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# EFFECT OF CHANGING THE COMPOSITION OF THE BATHING SOLUTIONS UPON THE ISOMETRIC TENSION–pCa RELATIONSHIP IN BUNDLES OF CRUSTACEAN MYOFIBRILS

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SUMMARY

1. The relative isometric tension–pCa relationship has been determined for isolated bundles of barnacle myofibrils under a variety of ionic conditions using [$Ca^{2+}$]-buffered solutions which also contained an ATP regenerating system (creatine phosphate and creatine kinase).

2. The results are in better agreement with the '*consecutive*' scheme of reaction rather than with the '*independent*' alternative (Ashley & Moisescu, 1972) for the co-operative action of two $Ca^{2+}$ ions in the process of tension activation in crustacean skeletal muscle.

3. Variations in the pH of the activating solutions did have a marked effect on the relative tension–pCa curve, although no effect was observed on the absolute maximum value for isometric tension. A shift in pH by 0·5 u. in the range 6·6–7·6 shifted the $Ca^{2+}$–activation curve by 0·5 log u. towards lower free $Ca^{2+}$ concentrations.

4. Changes in the free $Mg^{2+}$ concentration of the activating solutions in the millimolar range produced a pronounced shift of the relative tension–pCa curve along the pCa axis. Increasing [$Mg^{2+}$] from 1 to 5 mM shifted the curve by about 0·7 log u. to higher free $Ca^{2+}$ concentrations, without significantly modifying its steepness.

5. Changes in the MgATP concentration of the activating solutions in the range of 1–13 mM had no significant effect on the relative tension–pCa relationship.

6. Varying the $K^+$ concentration in the activating solutions was also

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observed to have a marked effect upon the tension–pCa relationship in barnacle. An increase in the $K^+$ concentration from 90 to 170 mM shifted the curve by some 0·6 log u. towards higher free $Ca^{2+}$ concentrations.

7. Cooling the standard activating solutions from room temperature to +4° C made no apparent difference to the *relative* tension–pCa relationship, but decreased significantly the *absolute* tension responses.

8. The results presented show that tonicity by itself has a marked effect upon the *absolute* steady-state tension levels in isolated bundles of myofibrils.

9. Maximum isometric tension in this preparation was not simply related to ionic strength, or to the monovalent cation concentration, but it depended, as well, upon the *anionic* composition of the activating solution. In addition, a change in ionic strength of 25 mM over the range of 245–270 mM did not appear to modify the *relative* tension–pCa relationship.

10. The effect of the physiologically occurring cations $H^+$, $K^+$, $Mg^{2+}$ upon the relative isometric tension–pCa relationship can be accounted for on the basis of a model of competitive inhibition between these cations and $Ca^{2+}$ for the functional unit for tension. This inhibitory effect appears to involve at least one $H^+$, one $Mg^{2+}$ and two $K^+$ per *each* $Ca^{2+}$ ion participating in the activation process of the functional unit for tension.

## INTRODUCTION

One way to examine the influence of $Ca^{2+}$ ions upon the properties of the contractile machinery is to employ large single muscle fibres which have been micro-injected with a $Ca^{2+}$-sensitive photoprotein such as aequorin, and to examine the simultaneous emission of light and mechanical response upon activation (Ashley & Ridgway, 1970; Ashley & Moisescu, 1972). Another method is to by-pass the membrane depolarization step of excitation–contraction coupling, and activate isolated bundles of myofibrils directly (Natori, 1954) in $Ca^{2+}$-buffered solutions (Hellam & Podolsky, 1969). This procedure has the advantage that one can control readily the ionic environment around the contractile elements.

In the present experiments, the effect of varying the external ionic conditions upon the steady-state relationship between $Ca^{2+}$ and tension in isolated bundles of myofibrils from the barnacle *Balanus nubilus* has been investigated. These experiments have been performed to obtain more information about the nature of the $Ca^{2+}$-dependent reactions for tension in crustacean muscle (Ashley & Moisescu, 1972; Ashley, Moisescu & Rose, 1974).

Preliminary accounts of some of these results have already been published (Ashley & Moisescu, 1973, 1974, 1975).

## METHODS

### *Dissection*

The myofibrils were prepared from single muscle fibres isolated from the lateral depressor muscles of the acorn barnacle *Balanus nubilus* Darwin (Hoyle & Smyth, 1963). The single muscle fibre was blotted to remove excess saline and placed on the bottom of a small Perspex cell. The fibre was covered with liquid paraffin (Heavy grade, BDH Reagent, Poole) and the sarcolemma was removed along its full length by employing a pair of No. 5 jeweller's forceps and fine hypodermic needles. Contraction was occasionally induced by the skinning operation, perhaps by the contamination of the myofibrils with external calcium, or by damaging some of the intracellular stores. These contractures soon disappeared, suggesting that the sarcoplasmic reticulum in this preparation was still active. The smaller bundles of myofibrils were prepared by paring down the thicker bundles obtained initially and this was usually performed by using a dissecting microscope. Alternatively, the fibres were skinned under oil, but surrounded by a droplet of 'relaxing solution' (solution of type B in Table 2) in which $[Ca^{2+}]$ was lower than $10^{-9}$ M. In these 'relaxing solutions', where the EGTA (ethanedioxy*bis*(ethylamine)tetraacetate) was present as $K_2EGTA$ ($K^+ > 90$ mM), a potassium contraction was observed in the pre-skinned, intact fibre. After the fibre relaxed, the bundles of myofibrils were readily prepared. Generally, this technique permitted bundles of 40–200 $\mu$m in diameter to be isolated.

### *Measurement and recording of tension*

The mechanical arrangement of the tension transducer is illustrated in Fig. 1. One end of the myofibrillar preparation was clamped between a pair of stainless steel jeweller's forceps, controlled by a screw clamp. The other end of the preparation was either clamped between the points of a modified pair of forceps (or tied with silk to a stainless steel rod) which were attached directly to the anode peg of an RCA 5734 mechano-electric transducer (see Fig. 1). Both the tension transducer and the unmodified forceps were fixed on the arms of a micromanipulator so that the length of the myofibrillar bundle could be adjusted readily. The micromanipulator was mounted on several layers of rubber foam to reduce spurious mechanical vibrations to a minimum. The circuit for the RCA mechano-electric transducer was standard and needs no detailed description. The output from the bridge circuit went either to a pen recorder (Devices Ltd, Welwyn Garden City or Schwarzer, Gmbh), or to the input stage of a Tektronix storage oscilloscope, where the record could be photographed readily. The sensitivity of the modified RCA transducer was about 300 V/N. Its natural oscillation period was 0·24 sec when it contained the modified forceps, and 0·006 sec when it had the stainless steel rod. The overall compliance was under 2 mm/N. The RCA force-transducer was located in a sturdy brass housing to reduce thermal drift to a minimum.

### *General procedure*

The diameter, and in many instances the average sarcomere spacing of the bundle, were determined under oil before and after clamping. The tension in the preparation was generally less than 10 $\mu$N after clamping, and this corresponds to average sarcomere lengths between 6 and 8 $\mu$m. The cross-sectional area was assessed assuming the bundle was circular. Silicone grease (BDH, Poole) was employed between the tips of both sets of forceps to minimize the amount of solution transferred from one chamber to the next by capillarity.

*Changing of solutions*

The method employed for changing solutions was similar to that of Hellam & Podolsky (1969). The bathing salines were contained in a series of cylindrical wells (volume 5 ml.) drilled out of a circular Perspex block (see Fig. 1). This block was assembled so that it could be rotated horizontally about its axis and was attached to a large adjustable Palmer stand so that it was readily raised or lowered. All solutions could be covered with paraffin oil, and the myofibrillar preparation could be changed from one well to the next without moving through air. This procedure, however, was found to be unnecessary, at least with the size of the preparations

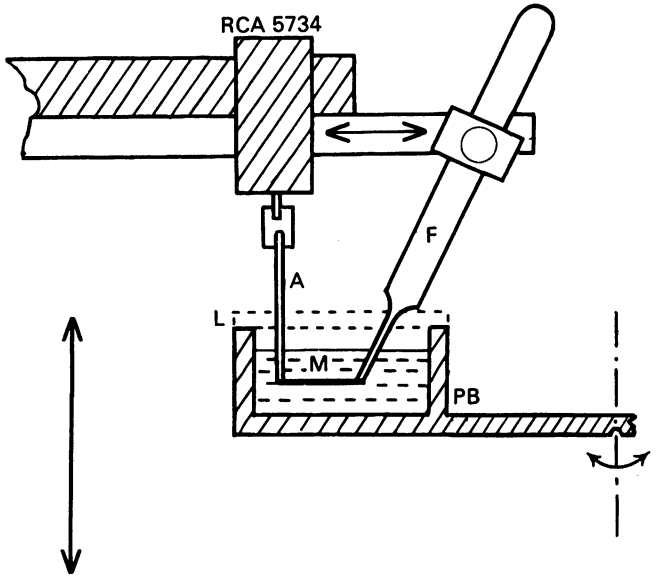


Fig. 1. Diagrammatic representation of the force measurement apparatus. F, jeweller's forceps; A, stainless steel arm attached to the anode peg of the RCA 5734 force transducer; PB, Perspex block with cylindrical chambers containing the solutions; L, Perspex lid; M, myofibrillar preparation. The arrows indicate the directions of movement.

utilized in these particular experiments (see also Ford & Podolsky, 1972). It was observed also that evaporation losses from the wells could be minimized by using a thin Perspex lid which could be rotated. The lid contained an opening through which the myofibrillar preparation could be immersed into the particular solution. When working at lower temperatures (+4° C), the solutions and the air above them were maintained to within ± 1° C.

