Contractile activation and force generation in skinned rabbit muscle fibres: effects of hydrostatic pressure

N. S. Fortune, M. A. Geeves* and K. W. Ranatunga

*Departments of Physiology and *Biochemistry, School of Medicine, University of Bristol, Bristol BS8 1TD*

1. Effects of hydrostatic pressure (range 0·1–10 MPa) on the isometric tension of skinned (rabbit psoas) muscle fibres were examined at 12 °C and at different levels of Ca^{2+} activation (pCa range 4–7); the effects on both the steady tension and the tension transients induced by rapid pressure release (< 1 ms) are described.

2. The steady tension was depressed by increased pressure ($\sim 1\ \% MPa^{-1}$) at a high level of Ca^{2+} activation (pCa ~ 4) whereas it was potentiated at lower Ca^{2+} levels (pCa > 6); the effects were reversible.

3. At maximal Ca^{2+} activation, the tension recovery following pressure release (10 MPa to atmospheric) consisted of a fast ($\sim 30\ s^{-1}$) and a slow ($2–3\ s^{-1}$) phase; the rate and the normalized amplitude (normalized to the steady tension at atmospheric pressure for a particular pCa) of the fast phase were invariant with changes in Ca^{2+} level.

4. The effects of changing Ca^{2+} level on the slow phase were complex; its positive amplitude at high Ca^{2+} levels changed to negative and the rate decreased to $\sim 1\ s^{-1}$ at low Ca^{2+} levels (pCa > 6·0).

5. Results are discussed in relation to previous studies on the effect of pressure on intact muscle fibres and the actin–myosin interaction. This work supports calcium regulation of cross-bridge recruitment rather than calcium regulation of the rate of a specific step in the cross-bridge cycle.

In a recent study, we examined the effects of increased hydrostatic pressure on the isometric contractions of intact rat fast muscle (Ranatunga & Geeves, 1991). The steady tension in a fused tetanic contraction was typically reduced at increased hydrostatic pressure and it was comparable to the pressure-induced tension depression obtained in maximally Ca^{2+}-activated skinned muscle fibres; this was attributed to an effect of high pressure on cycling cross-bridges (Geeves & Ranatunga, 1987; Fortune, Geeves & Ranatunga, 1989, 1991). Additionally, we argued that a small reduction in active tension in muscle fibres was expected from studies on the interaction between isolated actin and myosin sub-fragment-1 (S1) in solution (Coates, Criddle & Geeves, 1985), which showed that high pressure inhibits a particular isomerization between two acto–S1 complexes.

In contrast to the slight depression of tetanic tension, the peak tension of twitch contractions recorded from intact fibres was markedly potentiated under high pressure; this was associated with an increase of the rate of tension rise and a decrease of the rate of relaxation. We suggested that the twitch tension potentiation was due to pressure effects on one or more of the processes involved in excitation–contraction coupling.

In order to gain further understanding of the pressure effects on contractile activation, we have now examined the pressure-induced tension changes in skinned rabbit psoas muscle fibres at different levels of Ca^{2+} activation. This paper reports our findings, which show that, at certain levels of submaximal Ca^{2+} activation, the steady tension is potentiated by high pressure. As reported previously (Fortune *et al.* 1991), at maximal Ca^{2+} activation, the tension response to rapid pressure release (from 10 to 0·1 MPa in ~ 1 ms) consists of three phases; a tension decline in phase with pressure release followed by two phases (phase 2 and 3) of tension recovery. The Ca^{2+} dependence of these processes shows that only phase 3, the 'slower phase', changes directly as a result of changes in Ca^{2+} concentration.

A brief preliminary report of this work was presented to the European Muscle Conference (Fortune, Geeves & Ranatunga, 1992).

METHODS

The details of the experimental apparatus, techniques and the protocols used for recording tension changes in single skinned rabbit psoas muscle fibres under hydrostatic pressure have been reported previously. The basic design features of the pressure chamber were published by Geeves & Ranatunga (1987), the design of the transducer assembly by Fortune *et al.* (1989) and the methodology used in recording transient tension responses following rapid (< 1 ms) pressure release is described by Fortune *et al.* (1991).

Rabbits were sedated using an intramuscular injection of Hypnorm (a mixture of 0·317 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone; Janssen Pharmaceuticals, Oxford) at a dose of 1·5 ml (3 kg body weight)$^{-1}$. The rabbit was then rendered unconscious using a gaseous anaesthetic (halothane; Fluothane, ICI, Macclesfield, Cheshire) before being exsanguinated via the carotid artery.

Experiments used single muscle fibres, which were dissected out from chemically skinned and glycerinated fibre bundles of rabbit psoas. A segment (~ 4 mm) of a fibre was glued (using a nitrocellulose adhesive) between the tension transducer hook (AE 801, Akers, Horten, Norway) and another rigid hook fixed on the transducer plug assembly and inserted into the pressure chamber. The sarcomere length, as monitored by laser diffraction, was 2·2–2·6 μm. Full details of the composition of experimental solutions are given elsewhere (Fortune *et al.* 1989); they contained 50 mM imidazole as pH buffer (pH was 7·0, at 12 °C) and their ionic strength was 200 mM. Calcium-EGTA/EGTA in the activating solution was varied in order to obtain different levels of free calcium (pCa 4·5–7·5); the total EGTA concentration remained at 15 mM. Typically, three or four transient tension responses were recorded at each pCa. The pressure range used was 0·1–10 MPa and the temperature was 12 °C in all cases.

