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+46 46-222 00 00

# A mechanical stretch induces contractile activation in unstimulated developing rat skeletal muscle *in vitro*

Gabriel Mutungi, K. A. P. Edman\* and K. W. Ranatunga

Department of Physiology, School of Biomedical Sciences, University of Bristol, Bristol BS8 1TD, UK and \*Department of Physiological Sciences, Biomedical Centre, F11, University of Lund, S-221 84 Lund, Sweden

The effects of a stretch–release cycle (~25 % of the resting muscle fibre length,  $L_o$ ) on both tension and  $[Ca^{2+}]_i$  in small, unstimulated, intact muscle fibre bundles isolated from adult and neonatal rats were investigated at 20 °C. The results show that the effects of the length change depended on the age of the rats. Thus, the length change produced three effects in the neonatal rat muscle fibre bundles, but only a single effect in the adult ones. In the neonatal fibre bundles, the length change led to an increase in resting muscle tension and to a transient increase in  $[Ca^{2+}]_i$ . The stretch–release cycle was then followed by a twitch-like tension response. In the adult fibre bundles, only the increase in resting tension was seen and both the transient increase in  $[Ca^{2+}]_i$  and the stretch-induced twitch-like tension response were absent. The amplitude of the twitch-like tension response was affected by both 2,3-butanedione monoxime and sarcomere length in the same manner as active twitch tension, suggesting that it arose from actively cycling crossbridges. It was also reversibly abolished by 25 mM  $K^+$ , 1  $\mu M$  tetrodotoxin and 1.5 mM lidocaine (lignocaine), and was significantly depressed ( $P < 0.001$ ) by lowering  $[Ca^{2+}]_o$ . These findings suggest that a rapid stretch in neonatal rats induces a propagated impulse that leads to an increase in  $[Ca^{2+}]_i$ , and that abolishing the action potential abolishes the stretch-induced twitch-like tension response. In 5- to 7-day-old rats, the twitch-like tension response was ~50 % of the isometric twitch. It then decreased progressively with age and was virtually absent by the time the rats were 21 days old. Interestingly, this is the same period over which rat muscles differentiate from their neonatal to their adult types.

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**Corresponding author** G. Mutungi: Department of Physiology, University of Bristol, Bristol BS8 1TD, UK.  
Email: g.mutungi@bris.ac.uk

In most mammals, including rats, all of the limb muscles are slow contracting at birth and then differentiate progressively into the two distinct adult muscle types, fast and slow twitch (Buller *et al.* 1960; Close, 1964). In the rat, this changeover from neonatal to adult muscles occurs within the first 3 weeks after birth (Close, 1964; Whalen *et al.* 1981). Thus, at birth all of the limb muscles share the same myosin isoform, neonatal myosin, but by the time the rats are 21 days old, this type of myosin is completely replaced by the adult isoforms (Whalen *et al.* 1981). The contractile characteristics of the muscles also change accordingly (Close, 1964). However, the stimuli that trigger this postnatal muscle differentiation are unknown.

The aim of this study was to investigate the effects of a mechanical perturbation on resting tension in intact neonatal rat muscle fibres. The results show that when small muscle fibre bundles are subjected to a stretch–release cycle of ~25 % of resting muscle fibre length ( $L_o$ ), the stretch leads to: (i) an increase in resting muscle tension and (ii) a marked and transitory increase in  $[Ca^{2+}]_i$ . The length change is then followed by a twitch-like tension response. In 5- to 7-day-old rats, the amplitude of the

twitch-like response is ~50 % that of the isometric twitch; it then decreases with age and is virtually absent by the time the rats are 21 days old.

Some preliminary data from this study were communicated to The Physiological Society (Mutungi & Ranatunga, 2001; Mutungi & Edman, 2002, 2003) and to the European Muscle Congress (Mutungi & Ranatunga, 2002).

## METHODS

The experiments were performed at  $20 \pm 0.1$  °C on small muscle fibre bundles isolated from both the extensor digitorum longus (EDL, a fast-twitch muscle in adult rats) and the soleus (a slow-twitch muscle in adult rats) of neonatal (5- to 21-day-old) rats. All of the procedures conformed with the UK Animals (Scientific Procedures) Act, 1986. The rats were killed with an overdose of sodium pentobarbitone given intraperitoneally, and small bundles (~10 muscle fibres in 5- to 10-day old rats, average cross-sectional diameter ~200  $\mu m$ ) dissected with the aid of dark-field illumination. Care was taken to ensure that the bundles were clean and fibres that extended from end to end in each bundle were intact and electrically excitable. For comparison, adult rat muscle fibre bundles containing two to five fibres, average cross-sectional diameter ~150  $\mu m$ , isolated from a small toe muscle (the flexor

hallucius brevis, FHB) were used. The FHB was preferred to the adult rat EDL and soleus because of the length of its fibres; these fibres are ~2 mm long and are therefore similar to those of the neonatal EDL and soleus (~3.6 mm long in 7-day-old rats). For comparison, the muscle fibres of adult rats are ~10 mm long in EDL and ~12 mm in soleus.

During an experiment, a preparation was mounted horizontally between two stainless steel hooks, one attached to a force transducer and the other to a servomotor, in a flow-through muscle chamber with a glass bottom. The preparation was perfused at the rate of 0.5 ml min<sup>-1</sup> with Ringer solution, which was continuously bubbled with a mixture of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>. The standard Ringer solution contained (mM): NaCl 109, KCl 5.0, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 4, NaHCO<sub>3</sub> 24, NaHPO<sub>4</sub> 1 and sodium pyruvate 10, with 200 mg l<sup>-1</sup> bovine calf serum. The temperature of the muscle chamber was controlled using a Peltier device fitted underneath the muscle chamber and was monitored with a thermocouple that was placed inside the trough.

The initial sarcomere length of the muscle fibre bundles was set at ~2.4–2.5 μm by laser diffraction. The bundles were then stimulated with single supramaximal stimuli once every 90 s using two platinum plate electrodes placed symmetrically ~1–1.5 mm on either side. During some cycles, triangular length changes (~20–30 % of L<sub>0</sub>) were interposed between the twitches and the tension and sarcomere length responses to the stretches recorded. The sarcomere length change over a 0.5 mm region near the force transducer end of the bundle was monitored using a He–Ne laser diffractometer (for details on the diffractometer see Mutungi & Ranatunga, 1996a,b).