*Controlling the cationic composition of the solutions*

The concentration of $Mg^{2+}$ and $Ca^{2+}$ was controlled by two chelating systems, one based upon EGTA and the other upon ATP. The steady-state diffusion problems generated by the intrinsic ATPase activity of the myofibrillar bundles (Moisescu, 1976; Moisescu, D. G. & Thieleczek, R., in preparation) have been minimized by using a powerful ATP-regenerative system (creatine phosphate, CP; and creatine

kinase, CK; see Godt, 1974), and high concentrations of efficient [$Ca^{2+}$]- and pH buffers (TES: *N*-tris(hydroxymethyl)-2-aminoethane sulphonic acid; pH 7·5 at 20° C (Good, Winget, Winter, Connolly, Izawa & Singh, 1966)). In order to have solutions with appropriate [$Ca^{2+}$] and [$Mg^{2+}$], the *apparent* association constants of the ligands for all the cations used must be known. The *apparent* stability constants of the ligands for the binding of $Ca^{2+}$ and $Mg^{2+}$ can be calculated from the 'absolute' binding constants (see e.g. Sillén & Martell, 1964, 1970), using a method similar to that employed by Portzehl, Caldwell & Rüegg (1964).

However, it was necessary to measure directly the apparent affinity constants for ATP and EDTA for conditions similar to those used in the bathing solutions, as significantly different values have been quoted in the literature. This has been done by employing a pH metric method (see Miller & Moisescu, 1976; Moisescu, 1976; Moisescu, D. G. & Thieleczek, R., in preparation) and the constants used in the present experiments are listed in Table 1.

It is worth indicating that by mixing only two solutions A and B, one can obtain a set of bathing solutions with practically the same cationic composition, but with different buffered [$Ca^{2+}$]. This procedure simplifies considerably the work involved in preparing the activating salines, and has been used for most of the experiments. Both solutions A and B contain the same concentrations ($C_i$) of: EGTA ($C_1$), ATP ($C_2$), TES ($C_3$), CP ($C_4$), CK, $H^+$, $K^+$, $Na^+$, and $Cl^-$. Solution A has in addition Ca in an equimolar amount with EGTA ($C_1$). The *total* Mg concentration in each solution, $Mg_A$ and $Mg_B$, respectively, is calculated according to the following relations:

$$Mg_B = [Mg]_o + \sum_{i=1}^{4} C_i \cdot \beta_i^0 \cdot (1+\beta_i^0)^{-1}. \quad (1)$$

$$Mg_A = Mg_B - C_1 \cdot \beta_1^0 \cdot (1+\beta_1^0)^{-1}. \quad (2)$$

Here $[Mg]_o$ represents the *desired* free $Mg^{2+}$ level in the solutions, and $\beta_i^0 = K_{Mg}^{X_i} \cdot [Mg]_o$ ($i = 1, \ldots, 4$) where $K_{Mg}^{X_i}$ is the *apparent* affinity constant of the ligand $X_i$ ($X_1$ = EGTA; $X_2$ = ATP; $X_3$ = CP; $X_4$ = TES) for $Mg^{2+}$. If $a$ represents the fraction of solution A and $(1-a)$ the fraction of solution B in an intermediary solution, then the *actual* concentrations of free Ca, [$Ca^{2+}$], and free Mg, [$Mg^{2+}$], are obtained by solving the following two equations,

$$\sum_{i=1}^{4} C_i \cdot \alpha_i (1+\alpha_i+\beta_i)^{-1} + [Ca^{2+}] = a \cdot C_1. \quad (3)$$

$$\sum_{i=1}^{4} C_i \cdot \beta_i (1+\alpha_i+\beta_i)^{-1} + [Mg^{2+}] = a \cdot Mg_A + (1-a) \cdot Mg_B. \quad (4)$$

Here $\alpha_i = K_{Ca}^{X_i} \cdot [Ca^{2+}]$ and $\beta_i = K_{Mg}^{X_i} \cdot [Mg^{2+}]$, where $K_{Ca}^{X_i}$ is the *apparent* affinity constant of the ligand $X_i$ for $Ca^{2+}$.

If $\Delta$ is the relative error between the *actual* and the *desired* $Mg^{2+}$ level ($[Mg^{2+}] = [Mg]_o \cdot (1+\Delta)$), then by replacing eqns. (1) and (2) in (4), and eliminating $a$ between eqns. (3) and (4), one obtains the following relationship:

$$\Delta = \frac{(1+\beta_1^0)^{-1} \cdot \left\{ \sum_{i=2}^{4} C_i \cdot \alpha_i \cdot (\beta_i^0 - \beta_1^0) \cdot (1+\beta_1^0)^{-1} \cdot [1+\alpha_i+\beta_i^0 \cdot (1+\Delta)]^{-1} - \beta_1^0 \cdot [Ca^{2+}] \right\}}{[Mg]_0 + \sum_{i=1}^{4} C_i \cdot \beta_i^0 \cdot [1+\alpha_i+\beta_i^0 (1+\Delta)]^{-1} \cdot (1+\beta_i^0)^{-1}}. \quad (5)$$

Based on eqn. (5), it can be shown that the actual free $Mg^{2+}$ concentration should not differ in any mixture solution used in the present experiments by more than 3 % or 4 $\mu$M (whichever value is the larger) from the desired level, $[Mg]_0$. This error is negligible, and justifies the use of this simple procedure.

TABLE 1. Apparent affinity constants ($K^{app.}$) of $Ca^{2+}$ and $Mg^{2+}$ to the ligands used in these experiments

| Ligand | Cation | $K^{app.}$ ($M^{-1}$) | Experimental conditions: $K^+$ (mM) | $Na^+$ (mM) | pH | Temp. (°C) | Observations |
|---|---|---|---|---|---|---|---|
| EGTA | $Ca^{2+}$ | $(6 \pm 0.5)\ 10^{6}$* | 50–200 | 20–50 | 7·10 | $22 \pm 2$ | Apparent affinity constants measured* at pH 5·7–5·8 in the presence of TES (0–250 mM) and extrapolated to the respective pH according to Portzehl *et al.* (1964). These values are slightly lower than those calculated using the results of Schwarzenbach (1960) |
| | | $(6 \pm 0.5)\ 10^{5}$* | 50–200 | 20–50 | 6·60 | $22 \pm 2$ | |
| | | $(5.6 \pm 0.5)\ 10^{7}$* | 50–200 | 20–50 | 7·60 | $22 \pm 2$ | |
| | | $(5 \pm 0.5)\ 10^{6}$* | 50–200 | 20–50 | 7·10 | $2 \pm 1$ | |
| | $Mg^{2+}$ | $46 \pm 6$* | 50–200 | 20–50 | 7·10 | $22 \pm 2$ | Agreement with the value calculated from Schwarzenbach (1960) |
| | | $25 \pm 5$* | 50–200 | 20–50 | 7·10 | $2 \pm 1$ | |
| | | 14·4 | 90 | 30 | 6·60 | 20 | Values calculated according to Portzehl *et al.* (1964) |
| | | 244 | 90 | 30 | 7·60 | 20 | |
| ATP | $Mg^{2+}$ | $7500 \pm 500$* | 90 | 30 | 7·10 | $22 \pm 2$ | Agreement with the results indicating an 'absolute' affinity constant of $Mg^{2+}$ and $K^+$ to $ATP^{4-}$ of *ca.* $(1.5–2.0) \times 10^4$ $M^{-1}$ and 14 $M^{-1}$, respectively (see Sillén & Martell, 1964, 1970). Extrapolated values assuming an 'absolute' affinity constant of $H^+$ and $K^+$ to $ATP^{4-}$ of $8.9 \times 10^6$ $M^{-1}$ (Smith & Alberty, 1956) and 14 $M^{-1}$ (O'Sullivan & Perrin, 1964), respectively. |
| | | $4800 \pm 400$* | 170 | 30 | 7·10 | $22 \pm 2$ | |
| | | $6300 \pm 400$* | 90 | 30 | 7·10 | $2 \pm 1$ | |
| | | $4600 \pm 300$ | 90 | 30 | 6·60 | $22 \pm 2$ | |
| | | $9500 \pm 600$ | 90 | 30 | 7·60 | $22 \pm 2$ | |
| | $Ca^{2+}$ | $2500 \pm 300$* | 170 | 30 | 7·10 | $22 \pm 2$ | Corresponds to an 'absolute' affinity constant of $Ca^{2+}$ to $ATP^{4-}$ of *ca.* 8000–14000 $M^{-1}$ (see Sillén & Martell, 1964, 1970) |
| | | $3900 \pm 300$† | 90 | 30 | 7·10 | $22 \pm 2$ | |
| | | $3280 \pm 200$† | 90 | 30 | 7·10 | $2 \pm 1$ | |
| | | $2400 \pm 200$† | 90 | 30 | 6·60 | $22 \pm 1$ | |
| | | $4950 \pm 300$† | 90 | 30 | 7·60 | $22 \pm 1$ | |
| CP | $Mg^{2+}$ | 12 | 90–170 | 30 | 6–8 | 1–22 | Values measured* for 1° C and 130 mM $K^+$ (Moisescu, 1976) and extrapolated for the range indicated |
| | $Ca^{2+}$ | $\leqslant 20$ | 90–170 | 30 | 6–8 | 1–22 | |
| TES | $Mg^{2+}$ | $\leqslant 1$ | 90–170 | 30 | 6–8 | 1–22 | Agreement with Good *et al.* (1966) |
| | $Ca^{2+}$ | $\leqslant 1$ | 90–170 | 30 | 6–8 | 1–22 | |

* Determinations made using a pH-metric method (Moisescu & Thieleczek, in preparation; Moisescu, 1976; Miller & Moisescu, 1976).