It has been shown that high pressure has a negligible effect on the binding of calcium to EGTA buffers. Goldmann (1990), using arsenazo III as an indicator dye, found that the change in equilibrium constant for Ca^{2+} binding to EGTA at 10 MPa was < 0·3 %. Thus, a 10 MPa pressure rise increased the measured free [Ca^{2+}] by < 0·01 μM over the range pCa 5–6. A second assay, using the fluorescent dye dansylaziridine covalently attached to TnC (troponin-C), showed that a 10 MPa pressure rise changed the binding of Ca^{2+} to the protein by less than 1 %.

A small DC offset in the transducer output was obtained on pressurization to 10 MPa; this varied between ~ 5 and 50 μN in different transducer elements but remained constant in a given element. Therefore, the pressure sensitivity of the transducer (with or without a relaxed muscle fibre attached to it) was routinely recorded and the active muscle fibre tensions at high pressure were subsequently corrected (see Fortune *et al.* 1991).

Records of both tension and pressure were stored as 4000 12-bit data points on a digital oscilloscope (Nicolet 3901). Data were transferred to hard disk and subsequently analysed as 400 data points on a Hewlett Packard 310 computer. Data were fitted using a non-linear least-squares fitting routine in HP Basic (Hi-Tech Ltd, Salisbury, Wiltshire). Hill binding curves were fitted using the 'KFIT' programme of Dr N. C. Millar (Biophysics Department, King's College, London WC2B 5RL).

RESULTS

In experiments on a number of fibres, the pressure sensivity of the steady active tension was determined at a number of different Ca^{2+} levels and it was found that the pressure-induced tension change was dependent on the degree of Ca^{2+} activation. In contrast to a tension depression observed at maximal Ca^{2+} activation, a tension potentiation was obtained at low levels of Ca^{2+} activation. Figure 1*A* and *B* shows two sample tension records from a fibre at maximal (Fig. 1*A*) and submaximal Ca^{2+} activation (Fig. 1*B*); this illustrates the qualitatively different response induced by high pressure. In either case the tension change is fully reversible on pressure release.

Note that in both records, the inherent transducer offset was +30 μN (see Methods); this has the effect of visually reducing the amplitude of pressure-induced tension depression in Fig. 1*A* and increasing the tension potentiation in Fig. 1*B*.

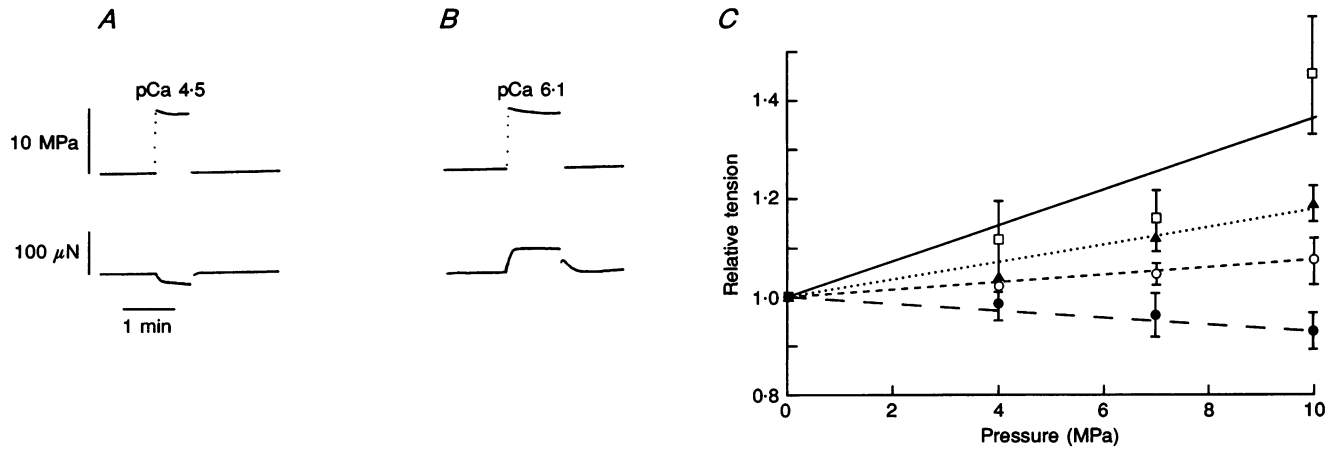


Figure 1.
A and *B*, sample experimental records from a fibre illustrating the effect of increase of pressure to 10 MPa (between the arrows) on the isometric tension; the fibre was maximally Ca^{2+} activated (pCa 4·5, steady tension 970 μN) in *A*, but submaximally activated (pCa 6·1, steady tension 260 μN) in *B*. Note that in either case the tension change is reversible on pressure release. In both records the inherent transducer offset was +30 μN; this has the effect of visually reducing the amplitude of pressure-induced tension depression in *A* and increasing the tension potentiation in *B*. *C*, data from another fibre showing the pressure sensitivity of tension at different levels of Ca^{2+} activation (●, pCa 4·52; ○, pCa 5·47; ▲, pCa 5·61; □, pCa 5·79. The steady tensions at atmospheric pressure ranged from 830 μN (~ 146 kN m^{-2}) at pCa 4·52 to 20 μN at pCa 5·79. Data were normalized to the initial tension (at atmospheric pressure) at a given pCa and each point represents the mean (± S.E.M.) in each case. The lines are fitted by eye.

