: normal Ringer solution, 53±3 ms ($n = 6$); 1·6× hypertonic solution, 41±3 ms ($n = 5$); 2·3× hypertonic solution, 55·3±4 ms ($n = 6$); 3·2× hypertonic solution, 36·4 ms ($n = 3$).

Thus, increases in tonicity up to 2·3 × normal had no obvious effect on the decay of the Arsenazo signal, but in 3·2 × hypertonic solution, the decay appeared to be slightly faster.

*Strength–duration curves*

Strength–duration curves for the threshold Arsenazo signal were obtained at various tonicities, since we believe these give information about a time-dependent

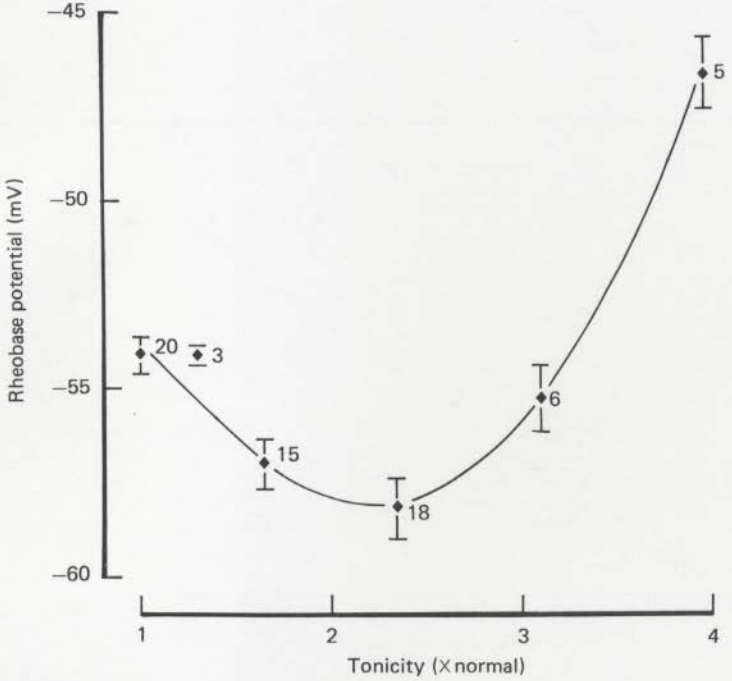


Fig. 5. Changes in rheobase potential for threshold Arsenazo signal at different tonicities. Ringer solution was made hypertonic by addition of sucrose. Rheobase potentials were measured with a pulse duration of 100 ms. Error bars indicate ±S.E. of mean. Numbers of fibres examined are indicated next to the data points.

process in e.–c. coupling (Miledi *et al.* 1983*b*). Fig. 4 shows curves obtained from fibres in one muscle, with measurements being made from records like those illustrated in Fig. 3 of Miledi *et al.* (1983*b*). Increasing tonicity to 2·3 × normal with sucrose shifted the rheobase potential (potential to give a threshold Arsenazo signal with long pulse durations) by about 3 mV to more negative values, but with short-duration (< 10 ms) pulses a greater depolarization was needed to elicit a threshold signal than in normal Ringer solution. In solution made 3·1 × hypertonic with sucrose the shift in rheobase potential was reversed, becoming 5 mV more positive than in normal Ringer solution, and the depolarization required at short intervals became much greater. For a pulse duration of 5 ms, a depolarization to −5 mV was required to give a threshold signal, compared to −50 mV in normal Ringer solution.

Mean values of the rheobase potential are plotted in Fig. 5 as a function of tonicity.