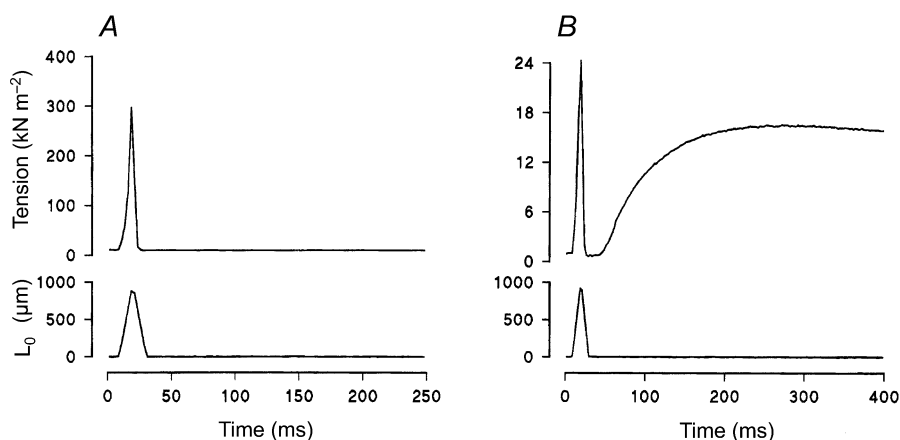
In some experiments, the intracellular Ca<sup>2+</sup> transients during twitch contractions and during length changes were monitored using the Ca<sup>2+</sup>-sensitive fluorescent dye fluo-3 AM, following the procedure described by Caputo *et al.* (1994). Briefly, each preparation was immersed in Ringer solution containing ~20 μM fluo-3 AM (Molecular Probes, Europe, Leiden, The Netherlands) for ~45–60 min. After loading, the fibre bundle was washed in

normal Ringer solution for ~20–30 min to remove all the free dye. The fluo-3 signal over a 1 mm region of the fibre bundle was monitored using a phototransistor whose output was fed into a current-to-voltage converter. We chose fluo-3 as our [Ca<sup>2+</sup>]<sub>i</sub> indicator because of the stability of its fluorescence signal, especially in muscle fibres undergoing contractions or being subjected to length changes (for details see Caputo *et al.* 1994).

[Ca<sup>2+</sup>]<sub>i</sub> was calculated from the fluo-3 signal by taking account of the on and off rate constants for the Ca<sup>2+</sup>–fluo-3 complex. For calibration of the fluo-3 signal, the maximum fluorescence signal was determined at the end of the experiment by rapidly exposing the fibre to a solution containing 0.1 mg ml<sup>-1</sup> saponin and 95 mM CaCl<sub>2</sub>. Immediately before this test, the fibre had been depolarised in an isotonic K<sup>+</sup> solution containing 20 mM 2,3-butanedione monoxime (BDM). For further details, see Sun *et al.* (1996).

In other experiments, muscle fibre bundles isolated from 5- to 7-day-old rats were bathed in either the standard Ringer solution or in Ringer solution containing any of three compounds: 1.5 mM lidocaine hydrochloride, 1 μM TTX or 25 mM KCl (the extra KCl replaced a corresponding amount of NaCl). The active twitch tension and the stretch-induced tension responses were then recorded before, during and after the exposure of the fibres to each of the compounds.

The length signal (from the motor), the sarcomere length signal (from the diffractometer) and the tension signal (from the force transducer) were then collected via a CED 1401 Micro laboratory interface using Signal 2.0 software (Cambridge Electronic Design, Cambridge, UK) and stored on a Genie-P3-500 computer (Viglen, Middlesex, UK) for further analysis. The initial analyses of the tension records, such as the half-rise time, the time to peak tension and the peak of the isometric twitch and the twitch-like tension response were made using the Signal 2.0 software. The calculation of [Ca<sup>2+</sup>]<sub>i</sub> was achieved using a program written in Matlab. In addition, the outputs from the force transducer and thermocouple were displayed continuously on an oscilloscope and a chart recorder and were used to monitor the viability of each preparation.



**Figure 1. Effects of a stretch–release cycle on resting muscle tension**

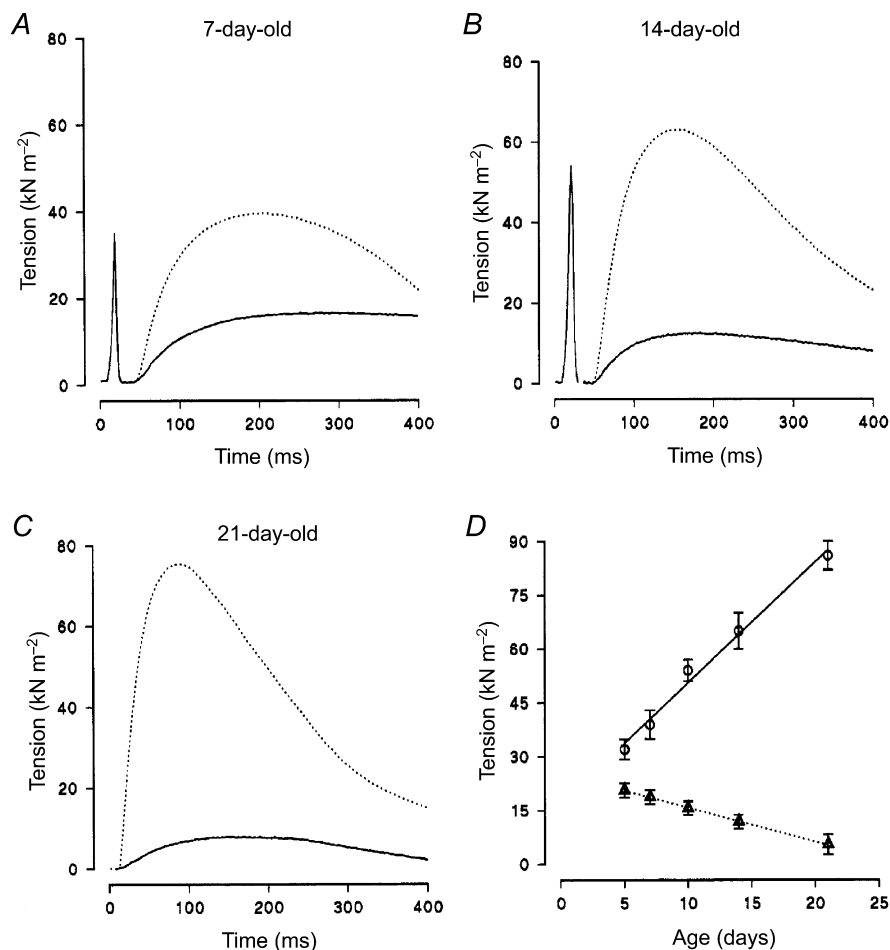
Typical tension responses to a stretch–release cycle in muscle fibre bundles isolated from the FHB of an adult male Wistar rat (A) and the EDL of a 7-day-old rat (B). Note that in both cases, the length change is accompanied by a complex transitory increase in resting tension that is larger in the adult than in the neonatal fibres. Moreover, in the 7-day-old rat, the length change is followed by a large twitch-like contraction (B). The fibre bundle in A was ~3.2 mm long and that in B, ~3.6 mm. The amplitude of the length change was ~980 μm in both A and B. The stretch–release speed was 93 mm s<sup>-1</sup> in A and 99 mm s<sup>-1</sup> in B.