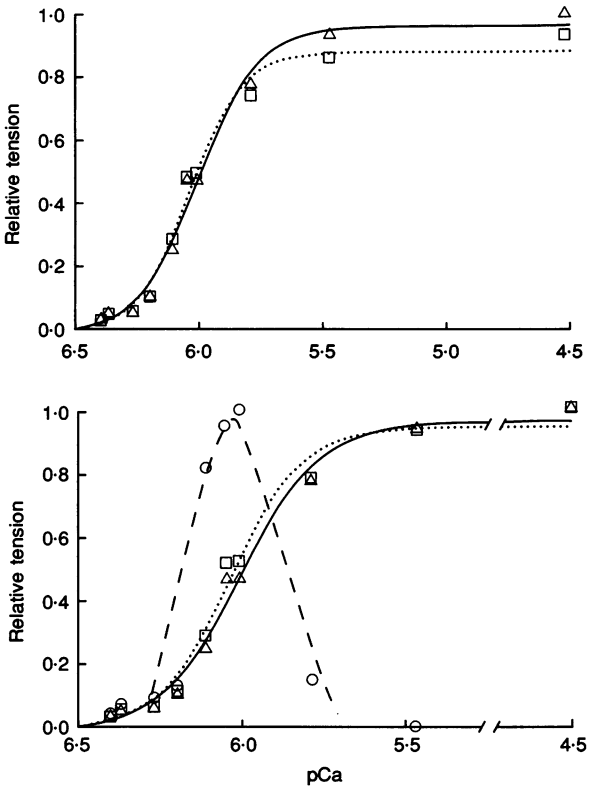
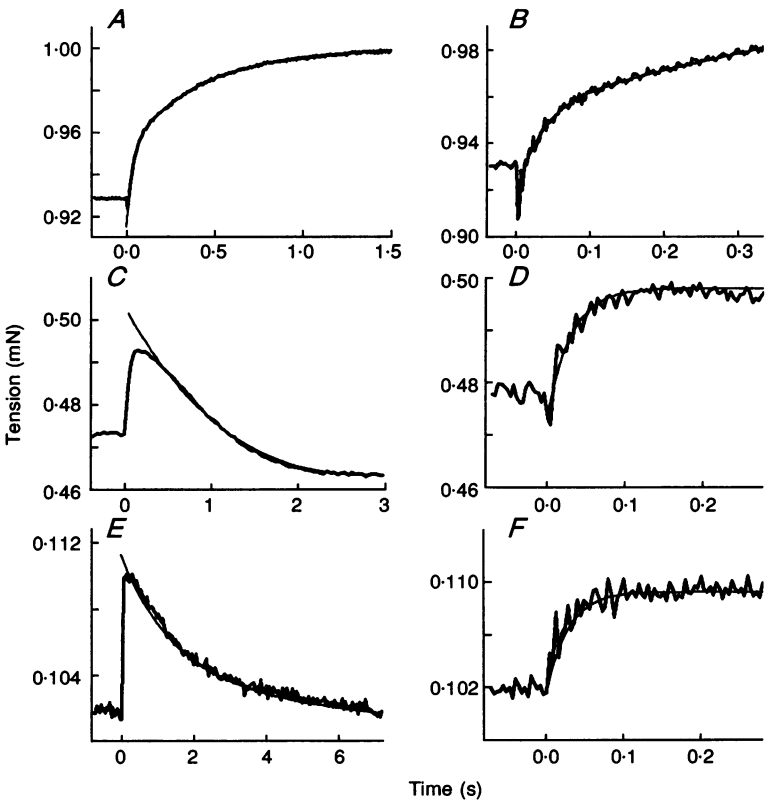


Figure 2.
The pCa–tension relations from a single muscle fibre, at atmospheric (△, continuous line) and at an increased hydrostatic pressure of 10 MPa (□, dotted line). The fibre specific tension was 158 kN m^{-2}. Each data point is the average of 3–4 individual responses and the curves are fitted using the Hill binding equation. *A*, tensions are normalized to the maximal active tension at atmospheric pressure. The fitted Hill curve at atmospheric pressure gives a pCa of 6·01 ± 0·02 (S.D.) in comparison to 6·04 ± 0·02 at 10 MPa. *B*, tensions in each curve are normalized to that at maximum activation. Circles with dashed curve represent difference between high and atmospheric pressure data after being normalized to the peak tension difference.

Figure 1*C* shows data from another fibre; at each of four levels of Ca^{2+} activation, the fibre was exposed to three different high pressures and the high pressure to atmospheric tension ratio is plotted. As reported in previous studies (Geeves & Ranatunga, 1987), pressure induces a depression of steady tension which is linearly related to pressure at maximal activation (pCa 4·52). However, a clear potentiation of tension is obtained as the free calcium concentration is reduced; the pressure dependence again is approximately linear. The linear change in tension at 10 MPa corresponds to a −8 % (tension depression) at pCa 4·5, whereas it corresponds to a +45 % (tension potentiation) at

Figure 3.
Tension transients induced by release of hydrostatic pressure (from 10 to 0·1 MPa, atmospheric pressure) at different levels of calcium activation, from the same muscle fibre as in Fig. 2. *A* and *B*, at pCa 4·52 (maximally Ca^{2+} activating). Note the tension decline in phase with pressure release (phase 1). The double exponential fit to the recovery (2 s time scale, *A*) gives $1/\tau_2$ (reciprocal relaxation time for phase 2) of 30 s^{-1} and $1/\tau_3$ of 2·36 s^{-1}; the amplitudes were 38 and 48 μN, respectively, for phase 2 and phase 3. A single exponential to the first 400 ms (*B*) of recovery gives $1/\tau_2$ of 28 s^{-1} and an amplitude of 39 μN. *C* and *D*, at pCa 6·01 (~ 50 % of maximal tension). At 2 s time scale (*C*), $1/\tau_3$ of 0·58 s^{-1} and an amplitude of 51 μN. Analysis of phase 2 (*D*), gives $1/\tau_2$ of 33 s^{-1} and an amplitude of 25 μN. *E* and *F*, a similar presentation to *C* and *D*, but at pCa 6·2, where the tension developed was 10 % of maximum tension; $1/\tau_3$ is 0·65 s^{-1} and its amplitude, 10 μN; $1/\tau_2$ is 35·5 s^{-1} and its amplitude, 10 μN.