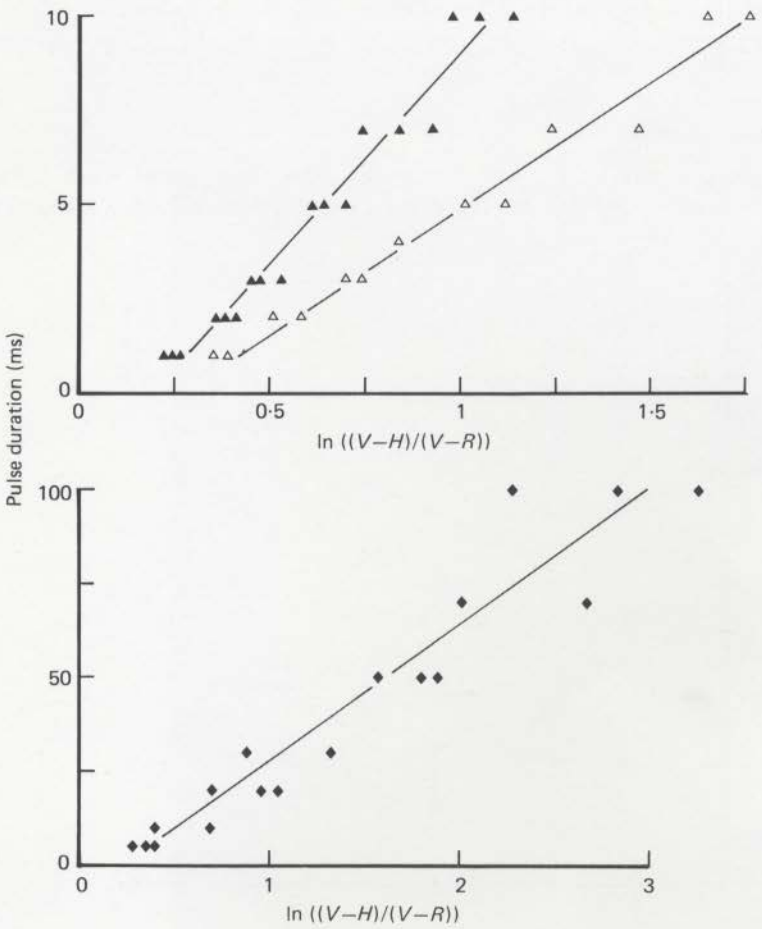


Fig. 6. Strength–duration relationships plotted showing pulse duration as a function of $\ln((V-H)/(V-R))$. See text for further details and explanation of symbols. Data are the same measurements as Fig. 4, made in normal Ringer solution (△), and solutions made 2·3× (▲) and 3·1× (◆) hypertonic with sucrose. Note change in scales of axes for data in 3·1× hypertonic solution. Lines were drawn by eye, and correspond to time constants for a 1/e change in $\ln((V-H)/(V-R))$ of: normal Ringer solution, 6·7 ms; 2·3× hypertonic solution, 11·2 ms; 3·1× hypertonic solution, 36 ms.

For tonicities up to about 2·3× normal the rheobase became progressively more negative, reaching a maximum value of −58 mV as compared to −54 mV in normal Ringer solution. At higher tonicities the rheobase began to shift to more positive potentials, and was −44 mV in 4× hypertonic solution. These values were obtained in solutions made hypertonic with sucrose, but similar results were also obtained in solutions made hypertonic with sodium chloride. Mean values for the rheobase in fibres from one muscle at different concentrations of sodium chloride were: normal Ringer solution, −51·6 mV ($n = 2$); 1·6× hypertonic solution, $-52{\cdot}5 \pm 0{\cdot}5$ mV ($n = 4$); 2·1× hypertonic solution, $-54{\cdot}8 \pm 0{\cdot}6$ mV ($n = 4$); 3·2× hypertonic solution, −47·1 mV ($n = 2$). Thus, it seems that the changes in rheobase arose because

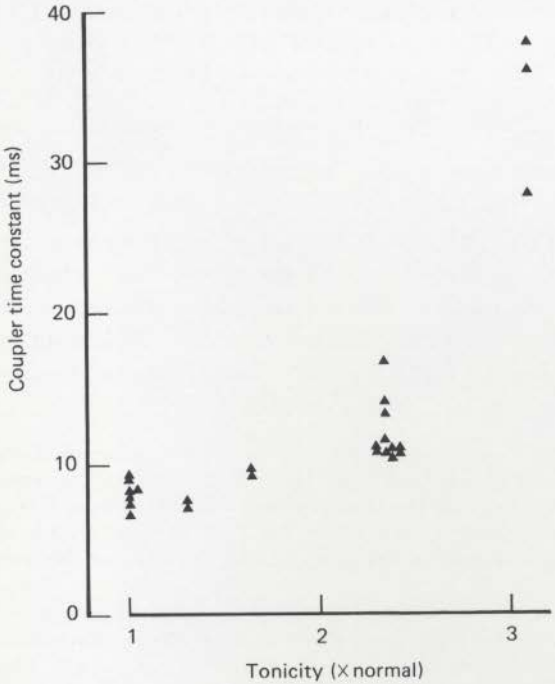


Fig. 7. Effect of tonicity on the estimated time constant for coupler build-up. Time constants were derived from the slope of lines fitted by eye to strength–duration relations plotted as in Fig. 6. Each point represents measurements from one fibre. Solutions were made hypertonic by addition of sucrose.