† Values obtained by dividing $K^{app.}$ for $Mg^{2+}$ for identical conditions by the ratio between the measured affinity constants for $Mg^{2+}$ and $Ca^{2+}$ in the presence of 170 mM $K^+$.

The *actual* free $Ca^{2+}$ level, $[Ca^{2+}]$, has been calculated for each particular solution by solving eqn. (3), with a relative error of less than 0·1 %.

*Estimate of the concentrations of Ca and EGTA*

It is important to know *very accurately* the ratio between the total EGTA and the total $Ca^{2+}$ concentration in the solutions of type A. Otherwise, an error of even 2–3 % would result in a very large inaccuracy in the estimation of the free $Ca^{2+}$ concentration, particularly in a mixture containing only a small fraction of solution B. Two methods were used to assess the relative concentrations of $Ca^{2+}$ to EGTA. The first was described by Keynes & Lewis (1956), and is based on the use of the $Ca^{2+}$ indicator, murexide (ammonium purpurate). This enabled the ratio of $Ca^{2+}$ to EGTA to be determined within 1–2 %. The reaction of $Ca^{2+}$ with murexide is little affected by $Mg^{2+}$ at pH 11, since the ratio of the $Mg^{2+}$ binding constant to murexide, compared to that for $Ca^{2+}$, is *ca.* $10^{-6}$. A more direct method, which is based on the release of $H^+$ following the binding of $Ca^{2+}$ to EGTA was also employed. The titration was performed at pH > 7·5 in the presence of a strong pH buffer, which does not interact strongly with $Ca^{2+}$ (Moisescu & Pusch, 1975). This latter method is more accurate than the former, and has the advantage that it enables one to determine very precisely the value of $a$ (eqn. (3)) for any experimental solution. All activating solutions were titrated at the end of an experiment with $CaCl_2$ in the presence of a relatively high $[Mg^{2+}]$ so that ATP was practically present as MgATP. The final pH was always higher than 7·5, and this enabled a precise estimate to be made of the amount of calcium-free EGTA, i.e. $(1-a)\cdot C_1$. Based on this value, one can determine $a$, and consequently the actual free $Ca^{2+}$ concentration from eqn. (3).

*General*

The stock solutions, A and B, were prepared 24 hr in advance, except for the presence of ATP, CP and CK, and were stored at 0–+5° C. Before carrying out the experiment, the solutions were removed from the store and brought to the working temperature. Then, ATP and CP were added as solids and the pH of the solutions was adjusted to the desired value with known amounts of KOH. Only at this point was CK added, and finally A and B were mixed in the appropriate proportions. If the total [Ca] in solution A did not exceed [EGTA], then the pH values of the whole set of solutions did not differ by more than 0·01 pH u.

Usually $Ca^{2+}$ was added to solution A as CaEGTA, and this was prepared from equimolar amounts of EGTA and $CaCO_3$ in water (final pH $\approx$ 4) aided by gentle heating (10–15 min at *ca.* 80° C) to displace the equilibrium of $H_2CO_3$ reaction towards $CO_2$ and $H_2O$.

The pH of all solutions was always assessed carefully before and after each experiment, and did not vary by more than $\pm$ 0·02 pH u. Calibration of the pH-meter and electrode against standard buffer solutions was performed at regular intervals, as shifts in the basic values occurred, brought about by both EGTA and TES in the experimental solutions.

The standard solutions have been chosen to have (mM): ATP, 5; CP, 10; CK 20 u./ml., $K^+ + Na^+ \approx 120$; EGTA, 30; TES, 60 (pH 7·10); caffeine 20; free $Mg^{2+} <$ 0·05, temp. 20° C (see Table 2).

## RESULTS

The experimental traces presented in Fig. 2 are typical of those from which the isometric tension–pCa curves are constructed. The myofibrillar bundle was incubated first for at least 10 min in a relaxing solution of

TABLE 2. Composition of the solutions used* for determining the relationship between the steady-state isometric tension response and pCa

| Solution | | $K^+$ (mM) | $Na^+$ (mM) | pH | $TES_t$ (mM) | $ATP_t$ (mM) | $Mg_t$ (mM) | CP (mM) | CK (u/ml.) | EGTA (mM) | Ca-EGTA (mM) | MgATP (mM) | $Mg^{2+}$ (mM) | $Cl^-$ (mM) | I/2 (mM) | Caffeine (mM) | Temp. (°C) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | A | 88–90 | 30 | 7·10 | 60 | 5 | 1·055 | 10 | 20 | — | 20 | 1† | 0·034† | 22·1 | *ca.* 165† | 20 | 20 |
| | B | 88–90 | 30 | 7·10 | 60 | 5 | 1·085 | 10 | 20 | 20 | — | | | 22·17 | | 20 | 20 |
| S2 | A | 88–90 | 30 | 7·10 | 60 | 5 | 1·055 | 10 | 20 | — | 20 | ≈1† | 0·04† | 29–30 | *ca.* 165† | 20 | 4 |
| | B | 88–90 | 30 | 7·10 | 60 | 5 | 1·085 | 10 | 20 | 20 | — | | | 29–30 | | 20 | 4 |
| S3 | A | 88–90 | 30 | 7·10 | 60 | 5 | 5·59 | 10 | 20 | — | 30 | 4·41† | 1† | 13 | *ca.* 165† | 20 | 20 |
| | B | 88–90 | 30 | 7·10 | 60 | 5 | 6·90 | 10 | 20 | 30 | — | | | 13·8 | | 20 | 20 |
| S4 | A | 88–90 | 30 | 7·10 | 60 | 5 | 10·76 | 10 | 20 | — | 25 | 4·87† | 5† | 31 | *ca.* 170† | 20 | 20 |
| | B | 88–90 | 30 | 7·10 | 60 | 5 | 15·43 | 10 | 20 | 25 | — | | | 35 | | 20 | 20 |
| S5 | A | 88–90 | 30 | 6·60 | 100 | 5 | 1·066 | 10 | 20 | — | 30 | 1† | 0·054† | 8·13 | *ca.* 170† | 20 | 20 |
| | B | 88–90 | 30 | 6·60 | 100 | 5 | 1·088 | 10 | 20 | 30 | — | | | 8·17 | | 20 | 20 |
| S6 | A | 88–90 | 30 | 7·10 | 60 | 5 | 1·055 | 10 | 20 | — | 30 | 1† | 0·034† | 2·1 | *ca.* 175† | 20 | 20 |
| | B | 88–90 | 30 | 7·10 | 60 | 5 | 1·1 | 10 | 20 | 30 | — | | | 2·2 | | 20 | 20 |
| S7 | A | 88–90 | 30 | 7·60 | 30 | 5 | 1·03 | 10 | 20 | — | 30 | 1† | 0·026† | 2·1 | *ca.* 170† | 20 | 20 |
| | B | 88–90 | 30 | 7·60 | 30 | 5 | 1·22 | 10 | 20 | 30 | — | | | 2·45 | | 20 | 20 |
| S8 | A | 168–173 | 30 | 7·10 | 60 | 5 | 1·061 | 10 | 20 | — | 30 | 1† | 0·052† | 82·2 | *ca.* 245† | 20 | 20 |
| | B | 168–173 | 30 | 7·10 | 60 | 5 | 1·13 | 10 | 20 | 30 | — | | | 82·3 | | 20 | 20 |
| S9 | A | 168–173 | 30 | 7·10 | 60 | 15 | 2·034 | — | — | — | 30 | 2† | 0·032† | 70·1 | *ca.* 270† | — | 20 |
| | B | 168–173 | 30 | 7·10 | 60 | 15 | 2·078 | — | — | 30 | — | | | 70·2 | | — | 20 |
| S10 | A | 168–173 | 30 | 7·10 | 60 | 15 | 13·59 | — | — | — | 30 | 12·41† | 1† | 90·2 | *ca.* 245† | — | 20 |
| | B | 168–173 | 30 | 7·10 | 60 | 15 | 14·91 | — | — | 30 | — | | | 92 | | — | 20 |

* All substances were of reagent grade.

† These values refer to a mixture solution from those of type A and B. The values are affected by experimental errors and were calculated based on the apparent affinity constants mentioned in Table 1. The affinity constants of $8 \times 10^6$ $M^{-1}$ and 10 $M^{-1}$ were also used as mean values for $H^+$ and the monovalent cations ($Na^+$ and $K^+$) in order to calculate I/2 (Sillén & Martell, 1964, 1970).

The subscript t refers to *total* amounts in the solutions.