pCa 5·8, which is 0·3 of a pCa unit below the mid-point of the pCa curve for this fibre. Data from four other fibres gave a 7·6 ± 3·09 % (mean ± S.D.) depression of tension at pCa 4·52 and a maximum potentiation of 21·3 ± 12·2 % at a pCa just below the mid-point (mid-point pCa range of 5·88–6·53).

pCa–tension relationship

Figure 2*A* shows data from an experiment on a single muscle fibre, in which tension was recorded at a series of different Ca^{2+} levels both at atmospheric pressure (9) and at one high pressure (10 MPa, 5). It can be seen that the pressure-induced tension depression seen at high Ca^{2+} levels (e.g. pCa < 5·5) is decreased and reversed at lower Ca^{2+} levels (> pCa 6·0). Hill curves fitted to the data gave a mid-point of 6·01 (±0·02, S.D.) at atmospheric pressure and 6·04 (±0·02, S.D.) at 10 MPa. In different fibres, the cross-over of the pCa–tension relations occurred at different Ca^{2+} levels (pCa 5·4–6·0), but typically at about the same relative tension level (~ 50 %) close to the mid-point. Data from three other fibres gave Hill curve mid-points at atmospheric pressure of 6·43 ± 0·03, 5·75 ± 0·04 and 6·02 ± 0·01. In each case pressure caused an increase in the pCa mid-point with an average increase for the four fibres of 0·033 ± 0·017 (S.D.) of a pCa unit at 10 MPa. The same data from Fig. 2*A* are plotted in Fig. 2*B*, after the high-pressure data were normalized to maximal Ca^{2+}-activated tension at 10 MPa. The data in Fig. 2*B* illustrate that the normalized pCa–tension relation of a muscle fibre is shifted to the left (mid-point 6·04 ± 0·02) on increase of pressure; the resultant difference curve (normalized) is shown by dashed line (○).

Tension transients following rapid release of pressure

The transient tension responses following rapid release of hydrostatic pressure (complete in < 1 ms) were examined in experiments on a number of fibres ($n = 7$) and over a range of calcium concentrations (pCa 4·52–7·5). A set of sample tension transient records from one fibre, in which 10 MPa pressure releases were applied, are shown in Fig. 3. Figure 3*A* shows a typical tension transient when the fibre was maximally Ca^{2+} activated (pCa 4·52). As reported previously (see Fortune *et al.* 1991), the tension decreased in phase with pressure release (phase 1) and then recovered to the prepressure control level. The recovery consists of two phases (phases 2 and 3) as identified by exponential curve fitting; a double exponential curve (Fig. 3*A*) gives a reciprocal relaxation time for phase 2 ($1/\tau_2$) of 30 s^{-1} and a reciprocal relaxation time for phase 3 ($1/\tau_3$) of 2·36 s^{-1}. Figure 3*B* shows the early part of the transient fitted with a single exponential; $1/\tau_2$ from this calculation is similar to above, 28 s^{-1}.

Figure 3*C* and *D* shows the pressure-release tension transient when the fibre was activated to develop ~ 50 % of maximal tension (pCa 6·01). Phase 1 is reduced in amplitude but still present; this is followed by a transient tension increase similar to that seen at pCa 4·5 and then a small slow tension decline. A single exponential fit over a 3 s time scale

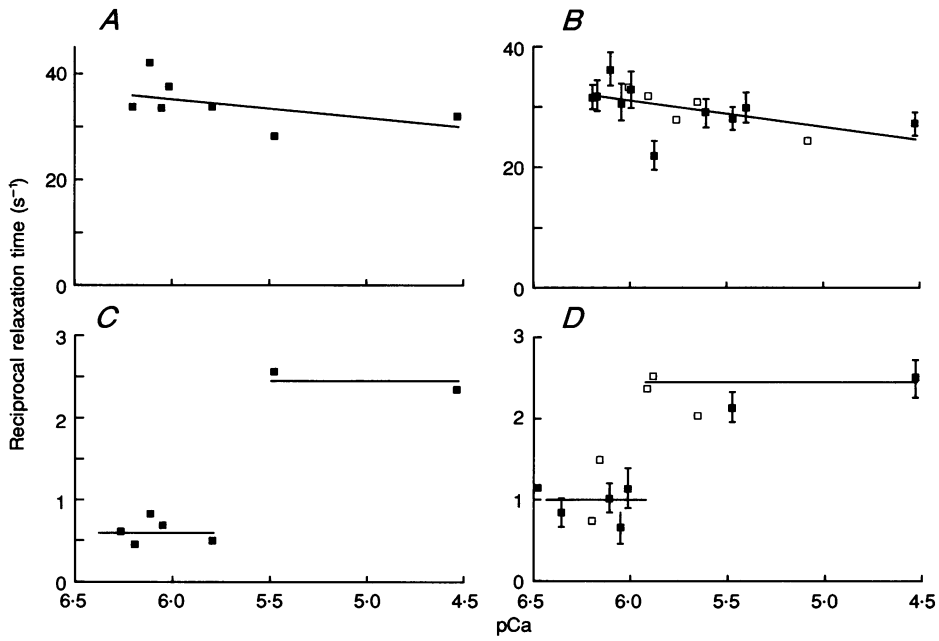


Figure 4.