All of the pooled data reported are given as means  $\pm$  S.E.M. Comparison of the data was performed using a standard Student's unpaired *t* test.

## RESULTS

The traces in Fig. 1 show the effects of a stretch–release cycle on passive (i.e. resting) tension in two muscle fibre bundles, one isolated from an adult and the other from a neonatal rat. As the traces illustrate, the resting tension in both the adult and neonatal fibre bundles increased and relaxed with the length change, as expected. However, in all of the fibres examined ( $n > 20$  fibres), the tension relaxed faster than it rose. Thus, plotting the instantaneous tension against the length change yielded a hysteresis loop at all the speeds. Moreover, the amplitude of the resting

tension responses was  $\sim 6$ – $10$  times higher in the adult than in the corresponding neonatal muscle fibre bundles. The length change was then followed, after a short delay, by a large tension increase (hereafter referred to as a stretch-induced twitch-like tension response) that was seen only in the neonatal muscle fibre bundles (Fig. 1B). The delay is probably due to the fact that the fibres are slackened on release and force only starts to develop after the fibres have taken up the slack. In 7-day-old rats and at speeds of  $> 9.8 L_o s^{-1}$ , this delay was  $34 \pm 2.8$  ms ( $n = 10$  fibres) in fibres isolated from the EDL muscle and  $46 \pm 3$  ms ( $n = 7$  fibres) in those isolated from the soleus muscle. It is also noteworthy that the active contractions recorded before and after a series of such stretches were qualitatively and quantitatively similar, suggesting that the stretches did not damage the fibres.



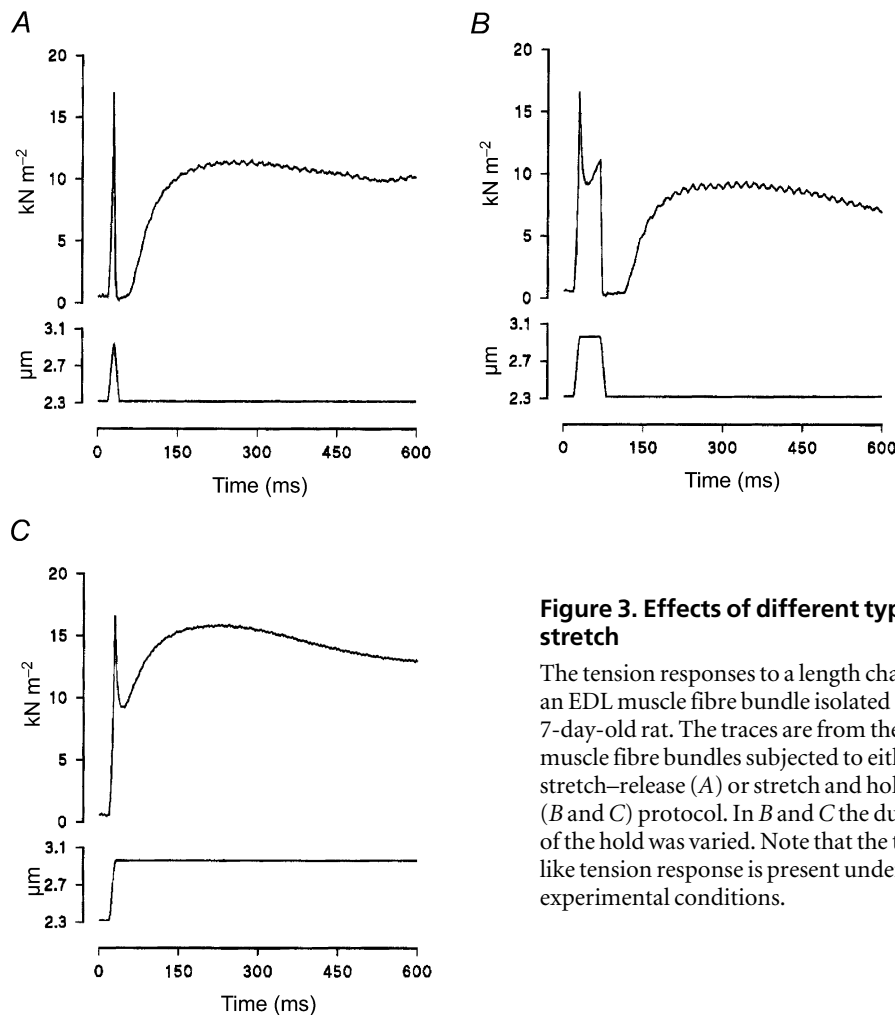
**Figure 2. Effects of age on the stretch-induced contractile activation**

A comparison between the tension responses to a stretch–release cycle (continuous line traces) and the isometric twitch (dotted line traces) in muscle fibres isolated from neonatal rats at 7 (A), 14 (B) and 21 days of age (C). Note that the twitch-like tension response decreases with age and is almost absent at 21 days of age. On the other hand, the amplitudes of both the transitory tension response accompanying the stretch and the isometric twitch force increase with age. Due to its size relative to the twitch tension and the stretch-induced twitch-like tension response, the transitory tension response is not shown in C. D, summary data from 12 EDL and nine soleus muscle fibre bundles isolated from rats of different ages (range 5–21 days). Note that the stretch-induced twitch-like tension response (triangles, dotted line) decreases with age, whereas the isometric twitch tension (circles, continuous line) more than doubles over the same period.