TABLE 3. Composition of the solutions used* for determining the dependency of the maximum $Ca^{2+}$-activated tension response upon ionic strength and tonicity

| Solution | $K^+$ (mM) | $Na^+$ (mM) | pH | $TES_t$ (mM) | $ATP_t$ (mM) | $MgCl_2$ (mM) | $CaCl_2$ (mM) | CaATP (mM) | MgATP (mM) | $Ca^{2+}$ (mM) | $Mg^{2+}$ (mM) | Γ/2 (mM) | Sucrose (mM) | Tonicity (mM) | Temp. (°C) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S11 | 46–47 | 30 | 7·60 | 30 | 15 | 18 | 1·7 | 1·04 | 13·63 | 0·62† | 4·1† | *ca.* 107 | — | *ca.* 165 | 20 |
| S12 | 63–64 | 30 | 7·60 | 60 | 15 | 5 | 0·5 | ≈0·5 | ≈5 | 0·01† | 0·05† | *ca.* 132 | 360 | *ca.* 530 | 20 |
| S13 | 108–110 | 30 | 7·60 | 60 | 15 | 5 | 0·5 | ≈0·5 | ≈5 | 0·012† | 0·06† | *ca.* 175 | 272 | *ca.* 530 | 20 |
| S14 | 109–112 | 30 | 7·60 | 60 | 27 | 5 | 0·5 | ≈0·5 | ≈5 | 0·007 | 0·035 | *ca.* 225 | 300 | *ca.* 526 | 20 |
| S15 | 240 | 30 | 7·10 | 60 | 5 | 1 | 0·5 | ≈0·5 | ≈1 | *ca.* 0·06 | *ca.* 0·06 | (see ‡) | | | 20 |

* All substances were of reagent grade.

† The addition of KCl (see Fig. 7) results in a decrease of the apparent affinity constant of ATP for $Ca^{2+}$ and $Mg^{2+}$, and subsequently in a slight increase of free $Mg^{2+}$ and free $Ca^{2+}$ concentrations.

‡ Solution S15 contained, in addition 10 mM-CP and 20 mM caffeine. Apart from TES, the anions were either $Cl^-$ (214 mM), proprionate (214 mM), or sulphate (107 mM) (see also Fig. 8). The version of solution S15 with sulphate contained, in addition, 107 mM-sucrose.

type B, and was then activated in solutions with a higher $Ca^{2+}$ concentration. The solutions were changed only after the tension had reached a steady-state level. The maximum tension developed by the preparation did not decrease significantly during the present experiment, and this permitted an accurate estimate to be made of the relative tension developed at each pCa value. The maximum tension developed in these experiments can be deduced from the trace in Fig. 2, and corresponds to *ca.* 34 N/cm², cf. 30–50 N/cm² for intact fibres *in vivo* (Hoyle & Smyth, 1963). In other

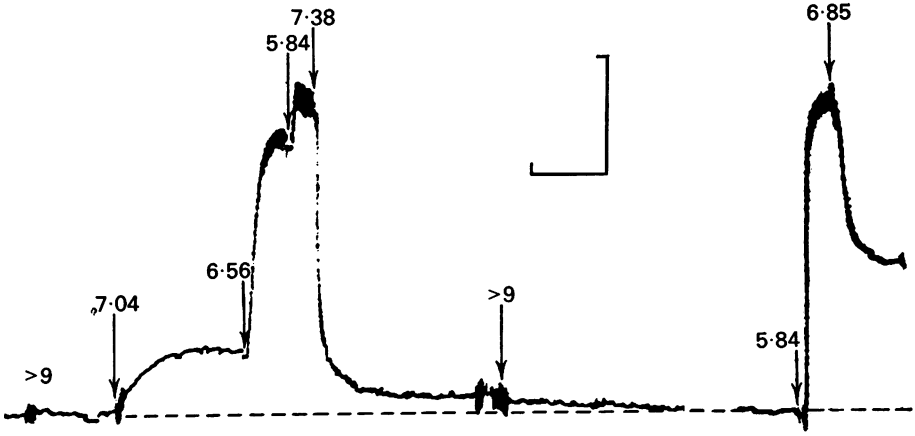


Fig. 2. Typical isometric recordings from a barnacle myofibrillar bundle activated in a set of solutions (S 7, Table 2) with different free $Ca^{2+}$ concentrations. The arrows indicate the moment when the bathing solutions were changed, and the associated numbers are the corresponding pCa values. Diameter 45 $\mu$m; average sarcomere length *ca.* 6 $\mu$m; preparation length 1·5 mm; temperature + 20° C. Calibration bars: vertical 0·2 mN, horizontal 60 sec. The dotted line represents the tension level in the relaxing solution. Note that the max. tension response did not change significantly between these two cycles of contraction.

preparations, the maximum tension did show a tendency to decrease during the cycles of contractions (by some 5–10 % from one maximal contracture to the next), and in this case an interpolation method similar to that described by Julian (1971) was employed, in order to estimate the relative tension values. The vertical bars associated with all the experimental points indicate the range over which the relative tension values are spread. The experimental points for each curve are average values from more preparations, all from the *same* single fibre, in order to reduce the scatter. An increase of the activity of CK from 20 to 40 u./ml., or higher, did not produce any change in the results.

*The influence of [$Mg^{2+}$] and temperature upon the relative isometric tension–pCa relationship*

The effect of three different $Mg^{2+}$ concentrations upon the steady-state isometric tension- pCa relationship for barnacle myofibrils is illustrated in Fig. 3. The free $Mg^{2+}$ and $Ca^{2+}$ concentrations were controlled in the manner described in the Methods under conditions that the ionic strength

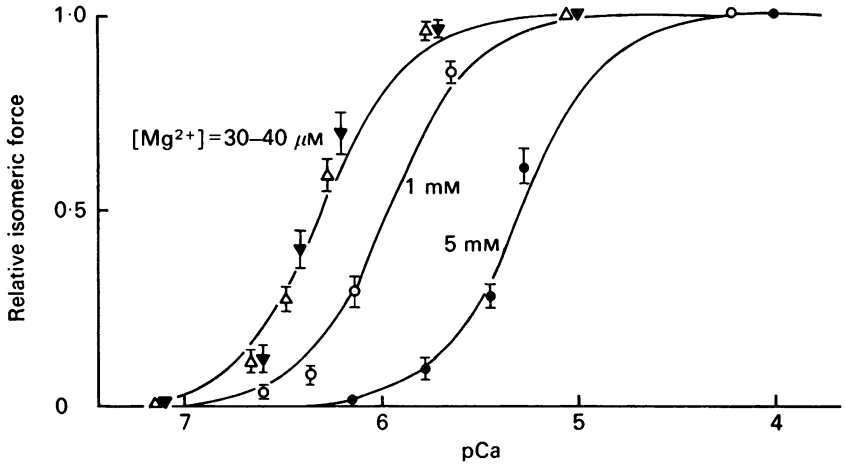


Fig. 3. Relative isometric tension–pCa relationships in barnacle myofibrillar preparations in four sets of solutions (S1 for (△), S2 for (▼), S3 for (○), S4 for (●); see Table 2) having essentially the same ionic strength and monovalent cation concentrations. Free $Mg^{2+}$ concentration: 30 μM for (△), 40 μM for (▼), 1 mM for (○) and 5 mM for (●). Temperature: 20 ± 1° C for (△), (○), (●) and 4 ± 1° C for (▼). The max. absolute tension is not significantly affected by the variation in [$Mg^{2+}$] (△, ○, ●) and in the corresponding [MgATP] (see Table 2). However, the drop in temperature from 20° C (△) to 4° C (▼) resulted in a diminished (less than 50 %) absolute tension response. The continuous lines are theoretical predictions from eqn. (9) for $K_1^{app} \ll K_2^{app}$ and $K_1^{app} \cdot K_2^{app}$. $4{\cdot}2 \times 10^{12}$ M$^{-2}$ for (△), (▼); $8{\cdot}6 \times 10^{11}$ M$^{-2}$ for (○); and $4 \times 10^{10}$ M$^{-2}$ for (●).

and $K^+$ concentration (see later) were essentially constant for all three curves (see Table 2). The main effect of increasing [$Mg^{2+}$] is to shift the curve relating isometric tension to pCa along the pCa axis towards higher [$Ca^{2+}$]. This effect is unlikely to be due to an increase in the concentration of MgATP since for 1 mM-[$Mg^{2+}$] and 5 mM-[$Mg^{2+}$], the MgATP concentration has increased only slightly from *ca.* 4·4 to *ca.* 4·8 mM, respectively, while the curves have been shifted by some 0·66 log u. In contrast to this, the curve for 1 mM-[$Mg^{2+}$] was shifted by only 0·3 pCa u. in comparison

with the curve for 34 $\mu$M, and here the MgATP concentration has changed from *ca.* 4·4 to *ca.* 1 mM, respectively. The same conclusion, namely that the shift is brought about chiefly by the change in $Mg^{2+}$ concentration, rather than by the change in [MgATP] is suggested by the results presented in Fig. 4. Here, the change in $[Mg^{2+}]$ from *ca.* 30 $\mu$M to 1 mM (at a constant monovalent cation concentration; see Table 2) produces a shift in the tension–pCa curves of an amount similar to that observed in Fig. 3 (curves with open triangle and open circle), although there was a much