A and *B*, data for $1/\tau_2$ at different pCa levels from a single fibre (*A*) and from the pooled data (*B*). *C* and *D* show corresponding data for $1/\tau_3$. Pooled data are from 7 fibres, the standard error bars are shown for points where $n > 1$; in some cases the bar was shorter than the height of the symbol. Data shown as □ represent single observations. Lines were fitted by regression analysis in *A* and *B*. In *C* and *D* the straight lines, drawn by eye, serve to illustrate the change in rate constant with $[Ca^{2+}]$ for both single and pooled data respectively.

gives $1/\tau_3$ of 0·58 s^{-1} and an amplitude of 51 μN (Fig. 3*C*). A single exponential fit to the early part of the trace (Fig. 3*D*) gives $1/\tau_2$ of 33 s^{-1}. It can be seen that, at this level of calcium activation, phase 3 no longer contributes to a tension increase as at pCa 4·5, but to a tension decrease. Figure 3*E* and *F* shows the tension transient when the fibre was activated to ~ 10 % maximal tension (pCa 6·2). Phase 1 is no longer apparent but may be hidden in the now much larger transducer noise; the transient tension rise (phase 2) is clearly present followed by a slow tension decrease in phase 3. Separate single exponential fits give $1/\tau_2$ of 35·5 s^{-1} and $1/\tau_3$ of 0·645 s^{-1}.

The reciprocal relaxation time for phase 2 is higher in the present experiments than we reported earlier (~ 20 s^{-1}). This may be due to the improved resolution in tension recording; tension oscillations resulting from pressure release lasted < 3 ms in these experiments as compared with ~ 6 ms previously.

It is seen from Fig. 3 that the initial decrease of tension (phase 1) is more marked at higher activation. However, because of the transducer noise, there is some uncertainity whether the small amplitude phase 1 would have been seen at low activation. It was shown in previous experiments that phase 1 (only) is seen in rigor fibres and it represents an effect of hydrostatic decompression on some normal elasticity in muscle fibres (see Ranatunga, Fortune & Geeves, 1990).

Figure 4*A* shows the reciprocal relaxation times for phase 2 ($1/\tau_2$) plotted against pCa from the same fibre as in Figs 2 and 3; $1/\tau_2$ is between 25–45 s^{-1} over the whole pCa range studied. Pooled data are shown in Fig. 4*B*, where each point represents data from between one and seven fibres. The mean ($\pm$ S.E.M.) $1/\tau_2$ is 27·4 $\pm$ 1·54 ($n = 23$) at pCa 4·5 and it is 31·6 $\pm$ 1·07 ($n = 5$) at pCa 6·2. Thus, as in the case of the single fibre data shown in Fig. 4*A*, the reciprocal relaxation time of phase 2 is relatively independent of pCa. The data from some fibres in Fig. 4*B* were collected from low to high [Ca^{2+}] (as in the case for single fibre data in Fig. 4*A*) whereas in others data were collected by starting at high levels of [Ca^{2+}] and carrying out activations at subsequent lower pCa levels. It is seen that the single fibre data and the pooled data show qualitative and quantitative similarities, indicating that there are no progressive changes in rate constants or amplitude during exposure to different pCa levels.

Figure 4*C* illustrates the variation in the reciprocal relaxation time for phase 3 ($1/\tau_3$) with pCa in a single fibre and Fig. 4*D* in the pooled data. There is a marked change in the value at around pCa 6, which is close to the mid-point of the pCa–tension relationship. This corresponds with the positive to negative change of the amplitude for phase 3 (see Fig. 6). We will refer to the component of phase 3 with a positive amplitude as phase 3*a* and the component with a negative amplitude as phase 3*b*. From the pooled data, $1/\tau_3$ at pCa 4·52 is 2·3 $\pm$ 0·36 s^{-1} ($n = 14$), whereas it is

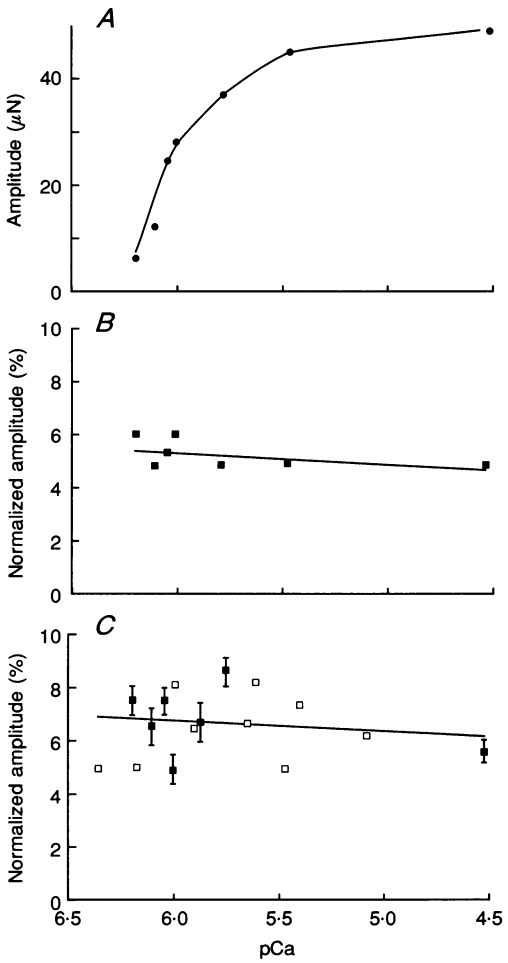


Figure 5.
The actual (*A*) and the normalized (*B*) amplitude of phase 2 from the single fibre experiment. The pooled data for normalized amplitude are shown in *C*. Data shown as □ represent single observations. Note that the normalized amplitude is relatively invariant with pCa. The lines in *B* and *C* are fitted by regression analysis.