of changes in tonicity *per se*, rather than because of differences in extracellular ionic strength.

*Quantitative analysis of strength–duration curves*

To study in more detail the changes in strength–duration curves, we analysed the results in terms of the coupler model of Miledi *et al.* (1983*b*), where the strength–duration curve is given by the equation (eqn. (2) of Miledi *et al.* (1983*b*)):

$$t = \tau \ln ((V-H)/(V-R)), \quad (1)$$

where $V$ is the threshold depolarization for any given pulse duration $t$, $H$ is the holding potential, $R$ is the rheobase potential, and $\tau$ is the time constant of coupler build-up. If this equation gives a correct description of the observations, then a straight line should be obtained by plotting $t$ against $\ln ((V-H)/(V-R))$, with a slope equal to $\tau$ (Miledi *et al.* 1984). The data of Fig. 4 are shown in this way in Fig. 6, where the three sets of results at different tonicities lie fairly well on straight lines. The slopes of the lines became steeper at higher tonicities, and for the data shown correspond to estimated coupler time constants ($\tau$) of 7 ms in normal Ringer solution, 11 ms in 2·3 × hypertonic solution, and 36 ms in 3·1 × hypertonic solution.

Pooled estimates of coupler time constants, obtained from fibres in three muscles from plots like those in Fig. 6, are shown in Fig. 7. Increasing tonicity to 2·3 × normal

caused a small but significant increase in time constants from $8{\cdot}1 \pm 0{\cdot}4$ ms ($n = 7$) in normal Ringer solution to $12{\cdot}0 \pm 0{\cdot}7$ ms ($n = 10$). Further increasing the tonicity to $3{\cdot}1\times$ normal caused a more dramatic increase to 34 ms ($n = 3$).

## DISCUSSION

### *Interpretation of Arsenazo signals*

We have previously discussed the evidence indicating that the standardized Arsenazo III absorbance change ($\Delta A/A_{570}$) gives a measure which is linearly proportional to the free calcium change in the muscle, and which is independent of the amount of dye injected and of differences in fibre diameters (Miledi *et al.* 1982). However, the present work raises the additional problem of whether the dye sensitivity remains constant when the muscle fibre is exposed to solutions of different tonicity, and several factors had to be considered.

(i) Increasing ionic strength has been shown to decrease the apparent dissociation constant of Arsenazo III for calcium (Brown & Rydqvist, 1981; Bauer, 1981). For example, Bauer (1981) found that increasing the buffer concentration (potassium HEPES) from 100 to 200 mM decreased the dissociation constant by a factor of about two. Thus, the increase in intracellular ionic strength of muscle fibres in hypertonic solutions would be expected to decrease the sensitivity of Arsenazo III.

(ii) In the present experiments, Arsenazo III was injected to give an absorbance increase (measured at 570 nm) which was roughly the same for all fibres. Since muscle fibres shrink in hypertonic solution, and would thus present a shorter optical path length, this implies that fibres in hypertonic solutions were injected to higher dye concentrations. However, the Arsenazo signal ($\Delta A$) during a twitch varies linearly with dye concentration over quite a wide range (Miledi *et al.* 1982), so the normalization of the response by dividing by the resting absorbance at 570 nm would correct for this effect.

In summary the net effect of hypertonic solutions is expected to be a slight decrease in sensitivity of Arsenazo III for calcium. The magnitude of the effect is difficult to quantify, however, and it is possible that factors other than those considered above could affect the dye (e.g. if intracellular pH was to change).