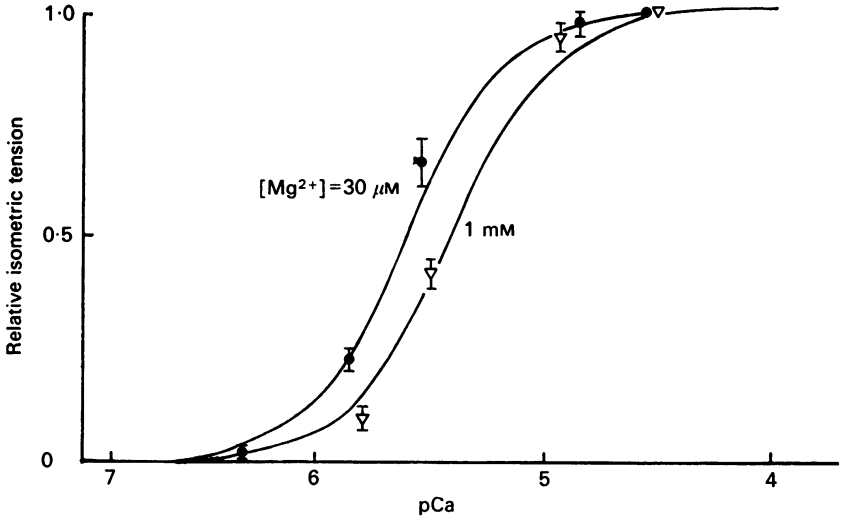


Fig. 4. The effect of $[Mg^{2+}]$ on the relative isometric tension–pCa relationship from barnacle myofibrils activated in solutions in which the ATP regenerating system (5 mM-ATP, 10 mM-CP, 20 u./ml. CK) was substituted by 15 mM-ATP. Free $Mg^{2+}$ concentrations: *ca.* 30 $\mu$M for ● (solutions S9); 1 mM for ▽ (solutions S10). Temp. 20 ± 1° C. The continuous lines are theoretical predictions from eqn. (9) for $K_1^{app.} \ll K_2^{app.}$ and $K_1^{app.} \cdot K_2^{app.}$: $1{\cdot}46 \times 10^{11}$ M$^{-2}$ for (●), and $6{\cdot}8 \times 10^{10}$ M$^{-2}$ for (▽). Note that the curves are shifted in comparison with those in Fig. 3, due to a higher $[K^+]$ in the solutions S9 and S10 than in S3 and S1 (see Fig. 6).

larger increase in [MgATP] (from *ca.* 2 mM to 13 mM) compared with that corresponding to the curves in Fig. 3. Other experiments performed in the absence of an ATP regenerative system (Ashley & Moisescu, 1974) also indicated that an increase in [MgATP] from 1 to 5 mM did not affect significantly the relative tension–pCa relationship for this preparation.

The effect of temperature on the relationship between relative isometric tension and $[Ca^{2+}]$ has been investigated by cooling the set of standard solutions to 4 ± 1° C (see Methods and Table 2). After lowering the temperature, the pH was re-adjusted with HCl to 7·10 ± 0·01, so that

no significant change in the ionic strength, [$Mg^{2+}$], or [MgATP] occurred. There was, however, a slightly higher [$Ca^{2+}$] in comparison with that at room temperature, since the apparent binding constant of $Ca^{2+}$ to EGTA appears to decrease by 20% (Moisescu, 1976). The results from this series of tension responses performed at $4 \pm 1°$ C are also included in Fig. 3 (see legend), and seem to overlap those obtained at 20° C. This indicates that the apparent affinity of the tension sites for the $Ca^{2+}$ ions is not significantly affected by changes in temperature over this range. In contrast to the *relative* tension, the *absolute* tension response at 4° C was only 50% (or lower) of that at room temperature.

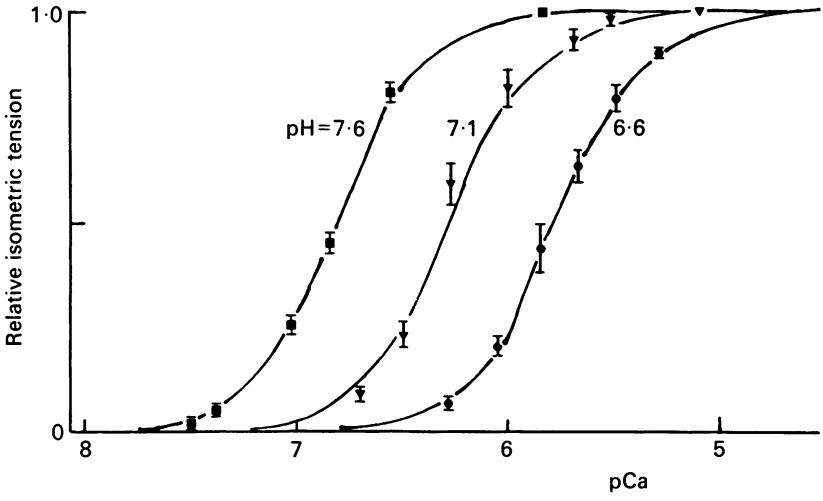


Fig. 5. The influence of pH on the relative isometric tension–pCa relationship in barnacle myofibrillar preparations. The sets of activating solutions (S5, S6, S7 in Table 2) had the following pH values, respectively: $6{\cdot}60 \pm 0{\cdot}01$ for (●), $7{\cdot}10 \pm 0{\cdot}01$ for (▼), and $7{\cdot}60 \pm 0{\cdot}01$ for (■). Temp. $20 \pm 1°$ C. The continuous curves are theoretical predictions from eqn. (9) for $K_1^{app.} \ll K_2^{app.}$ and $K_1^{app.} \cdot K_2^{app.}$: $4{\cdot}2 \times 10^{13}$ M$^{-2}$ for (■); $4 \times 10^{12}$ M$^{-2}$ for (▼); and $4{\cdot}2 \times 10^{11}$ M$^{-2}$ for (●).

*The influence of changing pH upon the isometric tension–pCa relationship*

The effect of varying the pH between 6·6 and 7·6 on the relationship between tension and pCa is illustrated in Fig. 5, where the composition of the activating solutions is indicated in Table 2. The results show that a change of pH has a marked effect on the curves; a change of 0·5 pH u. produces a shift in the curve by *ca.* 0·5 log u., the relationship becoming less sensitive to $Ca^{2+}$ as the $H^+$ concentration increased. The absolute max. values for tension were not, however, affected by changes in pH over this range, and results similar to these have been described previously with

this preparation under conditions where an ATP regenerating system was not present in the bathing solutions (Ashley & Moisescu, 1974).

*The effect of* $[K^+]$ *on the isometric tension–pCa relationship*

It was observed that changes in the $K^+$ concentration in the activating solutions over the range of 90–170 mM, at an essentially constant concentration of MgATP and $Mg^{2+}$, had a marked effect on the *relative* tension–pCa relationship. An increase in $[K^+]$ produced a shift in the relative isometric tension response towards higher $[Ca^{2+}]$ (*ca.* 0·6 log u. over the

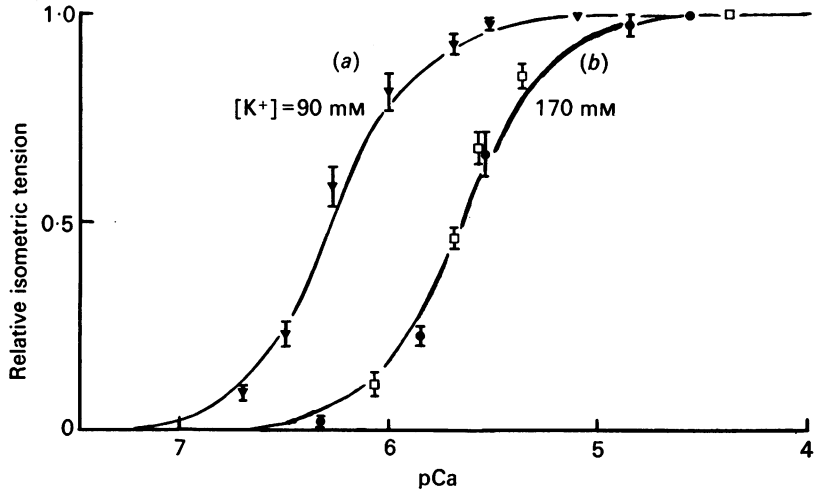


Fig. 6. The effect of increasing $[K^+]$ in the activating solutions from 90 mM (▼, S6) to 170 mM (□, S8; ●, S9) on the relationship between relative isometric tension response and pCa in barnacle myofibrils. Temp. 20 ± 1° C. The max. force response for (▼) was about 30 % larger than for (□), and *ca.* 40 % higher than for (●). The ionic strength for (●) was *ca.* 25 mM higher than for (□). The continuous lines are theoretical predictions from eqn. (9) for $K_1^{app} \ll K_2^{app.}$ and $K_1^{app.} \cdot K_2^{app.}$: $4{\cdot}2 \times 10^{12}$ M$^{-2}$ for (▼) and $2{\cdot}1 \times 10^{11}$ M$^{-2}$ for (□) and (●).

above-mentioned range of $[K^+]$; see Fig. 6). The replacement of the ATP-regenerating system with 10 mM-ATP, at low $[Mg^{2+}]$ and constant $[K^+]$, did not produce a significant change in the relationship (see Fig. 6*b*), although the ionic strength and osmolarity were appreciably changed (see Table 2). This latter observation indicates also that a change in the ionic strength of *ca.* 25 mM has no significant effect in *itself* on the relative tension–pCa relation in barnacle, and that the shift observed in Fig. 6 is due mainly to a specific effect of $K^+$ ions. As judged by the similarity of the two sets of results in Fig. 6*b*, it appears that the presence of 15 mM-

ATP in the activating solutions is as efficient as an ATP regenerating system (5 mM-ATP + 10 mM-(CP + CK)) in this preparation.