$1{\cdot}13 \pm 0{\cdot}10\ s^{-1}$ ($n = 7$) at pCa 6·01. At the lowest calcium concentrations studied (pCa 6·36) $1/\tau_3$ was $0{\cdot}84 \pm 0{\cdot}01\ s^{-1}$ ($n = 5$). Thus, unlike phase 2, the observed phase 3 shows a calcium dependence, giving two principal relaxation rates which change close to the mid-point in the pCa–tension relation.

It was observed that the actual amplitude of phase 2 scaled with the steady tension in a given fibre (Fig. 5*A*), so that the amplitude, when normalized to the steady tension at each pCa, remains relatively the same at all pCa levels (Fig. 5*B*). From this analysis and the pooled data in Fig. 5*C*, the contribution of phase 2 remains around 5–6 % of the steady tension over the whole pCa range studied. The mean ± S.D. of the normalized amplitude at pCa 4·52 was $5{\cdot}6 \pm 0{\cdot}27$ % ($n = 18$).

Figure 6*A* shows for the single fibre experiment and Fig. 6*B* for the pooled data, the amplitude of the observed phase 3 as a function of pCa. Despite scatter in the data, the change in direction of the amplitude at around pCa 6·0 is seen; the variation with pCa of the normalized amplitude ranges from (mean ± S.D.) $+5{\cdot}55 \pm 1{\cdot}37$ % ($n = 10$) at pCa 4·52 to a maximum value in the bell-shaped curve of $-21{\cdot}23 \pm 6{\cdot}06$ % ($n = 7$). For convenient discussion of the data, the amplitudes for phase 3 shown in Fig. 6*A* are reanalysed in Fig. 6C. Here we attempt to separate phase 3 into two components by assuming that phase 3*a* is calcium independent and its amplitude proportional to tension at a specific pCa. Subtraction of this calculated amplitude 3*a*, gives the component 3*b*. The reasoning behind this separation is given in the Discussion. In Fig. 6*C* squares represent the calculated amplitude of phase 3*a* and the triangles are the calculated amplitudes of phase 3*b*. Thus the amplitude of phase 3*b*, which represents a recovery from increased activation by high pressure, is maximal at pCa 6·0.

DISCUSSION

The principal finding reported here is that, while at maximal Ca^{2+} activation increased hydrostatic pressure results in a depression of steady isometric tension of a skinned muscle fibre, high pressure produces an elevation of tension at lower levels of activation. This compares with the observation on intact muscle fibres (Ranatunga & Geeves, 1991) in which high pressure typically depressed the tetanic tension but potentiated the twitch tension. The pressure-induced depression of maximal active tension has been attributed to the inhibition of a transition from low to high force state of the cross-bridge (Fortune *et al.* 1991) and parallel experiments in solution have revealed a pressure-sensitive isomerization of actomyosin subfragment-1 (Coates *et al.* 1985). As pressure induces a depression of tension at full activation, it seems likely that the tension potentiation at low [Ca^{2+}] is due to pressure affecting calcium activation of the thin filament or some process coupled to it.

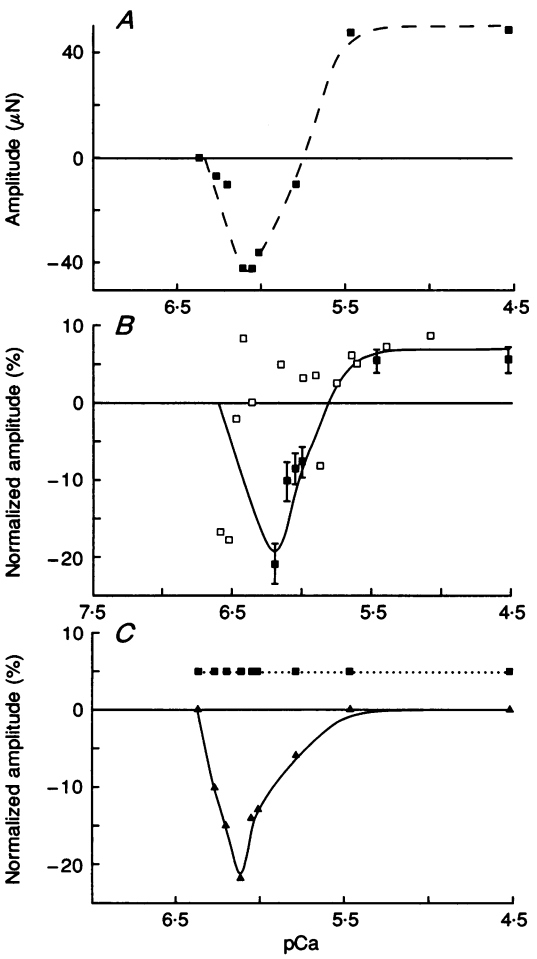


Figure 6.
The amplitude of phase 3 from the single fibre (*A*) and the normalized amplitude from the pooled data (*B*). Once again, data points shown as □ are single observations. Note the change in direction of amplitude at around pCa 6·0. The lines are fitted by eye. *C*, data in *A* are replotted for further analysis. Squares represent the amplitudes of phase 3*a* (as measured at high [Ca^{2+}]) if it is assumed to scale with steady tension (as phase 2 amplitude in Fig. 3*a*), and ▲ represent the amplitudes of phase 3*b* (seen at low [Ca^{2+}]). Note that phase 3*b* is maximal at ~ pCa 6·0 and represents the tension potentiating process under high pressure.

Characterization of the calcium dependence of tension transients induced by rapid pressure changes provides more detailed information about the underlying molecular processes.