### *Effect of hypertonic solutions on size of calcium transient*

The peak size of the normalized Arsenazo signal ($\Delta A/A_{570}$) evoked by a depolarizing pulse of 5 ms duration to 0 mV remained constant with increases in tonicity to $2\times$ normal, and then decreased at higher tonicities (Fig. 2). As discussed above, the sensitivity of Arsenazo III is expected, if anything, to decline with increasing ionic strength. Thus the constancy of the response up to $2\times$ tonicity indicates that the intracellular free calcium transient remained of constant size, or possibly increased slightly. At higher tonicities the abrupt decline in size of the Arsenazo signal, decreasing to almost zero at $4\times$ tonicity, is greater than might be expected from any decrease in Arsenazo sensitivity, and presumably reflects a reduction in size of the free calcium transient.

The lack of effect on the Arsenazo signal of tonicities up to $2\times$ normal is in agreement with results using aequorin as an intracellular calcium monitor in frog muscle fibres (Taylor *et al.* 1975). Also, the decline in response at higher tonicities supports the conclusions of Homsher *et al.* (1974), who found that activation heat (which is presumed to arise from the e.–c. coupling process) was reduced by more than 90% at a tonicity of $3{\cdot}1\times$ normal.

*Effects on kinetics of e.–c. coupling*

Hypertonicity slowed the kinetics of the e.–c. coupling process, as revealed by an increase in the delay between depolarization and onset of the Arsenazo signal (Fig. 3), and by an increase in the duration of depolarization to a given voltage required to elicit a threshold Arsenazo signal (Fig. 4). This confirms other work indicating that hypertonic solutions increased the delays between depolarization and the onset of latency relaxation (Mulieri & Alpert, 1982) and of intracellular calcium release (Rakowski, Best & James-Kracke, 1985).

Most, if not all, of the effect of hypertonic solutions on the size of the Arsenazo signal evoked by the standard depolarization (0 mV, 5 ms) could be explained by the change in strength–duration curve for threshold Arsenazo signal. In normal Ringer solution, the threshold for a 5 ms duration pulse was about −50 mV, but in 3·1 × hypertonic solution a much stronger depolarization to about −5 mV was needed to give a just-detectable signal. Thus, the standard test depolarization would have been only 5 mV above threshold. With longer pulses the effect was less marked; for example with a 50 ms pulse the threshold in 3·1 × hypertonic solution was only about 10 mV more positive than in normal Ringer solution.

We have previously proposed a model of e.–c. coupling in which depolarization is presumed to lead to the build-up of a hypothetical coupler, which in turn activates release of calcium from the sarcoplasmic reticulum once a certain threshold level is exceeded (Miledi *et al.* 1983*b*). On the basis of this model, the main effect of hypertonicity was to increase the time constant of coupler build-up. Time constants were estimated from strength–duration curves, and increased from about 7 ms in normal Ringer solution (see also Miledi, Parker & Zhu, 1983*b*) to more than 30 ms in 3·1 × hypertonic solution. This slowing of coupler build-up is also consistent with the increased delay to onset of the Arsenazo signal, since a longer time would be required before the level of coupler exceeded the threshold for calcium release.

*Relation with charge movement experiments*

Many of the early experiments measuring charge movement in muscle were made with 467 mM-sucrose added to the bathing solution to suppress contraction (e.g. Chandler, Rakowski & Schneider, 1976; Adrian & Almers, 1976). In this condition, the charge movement showed a single monotonic decay ($Q_\beta$), but in solutions of lower tonicity (350 mM-sucrose) a second component ($Q_\gamma$) became apparent (Adrian & Peres, 1979). Several properties of $Q_\gamma$ suggest that it is this component, rather than $Q_\beta$, which may be associated with e.–c. coupling (Adrian & Peres, 1979; Huang, 1981; Hui, 1983; Adrian & Huang, 1984). Our results support this conclusion, since the high tonicity (465 mM-sucrose = 3·4 × hypertonic solution) would almost suppress the calcium transient evoked by our test depolarization (Fig. 2). However, even with 350 mM-sucrose (2·75 × tonicity) calcium release would be reduced, and the kinetics of the calcium release process slowed appreciably (Fig. 7). If $Q_\gamma$ reflects an early stage in e.–c. coupling, then its kinetics might be altered in a similar way by moderate hypertonicity.

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