A similar specific effect of monovalent metallic cations on $Ca^{2+}$-activated systems has been observed with the photoprotein aequorin (Moisescu, Ashley & Campbell, 1975; Moisescu & Ashley, 1977).

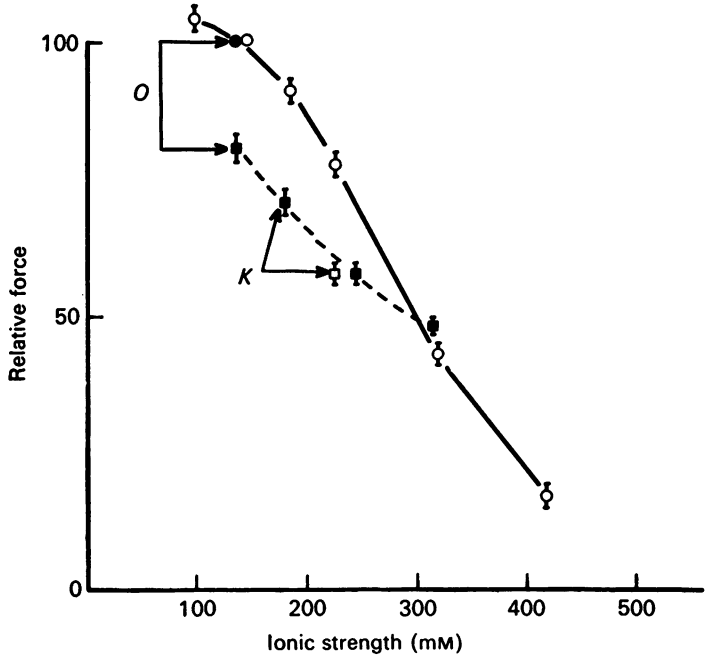


Fig. 7. The effect of adding KCl to the solution S11 (Table 3) on the max. isometric tension response from barnacle myofibrils (○). The dashed line (■) shows only the effect of increasing the ionic strength on the maximal tension response while maintaining the same tonicity in the activating solutions, by replacing iso-osmotically the sucrose in S12 (Table 3) by KCl. The two experimental points, indicated by the double arrow *O*, were obtained in solution S12 and in an otherwise identical solution which did not contain sucrose. The experimental points indicated by the double arrow *K* were obtained in solutions S13 and S14, respectively, which had the same concentrations of monovalent cations but different ionic strengths (see Table 3). We have chosen a pH value of 7·60 and a low $Mg^{2+}$ concentration for the solutions here in order to obtain maximal activation even for higher $K^+$ concentrations (see Figs. 3–6). The 100% relative tension corresponds to an ionic strength of *ca.* 140 mM. Temp. 20 ± 1° C.

*The influence of ionic strength, tonicity and anionic species on isometric tension*

As has been mentioned above, neither tonicity nor ionic strength *per se* appear to affect significantly the *relative* isometric tension–pCa relation-

ships, at least over the range of values investigated (see Table 2). However, an increase in tonicity or ionic concentrations has a marked effect on the *absolute* tension values. This is shown in Fig. 7, which illustrates the effect of increasing KCl concentration and tonicity on the maximal $Ca^{2+}$-activated tension response for the barnacle myofibrillar preparation. The experimental results presented in Fig. 7 do indeed represent max. tension values at the appropriate ionic strengths and tonicities, since an increase of $[Ca^{2+}]$ in the activating solutions did not produce any further increase in tension. The open circles (○) in Fig. 7 indicate the fall in the max. tension recorded for two preparations as the concentration of KCl in the activating solutions is increased, while maintaining a practically constant [MgATP] and $[Mg^{2+}]$. This decrease in max. tension, observed also by other workers in this field (see, e.g. April & Brandt, 1973; Gordon, Godt, Donaldson & Harris, 1973) is, however, only partly due to the increase in the ionic strength brought about by the increase in KCl (Moisescu, 1973). An increase in the osmolarity of the activating solutions by 320 m-osmole/l. with added sucrose, also produced a decline in max. tension response by about 25% (see also Moisescu, 1973). This is illustrated in Fig. 7 by the experimental points, indicated by the double arrow marked with *O*. If the tonicity of the activating solutions was maintained practically constant by replacing iso-osmotically the sucrose with KCl, then the effect of the ionic strength alone (dotted line in Fig. 7) appears to be less marked than had been initially expected from the continuous line in Fig. 7.

It was important to know if the decline in max. tension was simply associated with the increased ionic strength *per se*, or whether it was, in addition, dependent on the ionic species in the medium. In order to answer this question, the same bundles of myofibrils were activated in different solutions having essentially the same osmolarity, $Mg^{2+}$, MgATP and monovalent cation concentration, but different anionic species.

If one replaces, for example, 48 mM-$Cl^-$ with $ATP^{4-}$ (solutions S13 and S14, Table 3), a significantly lower tension response is obtained, and this is illustrated in Fig. 7 by the points marked with *K* double arrow. Initially, it seemed that the decline in max. tension could be explained simply by the higher ionic strength in S14, since both points lie on the hatched line in Fig. 8. Subsequent experiments suggested that this decline may at least partly be attributable to a specific influence of $ATP^{4-}$, the only ionic component that was changed significantly. A similar inhibitory effect of $ATP^{4-}$ on max. tension levels has also been observed on glycerinated muscle preparations (Rüegg, J. C., personal communication).

The composition of the three different versions of solution S15 (Table 3) differ only in the concentration of *anions* and/or *ionic strength*. A typical experimental result is presented in Fig. 8, where the replacement of $Cl^-$

(C) with proprionate (P) results in *ca.* 35 % increase in tension, although the ionic strength and the concentration of other components remained essentially constant. The replacement of $Cl^-$ (C) with $SO_4^{2-}$ (S), however, did not result in a significant change in tension, although in this case the ionic strength of the two solutions was presumably different.

Direct measurements with an osmometer indicated that the tonicity of 200 mM-$K_2SO_4$ solution was only 60–70 % of that of a 300 mM-KCl solution. This observation suggests that $K_2SO_4$ is not dissociated solely as $2K^+ + SO_4^{2-}$ (see also Sillén & Martell, 1970). Thus, the specific effect of $KSO_4^-$ on max. tension may be even greater than that of $Cl^-$.

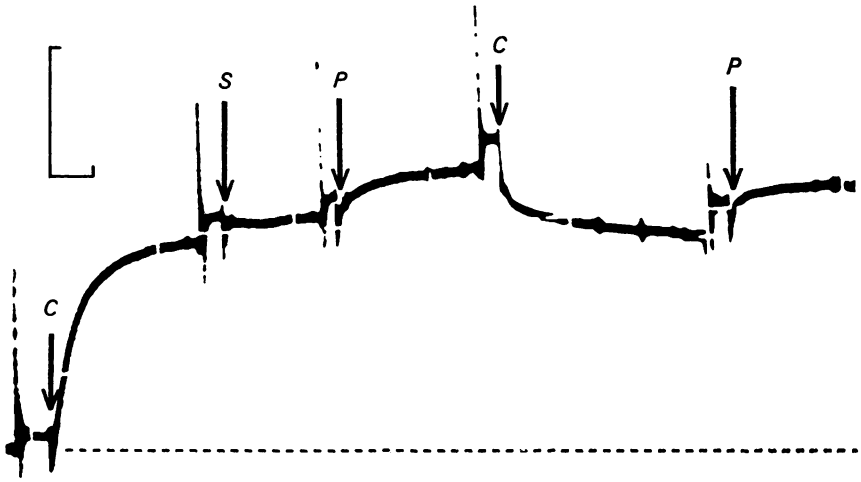


Fig. 8. Tension development in a bundle of barnacle myofibrils maximally activated in calcium-containing solutions (S15, Table 3). Prior to the activating step, the preparation had been equilibrated in a solution similar to S15 which contained instead of 0·5 mM-$CaCl_2$, 0·1 mM EGTA and $Cl^-$ as the main anion. The arrows indicate the moment the bathing solutions were changed and the letters *C*, *P*, and *S* refer to the solutions of type S15 containing either chloride, proprionate or sulphate respectively. Segment diameter: 30 $\mu$m, length 1·7 mm; temp.: *ca.* 22° C. Calibration bars: vertical 0·1 mN, horizontal 5 sec. The hatched line represents the level of tension in the relaxing solution.

These results suggest that the max. tension response in barnacle muscle is a complex function of tonicity, ionic strength and ionic species. A similar effect of anions was observed on skinned frog fibres, although the osmolarity of the solutions was not always balanced (Gordon *et al.* 1973).