In 5-day-old rats, the amplitude of the stretch-induced twitch-like tension response was  $60 \pm 3\%$  ( $n = 7$  fibres) of the isometric twitch tension (recorded at the same sarcomere length). The amplitude then progressively decreased with age so that it was  $10 \pm 0.9\%$  ( $n = 6$  fibres) of the isometric twitch tension in 14-day-old rats, and was almost absent by the time the rats were 21 days old (Fig. 2). It is relevant to note that although the length of the muscle fibre bundles increased from  $2.9 \pm 0.2$  mm (for both the EDL and soleus) in 5-day-old rats to  $6.5 \pm 0.3$  mm in the EDL and  $6.8 \pm 0.4$  mm in the soleus of 21-day-old rats, the amplitude of the length change in different experiments was adjusted so that it was  $\sim 25\%$  of  $L_0$ . Most of the experiments reported in this study were performed using 7-day-old rats. At this age the twitch-like tension response was  $48 \pm 2.5\%$  (range 35–62%;  $n = 21$  fibre bundles) of the isometric twitch tension (Fig. 2). In most muscle fibre bundles, the twitch-like tension response and the isometric twitch had similar time courses. However, in some preparations the stretch-induced twitch-like tension response lasted several seconds. The cause of this difference was not clear, but it was more frequent in EDL muscle fibre bundles from rats less than 10 days old.

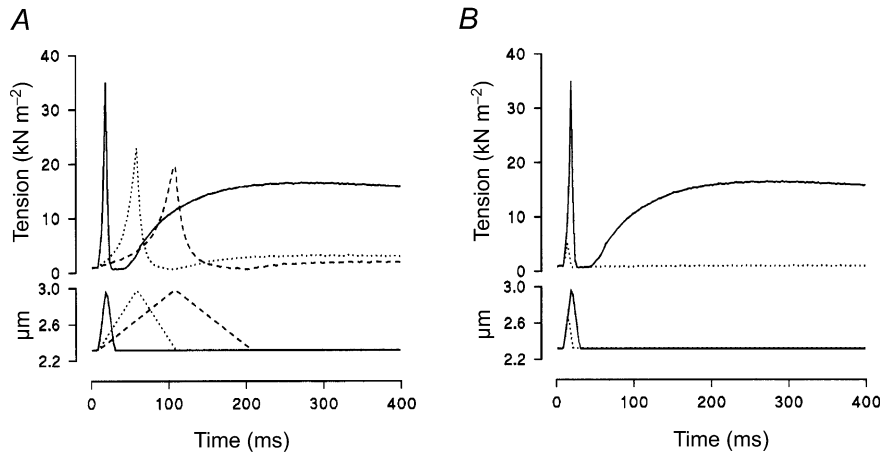
Figure 3 shows the tension responses recorded from a muscle fibre bundle from a 7-day-old rat subjected to different types of stretch–release protocols. In all of the records, the amplitude of the stretch was kept constant and only the duration of the period in which the fibre was held at the longer length was varied. The traces show that the twitch-like tension response was evident in all of the traces, irrespective of the stretch protocol adopted, even when the stretch was held at the longer length throughout the recording period, as shown in Fig. 3C. This was the case in all of the fibres investigated so long as the stretch was above a certain critical length and stretch speed (Fig. 4).

In 10 EDL and nine soleus muscle fibre bundles from 5- to 7-day-old rats, this critical stretch velocity was  $1.5 \pm 0.2 L_0 s^{-1}$  (range  $0.9$ – $2.1 L_0 s^{-1}$ ). At this speed, the stretch-induced twitch-like tension response was barely visible (Fig. 4A). Its amplitude then progressively increased with velocity, to reach a maximum at  $9.8 \pm 1.2 L_0 s^{-1}$  ( $n = 8$  fibres); thereafter, its amplitude remained relatively constant. Interestingly, the maximum shortening velocity of these fibres at  $20^\circ\text{C}$  is  $\sim 10 L_0 s^{-1}$  (Close, 1964). In the same preparations, no contractile activation was recorded at stretch amplitudes below 20% of  $L_0$  (Fig. 4B).



**Figure 3. Effects of different types of stretch**

The tension responses to a length change of an EDL muscle fibre bundle isolated from a 7-day-old rat. The traces are from the same muscle fibre bundles subjected to either a stretch–release (A) or stretch and hold (B and C) protocol. In B and C the duration of the hold was varied. Note that the twitch-like tension response is present under all the experimental conditions.

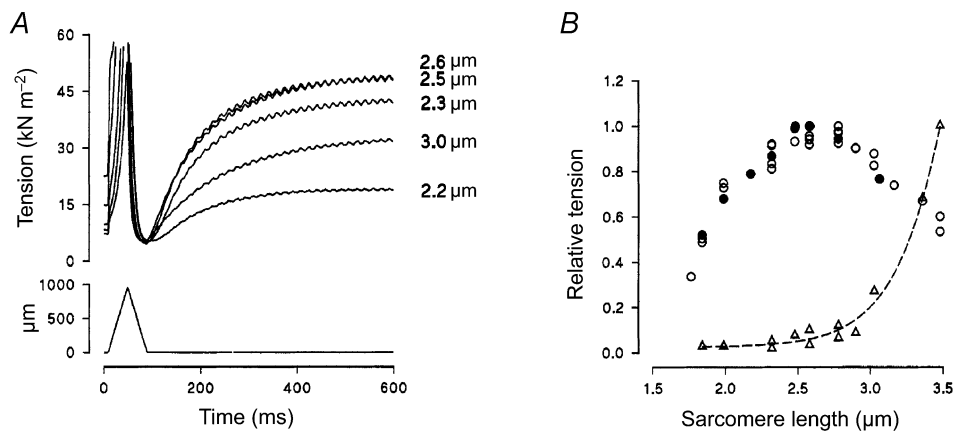


**Figure 4. Effects of the amplitude and velocity of stretch**

A, a family of traces recorded from an EDL muscle fibre bundle isolated from a 7-day-old rat, at three different stretch speeds: fast (continuous line trace), intermediate (dotted line trace) and near the critical velocity of this fibre bundle ( $\sim 2 L_0 s^{-1}$ ; dashed line trace). Note that the amplitude of the stretch-induced twitch-like response increases with velocity and is barely present at the lowest speed. B, tension records from the same muscle fibre bundle illustrating the amplitude dependency of the stretch-induced contractile activation in these fibres. Note that reducing the stretch amplitude from  $\sim 25\% L_0$  to around  $12.5\% L_0$  completely abolishes the stretch-induced twitch like response.