At maximal calcium activation, the tension response to rapid pressure release consists of three phases (phases 1, 2 and 3). Phase 1 is a decrease in tension in phase with the pressure change representing a decompression of an elastic element in series with the cross-bridges. This phase is difficult to characterize fully as the rate of the relaxation and the following recovery of tension in phase 2 are not fully separated. However, no calcium sensitivity of the series elasticity is expected.

Phase 2 is a relatively rapid recovery of tension at 20–30 s^{-1} and at full activation has been ascribed to a force-generating transition of the cross-bridges associated with the liberation of phosphate from the cross-bridge (Fortune *et al.* 1991). The present experiments show that the rate of phase 2 is essentially calcium independent and its amplitude scales with the level of activation (steady tension). As the level of calcium is reduced the number of cross-bridges maintaining the force is also reduced, yet the pressure-induced force-generating event (coupled to inorganic phosphate release) occurs at the same rate and the fraction of cross-bridges perturbed remains the same. The results therefore show that the rate of this force-generating event is not directly calcium regulated, a finding which is not compatible with the proposal of Chalovich & Eisenberg (1982) that phosphate release from the cross-bridge is calcium regulated. Our results are more easily accomodated with calcium regulation of cross-bridge recruitment.

A similar conclusion was reached by Millar & Homsher (1990), who identified a tension transient of $23{\cdot}5 \pm 1{\cdot}7$ s^{-1} at 10 °C induced by rapid photoliberation of 0·7 mM phosphate in muscle fibres (see also Dantzig, Goldman, Millar, Lactis & Homsher, 1992), the rate of which was independent of calcium concentration. In contrast, the work of both Brenner (1988) and Metzger, Greaser & Moss (1989) showed that the rate of force redevelopment following a period of unloaded shortening was calcium dependent. Millar & Homsher (1990) repeated these measurements and discussed possible reasons for the apparent discrepancy in the type of measurement. In our experiments the amplitude of the pressure-induced perturbation in phase 2 is always relatively small (6 % of the steady tension). The process observed is the response of those cross-bridges which are directly coupled to the transition perturbed by pressure. In the force redevelopment experiments the amplitudes may approach 100 % of the steady force. Additionally, the state of the cross-bridges at the start of the tension redevelopment is not as clearly defined. In some hands the velocity of shortening is calcium dependent (see Metzger & Moss, 1988 and references therein) and therefore the population of cross-bridge states preceding tension redevelopment may be calcium dependent. Thus, in their experiments the number of strongly attached cross-bridges may change during the transient and the observed process may therefore involve cross-bridge-induced changes in the level of thin filament activation.

Phase 3 is a slower recovery of tension occurring at a rate similar to the rate of turnover of the cross-bridges and may represent the response of a group of cross-bridges which must pass through the rate-limiting step of the cycle before again contributing to force generation (see Fortune *et al.* 1991). When examined at different levels of Ca^{2+} activation, the rate of phase 3 shows two characteristic values; $2{\cdot}3$ ($\pm 0{\cdot}36$) s^{-1} when the amplitude is positive (phase 3*a*) and $1{\cdot}13$ ($\pm 0{\cdot}1$) s^{-1} when the amplitude is negative (phase 3*b*). This complex behaviour of phase 3 suggests that it is composed of more than one component. The simplest interpretation is that there are two components: a component which dominates at high calcium (phase 3*a*), is calcium independent and has a positive amplitude which, like the amplitude of phase 2, scales with the level of activation; and a calcium-dependent component (phase 3*b*), which has a negative amplitude and dominates at mid to low calcium concentrations. When the observed amplitude of phase 3 is resolved into phase 3*a* and phase 3*b* (see Fig. 6*C*), the amplitude of phase 3*b* has the classic bell-shaped appearance of a concentration-dependent perturbation; the maximum amplitude should therefore correspond to the concentration of calcium where the two perturbed states are closest to equal occupancy. As the observed rate of the relaxation is independent of the concentration of calcium, the binding of calcium is not the event being perturbed but a transition between two states which is coupled to calcium binding.

Solution studies on isolated components of the thin filament have shown that pressure does not change the binding of calcium to dansyl aziridine-labelled troponin-C (free in solution, in a complex with whole troponin or in reconstituted thin filaments, Goldmann, 1990). Thus, if increased pressure causes activation of the thin filament, it must be at some point after calcium binding. Several versions of an activation model involving two states of the thin filament have been proposed (Huxley, 1972; Hill, Eisenberg & Greene, 1980; Geeves & Halsall, 1987; McKillop & Geeves, 1991). If pressure perturbs the equilibrium between the two states then an amplitude dependence, of the form observed in phase 3*b*, could be expected. Additionally, both the steric blocking model of Huxley and others (above) and the co-operative model of Geeves & Halsall (1987) are also compatible with a calcium-independent rate of phosphate release as observed in phase 2.

The rate constant for phase 3*b* (tension decline after pressure release at low Ca^{2+} levels) is less than 1 s^{-1}. This is much slower than the rate normally assumed for the switching on/off of the thin filament by calcium. A slow tension relaxation was also observed in temperature-jump (T-jump) experiments at certain levels of submaximal Ca^{2+} activation (see Fig. 13 of Goldman, McCray & Ranatunga, 1987); following a T-jump (+ T-clamp) from 20 °C, the tension transiently increased as with maximal activation, but then declined slowly (half-time ~ 2 s) to a new steady level. Goldman *et al.* (1987) suggested that although the processes of Ca^{2+} regulation of thin filament activation may be faster, the tension decline may be slow due to a very slow step in the cross-bridge cycle, the cycling cross-bridges

retarding thin filament deactivation. Alternatively, the slow process could arise from the co-operative nature of the interaction between myosin heads and the thin filament. If the turning off of the co-operative unit of the thin filament ($actin_7$.Tm.Tn) occurs only when the unit is free of strongly bound cross-bridges (Greene & Eisenberg, 1980; Geeves & Halsall, 1987) then the rate of tension decline represents the net rate at which all strongly bound bridges detach from co-operative units.