## DISCUSSION

One of the main aims of this present series of experiments was to investigate, in some detail, the dependence of relative isometric tension in barnacle myofibrils on free $Ca^{2+}$ concentration over a wider range of ionic conditions than is possible to achieve satisfactorily in intact muscle fibres. The results obtained have enabled a distinction to be made between the two alternative schemes for the co-operative action of the two $Ca^{2+}$ ions involved in the functional unit for tension in barnacle, which were deduced initially from the transient state experiments employing aequorin in intact muscle fibres (Ashley & Moisescu, 1972).

The steady-state isometric tension results presented in this paper all show a sigmoidal relationship with free $Ca^{2+}$ concentration, when plotted

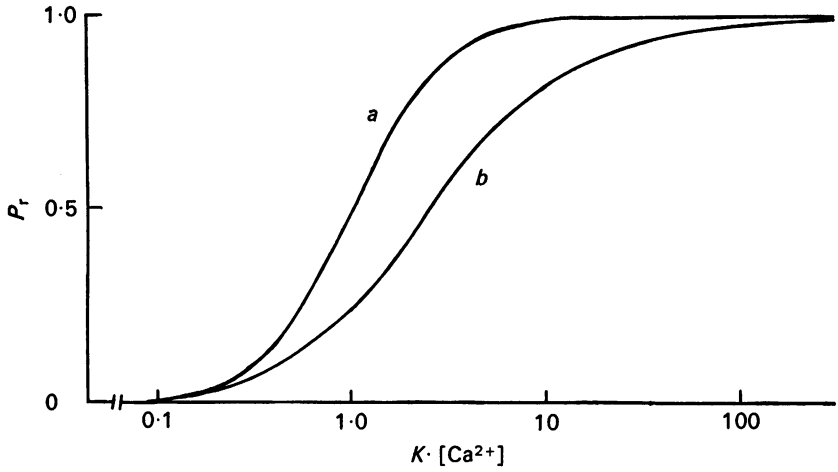


Fig. 9. The steepest curves relating relative tension, $P_r$, to free $Ca^{2+}$ concentration which can be obtained, assuming the participation of two $Ca^{2+}$ ions in a *consecutive* scheme (*a*) and in an *independent* scheme of reaction (*b*) to produce tension (see Ashley & Moisescu, 1972 and Discussion). Curve *a* has been predicted by eqn. (9) with $K_1^{app.} \ll K_2^{app.}$ and $K_1^{app.} \cdot K_2^{app.} = K^2$, and curve *b* was calculated from eqn. (8) with $'K_1^{app.} = 'K_2^{app.} = K$. The curves have been drawn such as to overlap for relatively low $[Ca^{2+}]$, i.e. to show the same *apparent* $Ca^{2+}$ threshold for activation.

semi-logarithmically. The steepness suggested by the experimental results is significantly greater than can be predicted by the co-operative action of two $Ca^{2+}$ ions in an '*independent*' scheme (Ashley & Moisescu, 1972) and the curves are better fitted by the '*consecutive*' alternative. This point is illustrated theoretically in Fig. 9, where the steepest curves obtainable from the two schemes are represented.

In the *independent* scheme, the two sites $M_1$, $M_2$ in the functional unit are considered to be independent in their calcium-binding ability, but only when both are occupied with $Ca^{2+}$, can the functional unit give rise to isometric tension, $P$:

$$Ca^{2+} + M_1^* \overset{{}'K_1^{app.}}{\rightleftharpoons} CaM_1,$$

$$Ca^{2+} + M_2^* \overset{{}'K_2^{app.}}{\rightleftharpoons} CaM_2,$$

$$P \propto [CaM_1] \cdot [CaM_2]. \tag{7a}$$

In the *consecutive* scheme, the second $Ca^{2+}$ can only be bound after the first, and this is indicated as follows:

$$Ca^{2+} + {}^*M_1 - M_2 \overset{K_1^{app.}}{\rightleftharpoons} \underset{+}{CaM_1 - M_2^*} + Ca^{2+} \overset{K_2^{app.}}{\rightleftharpoons} CaM_1 - M_2Ca \propto P. \tag{7b}$$

${}'K_1^{app.}$, ${}'K_2^{app.}$ and $K_1^{app.}$, $K_2^{app.}$ are the *apparent* equilibrium constants of the $Ca^{2+}$-binding steps in the independent and in the consecutive scheme, respectively. The active $Ca^{2+}$-binding sites are indicated by an asterisk.

The relationships for steady-state tension can be derived readily by making use of the conservation equation, which expresses the plausible assumption that there is a constant number of $Ca^{2+}$-regulated functional units for tension ($C_T$) at a given sarcomere length. This equation can be written as

$$[M_1^*] + [CaM_1] = [M_2^*] + [CaM_2] = C_T,$$

for the independent scheme, or as

$$[{}^*M_1 - M_2] + [CaM_1 - M_2^*] + [CaM_1 - M_2Ca] = C_T,$$

for the consecutive scheme. Based on eqns. (7), it follows that the relative isometric tension, $P_r$, in the independent scheme is given by:

$$\begin{aligned} P_r &= [CaM_1] \cdot [CaM_2] \cdot C_T^{-2} \\ &= {}'K_1^{app.} \cdot {}'K_2^{app.} \cdot [Ca^{2+}]^2 \cdot (1 + {}'K_1^{app.} \cdot [Ca^{2+}])^{-1} \cdot (1 + {}'K_2^{app.} \cdot [Ca^{2+}])^{-1}. \end{aligned} \tag{8}$$

The consecutive scheme, eqn. (8), leads to the following expression for $P_r$:

$$\begin{aligned} P_r &= [CaM_1 - M_2Ca] \cdot C_T^{-1} \\ &= K_1^{app.} \cdot K_2^{app.} \cdot [Ca^{2+}]^2 (1 + K_1^{app.} \cdot [Ca^{2+}] + K_1^{app.} \cdot K_2^{app.} \cdot [Ca^{2+}]^2)^{-1}. \end{aligned} \tag{9}$$

It is important to mention that the consecutive scheme is more general than the independent scheme, but only for *steady-state* conditions. Thus, for any pair of binding constants ${}'K_1^{app.}$, ${}'K_2^{app.}$ in the independent scheme, one can find a pair of binding constants $K_1^{app.}$, $K_2^{app.}$ in the consecutive scheme to provide an *identical* relationship of $P_r$ on $[Ca^{2+}]$:

$$K_1^{app.} = {}'K_1^{app.} + {}'K_2^{app.}; \; K_2^{app.} = {}'K_1^{app.} \cdot {}'K_2^{app.} \cdot ({}'K_1^{app.} + {}'K_2^{app.})^{-1}. \tag{10}$$

The reciprocal is not true.

The steepest sigmoidal curves that can be obtained with the independent scheme (eqn. (9)) is for ${}'K_1^{app.} = K_2^{app.}$, and is plotted in Fig. 9*b*. The consecutive scheme (eqn. (9)) will provide the steepest sigmoidal curve for $P_r$ when $K_1^{app.} \ll K_2^{app.}$, and this curve is shown in Fig. 9*a*.

The lines drawn in Figs. 3–6, which fit reasonably well the experimentally determined tension responses, are predictions derived from eqn. (9) for different values of the product $K_1^{app.} \ll K_2^{app.}$ where $K_1^{app.} \ll K_2^{app.}$. These results indicate that the process of tension production in skeletal muscle fibres from barnacle might involve at least *two* $Ca^{2+}$ ions acting in a *consecutive* scheme of reaction.

However, the *consecutive* scheme is only more likely than the *independent* scheme for the action of calcium in barnacle muscle, if *only* $2Ca^{2+}$ are involved in the activation process. If the over-all number of calciums involved is greater than the *apparent* two required on the basis of the present experiments, as recent biochemical evidence in vertebrate muscle seems to suggest (Bremel & Weber, 1972; Potter, Leavis, Seidel, Lehrer & Gergely, 1975), then the *independent* scheme cannot be excluded definitively, based solely upon these experiments.

It seems possible, however, that the two *apparent* calcium ions required for the present steady-state experiments, may well be the same $2Ca^{2+}$ observed from an analysis of transient state experiments on barnacle fibres with aequorin (Ashley & Moisescu, 1972). In that analysis, Fig. 1*a* illustrated a linear relationship between log $dP/dt$ and log $Ca/Ca_r$ where $Ca_r$ is the fibre resting free calcium ion concentration. The axes in that fig. were, unfortunately, inverted but the original line had a slope of 2, which suggested that two calcium ions were involved mainly per activation site for tension over the range of pCa values investigated. The calculation of the relative free calcium concentration ($Ca/Ca_r$) was based on the apparent calcium-aequorin stoichiometry for emission of light, as being close to 2 over a wide range of pCa values (Ashley, 1970; Baker, Hodgkin & Ridgway, 1971; Moisescu *et al.* 1975; Ashley & Moisescu, 1975). At pCa values 76·4, in the presence of a simulated internal ionic environment ($Mg^{2+}$, 5 mM) the participation of the third calcium ion (Shimomura & Johnson, 1970) becomes apparent, and the power of the relationship between light intensity and $Ca^{2+}$ is now $> 2$ (Moisescu & Ashley, 1977). However, at pCa 6·4 steady-state isometric tension should be $< 1\ \%\ P_o$ (see Fig. 3, 5 mM-$Mg^{2+}$), while in the transient state the resting force, $P_r$, was considered as $1{\cdot}7\ \%\ P_o$ (Ashley & Moisescu, 1972). Thus the intensity of the light emitted by aequorin at pCa's $< 6{\cdot}4$ and transient tensions $> 1{\cdot}7\ \%\ P_o$ should be $[Ca^{2+}]^2$ during the complete response (see Ashley & Moisescu, 1972).