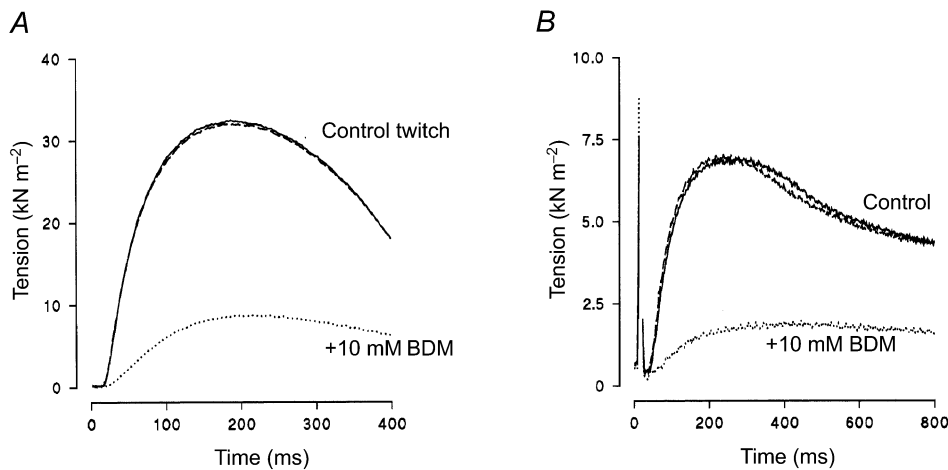
To determine whether the twitch-like tension response was due to cycling crossbridges or stress in another sarcomeric structure, we examined its sarcomere length dependency. It was assumed that if the twitch-like tension response arose from cycling crossbridges, its amplitude would scale with increasing sarcomere length in the same

way as peak isometric twitch tension. A summary of typical results obtained from muscle fibres isolated from 7-day-old rats is shown in Fig. 5. As the data shows, the stretch-induced twitch-like tension response and the isometric twitch tension from these fibres scale with sarcomere length in a similar manner, suggesting that they both arise



**Figure 5. Effects of initial sarcomere length on the tension response to a large triangular length change**

A, a family of tension traces recorded from a slow muscle fibre bundle isolated from a 7-day-old rat and subjected to a stretch-release cycle. The preparation was held at the initial sarcomere lengths shown. Note that at each sarcomere length, the length change is followed by a pronounced increase in tension, and that the amplitude of this tension increase is maximal at sarcomere lengths of between 2.5 and 2.6  $\mu m$ . B, the sarcomere length dependency of steady-state resting muscle tension (open triangles, dashed line curve), twitch tension (open circles) and the stretch-induced twitch-like tension response (filled circles) in four muscle fibre bundles (2 isolated from the EDL and 2 from the soleus). All of the tensions were normalised to their amplitudes at optimum sarcomere length. Note that tetanic tension and the delayed tension rise have similar sarcomere length dependencies.



**Figure 6. The effects of BDM on twitch tension and the stretch-induced twitch-like response**

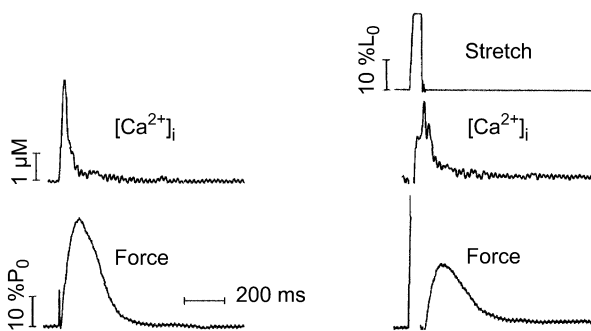
The traces are from the same muscle fibre bundles isolated from a 7-day-old rat before (continuous line traces), during (dashed line traces) and after exposure to BDM (dotted line traces). *A*, the effects of BDM on twitch. *B*, the effects of BDM on the stretch-induced tension response. Note that the addition of 10 mM BDM reversibly reduces both responses by a similar magnitude.

from cycling crossbridges. Both the isometric twitch tension and the stretch-induced twitch-like tension were found to have an optimum sarcomere length of  $\sim 2.5 \mu\text{m}$  (range  $2.4\text{--}2.6 \mu\text{m}$ , Fig. 5).

In another series of experiments, the effects of the ATPase and  $\text{Ca}^{2+}$ -release inhibitor BDM on both the isometric twitch and the stretch-induced twitch-like tension response were investigated. As Fig. 6 shows, both the isometric twitch and the stretch-induced twitch-like tension response were reversibly inhibited to more or less the same extent ( $\sim 70\%$  inhibition) by 10 mM BDM. In mammalian muscle fibres, BDM in millimolar concentrations is known to reversibly inhibit twitch tension (Fryer *et al.* 1988; Mutungi & Ranatunga, 1996c) by suppressing both  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum (SR; Fryer *et al.*

1988) and crossbridge cycling. In six EDL and six soleus muscle fibre bundles isolated from 5- to 7-day-old rats, the addition of 10 mM BDM led to a  $75 \pm 5\%$  reduction in the isometric twitch tension and to a  $70 \pm 6\%$  drop in the stretch-induced twitch-like tension response.