The results reported here show that a high-pressure effect on the thin filament induces cross-bridge recruitment leading to an increase of tension of $\sim 2\,\%\,MPa^{-1}$ in a submaximally calcium-activated muscle fibre. Clearly, this must be a contributory factor to the pressure-induced twitch tension potentiation observed in intact muscle fibres. However, the extent of twitch tension potentiation was considerably higher ($5–6\,\%\,MPa^{-1}$) and was associated with characteristic changes in the twitch time course (see Ranatunga & Geeves, 1991). The implication is that high pressure perturbs additional processes (e.g. Ca^{2+} release and uptake) which enhance contractile activation and tension development in submaximally activated intact muscle fibres.

REFERENCES

Brenner, B. (1988). Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibres: Implications for regulation of muscle contraction. *Proceedings of the National Academy of Sciences of the USA* **85**, 3265–3269.

Chalovich, J. M. & Eisenberg, E. (1982). Inhibition of actomyosin ATPase activity by troponin–tropomyosin without blocking the binding site of myosin to actin. *Journal of Biological Chemistry* **257**, 2432–2437.

Coates, J. H., Criddle, A. H. & Geeves, M. A. (1985). Pressure relaxation studies of pyrene-labelled actin and myosin subfragment-1 from rabbit skeletal muscle. *Biochemical Journal* **232**, 351–356.

Dantzig, J. A., Goldman, Y. E., Millar., N. C., Lactis, J. & Homsher, E. (1992). Reversal of the cross-bridge force-generating transition by photogeneration of phosphate in rabbit psoas muscle fibres. *Journal of Physiology* **451**, 247–278.

Fortune, N. S., Geeves, M. A. & Ranatunga, K. W. (1989). Pressure sensitivity of active tension in glycerinated rabbit psoas muscle fibres: effects of ADP and phosphate. *Journal of Muscle Research and Cell Motility* **10**, 113–123.

Fortune, N. S., Geeves, M. A. & Ranatunga, K. W. (1991). Tension responses to rapid pressure release in glycerinated rabbit muscle fibres. *Proceedings of the National Academy of Sciences of the USA* **88**, 7323–7327.

Fortune, N. S., Geeves, M. A. & Ranatunga, K. W. (1992). Effect of hydrostatic pressure on skinned muscle fibre tension at submaximal levels of calcium activation. *Journal of Muscle Research and Cell Motility* **13**, 233a.

Geeves, M. A. & Halsall, D. J. (1987). Two-step ligand binding and cooperativity. A model to describe the cooperative binding of myosin S1 to regulated actin. *Biophysical Journal* **52**, 215–220.

Geeves, M. A. & Ranatunga, K. W. (1987). Tension responses to increased hydrostatic pressure in glycerinated rabbit psoas muscle fibres *Proceedings of the Royal Society* B **232**, 217–226.

Goldmann, W. H. (1990). Pressure and temperature perturbation studies of the interaction between actin and myosin and between calcium and troponin-C. PhD Thesis, University of Bristol.

Goldman, Y. E., McCray, J. A. & Ranatunga, K. W. (1987). Transient tension changes initiated by laser temperature jumps in rabbit psoas muscle fibres. *Journal of Physiology* **392**, 71–95.

Greene, L. E. & Eisenberg, E. (1980). Cooperative binding of myosin subfragment-1 to the actin–troponin–tropomyosin complex. *Proceedings of the National Academy of Sciences of the USA* **77**, 2616–2620.

Hill, T. L., Eisenberg, E. & Greene, L. E. (1980). Theoretical model for the cooperative equilibrium binding of myosin subfragment-1 to the actin–troponin–tropomyosin complex. *Proceedings of the National Academy of Sciences of the USA* **77**, 3186–3190.

Huxley, H. E. (1972). Structural changes in the actin and myosin containing filaments during contraction. *Cold Spring Harbor Symposia on Quantitative Biology* **37**, 361–371.

McKillop, D. F. A. & Geeves, M. A. (1991). Regulation of the actin and myosin interaction by troponin. Evidence of control of a specific isomerisation of acto.S1. *Biochemical Journal* **279**, 711–718.

Metzger, J. M., Greaser, M. L. & Moss, R. L. (1989). Variations in crossbridge attachment rate and tension with phosphorylation of myosin in mammalian skinned sleletal muscle fibres: Implications for twitch potentiation in intact muscle. *Journal of General Physiology* **93**, 855–883.

Metzger, J. M. & Moss, R. L. (1988). Thin filament regulation of shortening velocity in rat skinned skeletal muscle: effects of osmotic compression. *Journal of Physiology* **398**, 165–175.

Millar, N. C. & Homsher, E. (1990). The effect of phosphate and calcium on force generation in glycerinated rabbit skeletal muscle fibres. A steady state and transient kinetic study. *Journal of Biological Chemistry* **265**, 20234–20240.

Ranatunga, K. W., Fortune, N. S. & Geeves, M. A. (1990). Hydrostatic compression in glycerinated rabbit muscle fibres. *Biophysical Journal* **58**, 1401–1410.

Ranatunga, K. W. & Geeves, M. A. (1991). Changes produced by increased hydrostatic pressure in isometric contractions of rat fast muscle. *Journal of Physiology* **441**, 423–431.

Acknowledgements

The work was supported by a Wellcome Trust project grant. M. A. G. is a Royal Society Research Fellow.

Received 22 February 1993; accepted 23 June 1993.