In addition, in these transient-state experiments (Ashley & Moisescu, 1972), both *rapidly* and *slowly* equilibrating calcium ions with high binding affinities should have been detected, since tension responses of different magnitudes were predicted accurately, solely on the basis of the individual free calcium ion change.

It was pointed out also that the transient experiments do not give any indication as to the exact location of the calcium ion binding sites, which may well be on troponin but could also be on myosin, the myosin site being induced *in vivo* (Ashley & Moisescu, 1972). The induction of the myosin site could involve, for example, an increase in calcium ion binding affinity, converting it essentially from a low affinity to a higher affinity site as a result of calcium ion binding on troponin. Alternatively, a higher affinity site could be induced on troponin by calcium ion binding to a first site on myosin. This transient kinetic analysis was not, however, able to distinguish between these and other possible alternatives.

The effects of $[H^+]$, $[K^+]$ and $[Mg^{2+}]$ on the relative isometric tension–pCa relationship can be accounted for on the basis of a model of competitive inhibition between these cations and $Ca^{2+}$ for the functional unit

(see Moisescu & Ashley, 1977). The functional unit for tension will only switch 'on' if the calcium ions are bound on to it, and will remain switched 'off' if $H^+$, $K^+$ or $Mg^{2+}$ are occupying the reactive sites. The effect of $Na^+$ on the functional unit for tension was not examined in these experiments.

A change in pH around 7·10 by 0·5 u. resulted in a symmetrical shift of the isometric tension–pCa relationship along the pCa axis by 0·5 log u. without affecting significantly the steepness of the curve (Fig. 5). This result suggests that the functional unit for tension could react with one $H^+$ for every $Ca^{2+}$ required for its activation. If two $Ca^{2+}$ ions were involved in barnacle to produce tension (Ashley & Moisescu, 1972 and later), then the product of the *apparent* affinity constants for the corresponding two protons must be at least of the order of $10^{17}$–$10^{18}$ $M^{-2}$ for our standard solution. This pH effect upon the $Ca^{2+}$-activation curve of the contractile apparatus in skeletal muscle fibres, observed initially by us in solutions which did not contain an ATP-regenerating system (Ashley & Moisescu, 1974), has also been found recently in heart cells (Ebashi, Nonomura, Kitazawa & Toyo-oka, 1975).

A decrease in the $Mg^{2+}$ concentration from 5 to 1 mM produces a shift of the $Ca^{2+}$-activation curve by some 0·7 u. towards lower $Ca^{2+}$ concentrations (Fig. 3). This shift is equivalent to 1 log u. per 10 times change in $[Mg^{2+}]$, which is the same as that observed by varying $[H^+]$, and can be accounted for by assuming similarly that the functional unit for tension can react with one $Mg^{2+}$ for every $Ca^{2+}$ required for its activation. However, a further decrease in $[Mg^{2+}]$ from 1 mM to *ca.* 30 $\mu$M shifts the $Ca^{2+}$-activation curve by only 0·3 pCa u. This implies that the product of the apparent affinity constants for the corresponding two $Mg^{2+}$ ions should be only of the order of $10^6$ $M^{-2}$ for our standard conditions. The experiments described here indicate also that a variation in the MgATP concentration over the range 1–13 mM has no significant effect on the isometric tension–pCa relationship, and this result is consistent with the findings of Kerrick & Donaldson (1972) on frog skinned fibres and with those of Fabiato & Fabiato (1975) on skinned cardiac cells. From our results, it appears also that the steepness of the $Ca^{2+}$-activation curve is not affected significantly by changes in $[Mg^{2+}]$. This observation is in agreement with the data obtained by Fabiato & Fabiato (1975), but contradicts the results of Donaldson & Kerrick (1975). The disagreement might be due to the fact that the free $Ca^{2+}$ concentration was no longer buffered adequately for the pCa range required to activate the contractile apparatus, when $Mg^{2+}$ concentration in their solutions, was higher.

The interesting inhibitory effect of $K^+$ ions on the isometric tension–pCa relationship observed in this preparation can be explained also by a com-

petition between these monovalent cations and $Ca^{2+}$ for the reactive sites of the functional unit for tension. However, it is necessary to assume at least *two* $K^+$ ions for every $Ca^{2+}$ required for tension, since an increase of the $K^+$ concentration in the activating solutions from 90 to 170 mM has shifted the $Ca^{2+}$-activation curve to four times higher $Ca^{2+}$ concentrations. This co-operative inhibitory effect of potassium ions on the isometric tension–pCa relationship may contribute to the decline in the tension response of skeletal muscle fibres when immersed in hypertonic salines (Caputo, 1966; April, Brandt, Reuben & Grundfest, 1968), although this effect would not account for a decrease in maximum tension.

The finding that $K^+$ has a similar inhibitory effect on $Ca^{2+}$-induced light emission from aequorin (Moisescu & Ashley, 1977) could contribute to the decline in the height of the aequorin light response in hypertonic media produced by electrical stimulation (Ashley & Ridgway, 1970). However, single muscle fibres exposed to hypertonic salines, although unable to elicit a calcium transient in response to electrical stimulation, are still able to respond with an aequorin light response upon the external application of caffeine or sodium-free salines (Griffiths, P. J., personal communication). Both these agents are known to be able to increase the free calcium ion concentration within the cell. Thus, it seems that the decline in the height of the calcium transient evoked by electrical stimulation in hypertonic media cannot be explained simply by a change in the calcium ion sensitivity of aequorin within the cell.

This marked effect of $K^+$ on tension might account for the inability of some biochemists (Potter & Gergely, 1975; Potter *et al.* 1975) to observe any effect of $[Mg^{2+}]$ on rabbit myofibrillar ATPase–pCa curves, since in these experiments an increase in the $Mg^{2+}$ concentration was accompanied by a decrease in $K^+$ concentration, in order to maintain a constant ionic strength.

From these experiments, it follows directly that there is no *absolute* threshold for $[Ca^{2+}]$ to initiate contraction in muscle, and that, for a given $[Ca^{2+}]$, one can obtain either full activation or complete relaxation if the other parameters pH, $[Mg^{2+}]$ or $[K^+]$ are modified accordingly.

From our experiments, it appears that a change in the ionic strength, by itself, could modify the *absolute* value of the force per functional unit, but does not modify the relationship between *relative* isometric tension and free $Ca^{2+}$ concentration (see Figs. 6 and 7). An increase in ionic strength from *ca.* 100 to *ca.* 300 mM (Fig. 7) caused a decrease of 50 % in max. tension. This effect may also partly be responsible for the marked decline in the absolute tension response observed in intact muscle fibres when exposed to hypertonic salines. The decrease in max. tension observed in these experiments with isolated myofibrils, when exposed to activating

solutions of high ionic strength, may be due to the decrease in the ATPase activity of the acto-myosin systems, which was observed under similar conditions (Perry, 1956; Weber & Herz, 1962). This effect has been attributed, more recently, to a change in the dissociation constant of the acto-heavy meromyosin complex as a result of an increase in the ionic strength of the medium (Eisenberg & Moos, 1970).

Experiments presented in this paper have shown that tonicity by itself (Fig. 7) has also a significant effect on the maximal isometric tension response in barnacle myofibrils. It is not yet clear what sort of process might cause this effect, but it is important to be considered together with other effects of ionic strength, pH, $[Mg^{2+}]$ and $[K^+]$ on the isometric tension response, when replacing EGTA with other anionic species in order to produce quick $[Ca^{2+}]$ changes in myofibrillar bundles (Ashley & Moisescu, 1973, 1975; Moisescu, 1976).

In general, the effects of ionic strength and tonicity upon max. isometric tension have not been examined independently of each other (see, e.g. April & Brandt, 1973; Gordon *et al.* 1973). In this present paper, when both tonicity and ionic strength are increased together there was a more pronounced decline in the max. isometric tension response (see Fig. 7). Similar results to this have been reported on myofibrillar preparations isolated from frog (Matsubara & Elliott, 1972) and crayfish (April, Brandt & Elliott, 1972).

It was also noteworthy that a decrease in temperature from $+22°$ C to $+4°$ C did not produce any appreciable effect on the calcium ion sensitivity of the *relative* tension response. In the transient state, experiments with aequorin in intact barnacle muscle fibres suggest that the calcium kinetic parameters associated with the isometric tension response change significantly over this temperature range (Ashley, C. C. & Hill, J., unpublished observations). It is possible, therefore, that the change in temperature affects both the forward and reverse rates of the calcium ion reactions for tension to a similar extent, so that the values of the equilibrium constants are not appreciably modified.

Finally, the present experiments indicate that the activation process of the contractile apparatus in barnacle may involve two $Ca^{2+}$ ions per functional unit, acting in a consecutive scheme of reaction (Ashley & Moisescu, 1972) and that the effect of all the other physiologically occurring cations on the $Ca^{2+}$-activation curves is consistent with a competitive inhibition process.

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