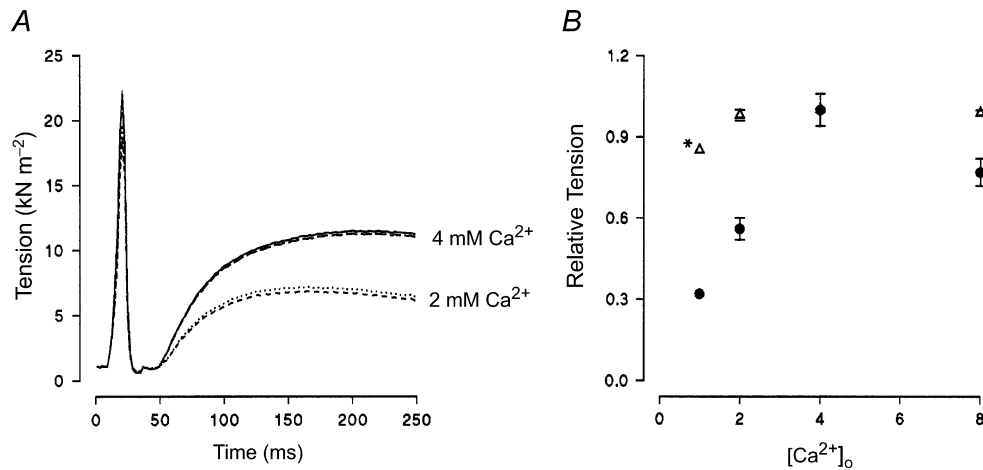
From these observations we hypothesised that the length change elicited an increase in  $[\text{Ca}^{2+}]_i$  that led to the activation of the contractile system and hence the twitch-like tension response. To examine whether this was indeed the case, the  $[\text{Ca}^{2+}]_i$  during both active contractions and stretch-release cycles in four fast-twitch and four slow-twitch muscle fibre bundles isolated from neonatal rats aged between 5 and 12 days, was monitored using the  $\text{Ca}^{2+}$ -sensitive dye fluo-3. As the results in Fig. 7 show, in a 7-day-old rat, the stretch was indeed accompanied by a large transient increase in  $[\text{Ca}^{2+}]_i$ . In all of the muscle fibre bundles examined, there was a good correlation between the amplitude of the twitch-like tension response and the amount of intracellular  $\text{Ca}^{2+}$  released.



**Figure 7. Effects of a stretch-release cycle on force and  $[\text{Ca}^{2+}]_i$**

The traces are from an EDL fibre bundle isolated from a 5-day-old rat. Left panel: isometric twitch. Right panel: passive stretch-release movement. Note that the length change is accompanied by a pronounced increase in  $[\text{Ca}^{2+}]_i$  and a by a twitch-like tension response after the stretch.  $P_0$ , maximum isometric tension.

Is the stretch-induced twitch-like tension response sensitive to the levels of  $[\text{Ca}^{2+}]_o$ ? To investigate this, the effects of varying  $[\text{Ca}^{2+}]_o$  on both the isometric twitch and the twitch-like tension response were examined in six (4 neonatal and 2 adult) muscle fibre bundles. The bundles were exposed to Ringer solution containing 2, 4 or 8 mM  $\text{Ca}^{2+}$  and the isometric twitch tension and the tension responses to a  $25\%$   $L_0$  length change were recorded in control, test and finally in control Ringer solution. In one experiment (4 observations), involving a neonatal fibre bundle, the effects of 1 mM  $\text{Ca}^{2+}$  on both the isometric twitch and the twitch-like tension response were also examined. The results shown in Fig. 8 illustrate that decreasing the  $[\text{Ca}^{2+}]_o$  from 4 to 2 mM led to a  $43 \pm 3\%$  inhibition of the stretch-induced twitch-like tension



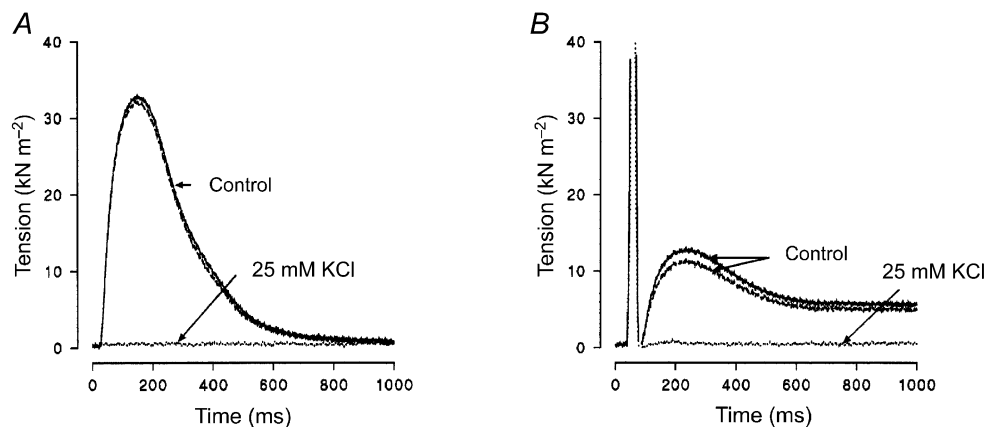
**Figure 8. Effects of lowering  $[Ca^{2+}]_o$ .**

*A*, tension traces recorded from a soleus muscle fibre bundle of a 7-day-old rat in Ringer solution containing either 4 (continuous and long-dash line traces) or 2 mM (dotted and dashed line traces)  $Ca^{2+}$ . The traces were recorded in 4 (continuous line trace), 2 (dashed line trace), 2 (dotted line trace) and finally 4 mM (long-dash line trace)  $[Ca^{2+}]_o$ . Note that lowering the  $[Ca^{2+}]_o$  to 2 mM significantly reduced the stretch-induced twitch-like tension response, and that the observations were highly repeatable. *B*, summary data showing the effects of varying the  $[Ca^{2+}]_o$  on the isometric twitch (triangles) and the stretch-induced twitch-like tension response (circles) in four muscle fibre bundles isolated from 7-day-old rats. The data have been normalised to that recorded in 4 mM  $Ca^{2+}$ . The data points for 1 mM  $[Ca^{2+}]_o$  were recorded from a single preparation only. Note that lowering or increasing the  $[Ca^{2+}]_o$  from 4 to 2 or to 8 mM significantly depresses the amplitude of the twitch-like tension response ( $P < 0.001$ ), but not that of the isometric twitch. The isometric twitch was only significantly ( $P < 0.001$ ) affected when the  $[Ca^{2+}]_o$  was reduced to 1 mM (\*).

response, but had little or no effect on the isometric twitch in both the adult and neonatal muscle fibre bundles. Further reduction of the  $[Ca^{2+}]_o$  to 1 mM led to a 68% depression of the stretch-induced twitch-like tension response and a 15% drop in the isometric twitch tension. This inhibition of isometric twitch tension is similar to that reported previously in neonatal mouse skeletal muscles (Dangain & Neering, 1991). On the other hand,

increasing the  $[Ca^{2+}]_o$  from 4 to 8 mM led to a  $23 \pm 3\%$  depression of the stretch-induced twitch-like tension response without significantly ( $P > 0.05$ ) affecting the isometric twitch in either the neonatal or adult muscle fibres.

The results shown in Fig. 8 suggest that  $[Ca^{2+}]_o$  is essential for the stretch-induced increase in  $[Ca^{2+}]_i$ . Moreover, as the results in Fig. 7 show, the increase in  $[Ca^{2+}]_i$  occurs in



**Figure 9. The effects of 25 mM KCl**

Active twitch myograms (*A*) and the stretch-induced twitch-like tension responses (*B*) recorded from a 7-day-old EDL fibre bundle. Each frame shows a family of three records, two in control Ringer solution (before and after high KCl exposure) and the other in 25 mM KCl. Note that the depolarisation of the fibre bundle with KCl reversibly abolishes both the active twitch tension and the stretch-induced twitch-like tension response.



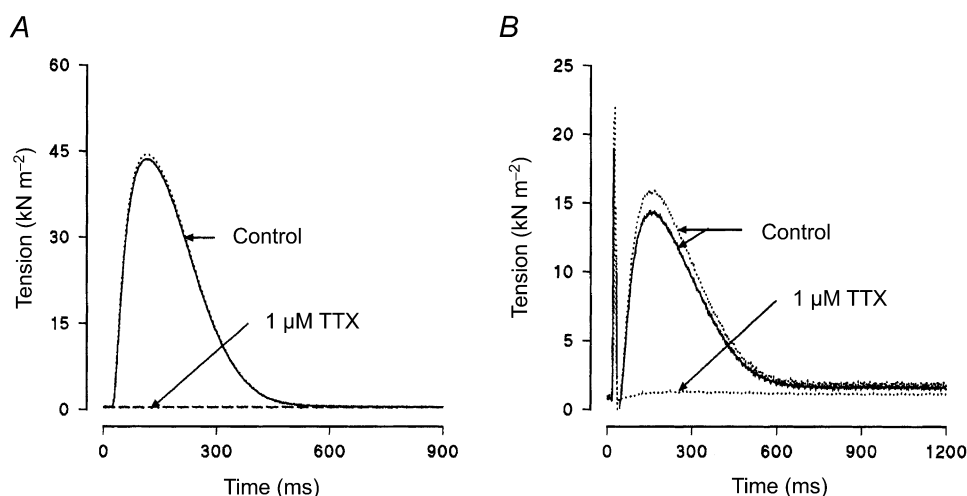
milliseconds and its characteristics are qualitatively similar to those of the  $\text{Ca}^{2+}$  transient induced by the electrical stimulus. Therefore, to investigate whether membrane excitation is essential for the twitch-like tension response to occur, the resting potential in 10 muscle fibre bundles (5 EDL, 5 soleus) of 5- to 7-day-old rats was reduced below its excitability level by exposing the fibres to Ringer solution containing high  $[\text{K}^+]_o$ . Preliminary experiments using various amounts of  $[\text{K}^+]_o$  showed that 25 mM KCl reversibly abolished the isometric twitch in both adult and neonatal rat muscle fibre bundles. As the results in Fig. 9 show, exposing the fibre bundles to high  $[\text{K}^+]_o$  completely but reversibly abolished both the active twitch tension and the stretch-induced twitch-like tension response. For the twitch-like tension response to be completely abolished, the bundle had to be totally electrically unexcitable (i.e. a stimulus could not elicit a twitch contraction).

The high- $\text{K}^+$  experiments described herein showed that a change in resting membrane potential was essential for the stretch-induced twitch-like tension response to occur, but not the ions involved. Under physiological conditions,  $I_{\text{Na}}$  (a voltage-dependent inward  $\text{Na}^+$  current) is the dominant inward current in neonatal rat skeletal muscle fibres (Beam & Knudson, 1988). Therefore, to investigate whether  $\text{Na}^+$  were involved in the development of the twitch-like tension response, we examined the effects of TTX in six muscle fibre bundles, three isolated from the EDL and three from the soleus of 5- to 7-day-old rats. The fibre bundles were bathed continuously in either the standard Ringer solution or in one containing  $1 \mu\text{M}$  TTX. Although neonatal muscle fibres have been shown to contain TTX-

insensitive  $\text{Na}^+$  channels (Thesleff *et al.* 1974), preliminary experiments showed that  $1 \mu\text{M}$  TTX completely and reversibly abolished active twitch tension in muscle fibre bundles isolated from both adult and neonatal rats. However, its effects at higher doses were irreversible. Typical results from these experiments are shown in Fig. 10. As the results show, TTX completely abolished the isometric twitch tension and stretch-induced twitch-like tension response in neonatal muscle fibres, and its effects were reversible. As in the case of high KCl, all of the fibres in a bundle had to be rendered completely unexcitable for the twitch-like tension response to be totally abolished. Similar results were obtained in 10 fibre bundles (5 EDL and 5 soleus) isolated from 5- to 7-day-old rats exposed to 1.5 mM lidocaine, another  $\text{Na}^+$  channel blocker (results not illustrated).

## DISCUSSION

The results reported here show that the effects of a length change of  $\sim 25\%$   $L_o$  in unstimulated intact mammalian muscle fibre bundles depend on the age of the rats from which they were isolated. Thus, the length change had two effects on tension in neonatal rats, but only a single effect in adult ones. In both the adult and neonatal rats, the length change led to a complex increase in resting muscle tension that yielded a tension hysteresis loop when plotted against the length change. Resting intact mammalian muscle fibres have been shown to possess both viscous and viscoelastic properties (Mutungi & Ranatunga, 1996a,b) and it is therefore not surprising that the resting tension relaxes faster than it develops. What is more interesting is the



**Figure 10. The effects of TTX**

Twitch myograms (A) and the stretch-induced twitch-like tension responses (B) recorded from 7-day-old rat EDL before (continuous line traces), during (dotted line traces) and after (dashed line traces) the exposure to Ringer solution containing  $1 \mu\text{M}$  TTX. Note that, like high KCl, TTX reversibly abolishes both the active twitch myogram and the stretch-induced twitch-like tension response.

observation that the resting tension responses were an order of magnitude higher in the adult than in neonatal rats.

In the adult sarcomere, most of the steady-state resting force resides in the I-band region of the titin (also known as connectin) molecule known as the gap filament (Horowitz *et al.* 1986). Each gap filament consists of six titin molecules, which in the A-band region are closely associated with the thick filament (Liversage *et al.* 2001). The psoas muscle of neonatal rats also contains ~80 % of the myofibrillar density (per fibre) and twice the ground substance (i.e. the cell matrix interspersed among myofibrils, sarcotubular elements and mitochondria) compared with adult rat muscle (Schiaffino & Margreth, 1969). Therefore, the lower resting tension responses in neonatal rats may reflect these structural differences.

The main observation in this study was that the length change in the neonatal rat muscle fibres was followed, after a short delay, by a large twitch-like tension response. It is important to bear three things in mind: (1) the present studies were performed at 20 °C, (2) the muscle fibre bundles were unstimulated (i.e. relaxed) and (3) in 5- to 7-day-old rats, the stretch-induced twitch-like tension response was ~50 % of the isometric twitch tension recorded at the same sarcomere length. Although a delayed tension rise has been reported previously in relaxed, chemically skinned rabbit psoas (Ranatunga, 1994) and resting intact rat muscle fibres (Mutungi & Ranatunga, 1996c), it is noteworthy that these fibres were from adult animals and the fibres were subjected to ramp stretches of 2–3 %  $L_o$ . Furthermore, this kind of stretch activation manifested itself as a small tension rise (~1–2 % of isometric twitch in the intact fibre bundles) and was only seen at temperatures above 28 °C. The chemically skinned muscle fibres were also immersed in relaxing solution containing the  $Ca^{2+}$  buffer EGTA. Therefore, it is likely that this kind of stretch-induced contractile activation occurs due to thermal stress in the gap filament and does not require an increase in  $[Ca^{2+}]_i$  (Ranatunga, 1994; Mutungi & Ranatunga, 1996c).

Another novel finding in the present study was that in the neonatal fibre bundles, the stretch was accompanied by a transient increase in  $[Ca^{2+}]_i$  (Fig. 7). In addition, the stretch-induced twitch-like tension response was reversibly abolished by TTX and lidocaine, both of which are  $Na^+$  channel blockers, and by high  $[K^+]_o$ , which reduced the resting membrane potential below its excitability level. It was also significantly depressed by either lowering or increasing the  $[Ca^{2+}]_o$ . Although the exact mechanism(s) underlying these observations remains uncertain, neonatal rat muscle fibres have been shown to possess two inward, voltage-dependent  $Ca^{2+}$  currents,  $I_{fast}$  and  $I_{slow}$ . Moreover, peak  $I_{fast}$  decreases with age in the same manner as the stretch-induced

twitch-like tension response (the present study), and is absent in adult rat muscle fibres. On the other hand, peak  $I_{slow}$  increases with age and is the dominant inward  $Ca^{2+}$  current in adult rat fibres (Beam & Knudson, 1988). Therefore, one possible mechanism is that the length change induces membrane depolarisation, probably by perturbing  $Na^+$  channels located either on the sarcolemma or on T-tubules, and this leads to  $Ca^{2+}$  influx via  $I_{fast}$ .

In skeletal muscles, the ryanodine receptor (RyR) is responsible for the transient increase in  $[Ca^{2+}]_i$  that follows cell depolarisation. Of the three isoforms of the receptor described so far, only type 1 (RyR1) and type 3 (RyR3) are expressed in skeletal muscles (see reviews by Franzini-Armstrong & Protasi, 1997, and Sorrentino & Reggiani, 1999). RyR1 is the main isoform expressed in adult mammalian skeletal muscles (Block *et al.* 1988), whilst neonatal mammalian muscles contain equal amounts of RyR1 and RyR3 (Tarroni *et al.* 1997; Bertocchini *et al.* 1997). RyR1 is coupled to the dihydropyridine receptor (DHPR) and plays a central role in voltage-dependent  $Ca^{2+}$  release from the SR. On the other hand, RyR3 is not coupled to the DHPR and is thought to amplify the effects of RyR1 via  $Ca^{2+}$ -induced  $Ca^{2+}$  release (Bertocchini *et al.* 1997; Yang *et al.* 2001). Therefore, another possibility is that the membrane depolarisation caused by the length change, in the neonatal rat muscle fibre bundles, induces some  $Ca^{2+}$  release from the SR via the DHPR-coupled RyR1. The increase in  $[Ca^{2+}]_i$  then activates RyR3, leading to further  $Ca^{2+}$  release. Indeed, the lack of RyR3 and the presence of a well-developed  $[Ca^{2+}]_i$ -buffering system in the adult fibre bundles may explain why the length change does not lead to a twitch-like tension response in these fibres. However, to what extent each of these mechanisms contributes to the stretch-induced increase in  $[Ca^{2+}]_i$  is uncertain.

As the observations reported here show, the amplitude of the stretch-induced twitch-like tension response progressively decreased with age and was practically absent by the time the rats were 21 days old (Fig. 2). It is noteworthy that: (1) this is the same period over which rat muscles differentiate from their neonatal to adult types (Close, 1964; Whalen *et al.* 1981) and (2) the length changes used in this study are within the range shown to occur when mammalian muscles are passively stretched *in situ* (Herbert *et al.* 2002). Is this a coincidence, or are postnatal muscle differentiation and the stretch-induced increases in  $[Ca^{2+}]_i$  interlinked? We speculate that an increase in  $[Ca^{2+}]_i$  in neonatal rat muscle fibres may be somehow coupled to postnatal muscle differentiation/myogenesis. The stretch-induced  $Ca^{2+}$  release and twitch-like tension response in the isolated neonatal fibre bundles may indeed be an integral part of this coupling.

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