

## Skeletal muscle function: the role of ionic changes in fatigue, damage and disease

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### Summary

1. Repeated activity of skeletal muscle causes a variety of changes in its properties; muscles become weaker with intense use (fatigue), may feel sore and weak after repeated contractions involving stretch, and can degenerate in some disease conditions. This review considers the role of early ionic changes in the development of each of these conditions.

2. Single fibre preparations of mouse muscle were used to measure ionic changes following activity-induced changes in function. Single fibres were dissected with intact tendons and stimulated to produce force. Fluorescent indicators were micro-injected into the fibres to allow simultaneous ionic measurements together with mechanical performance.

3. One theory to explain muscle fatigue is that it is caused by accumulation of lactic acid producing an intracellular acidosis which inhibits the myofibrillar proteins. In contrast we found that during repeated tetani there was little or no pH change but failure of calcium release was a major contributor to fatigue. Currently it is proposed that precipitation of calcium and phosphate in the sarcoplasmic reticulum contributes to the failure of calcium release.

4. Muscles can be used to shorten and produce force or they can be used to decelerate loads (stretched or eccentric contractions). A day after intense exercise involving stretched contractions muscles are weak, sore and tender and this damage can take a week to recover. In this condition sarcomeres are disorganised and there are increases in resting intracellular  $\text{Ca}^{2+}$  and  $\text{Na}^+$ . Recently we demonstrated that the elevation of  $\text{Na}^+$  occurs through a stretch-activated channel which can be blocked by either gadolinium or streptomycin. Preventing the rise of  $[\text{Na}^+]_i$  with gadolinium also prevents part of the muscle weakness after stretched contractions.

5. Duchenne muscular dystrophy is a lethal degenerative disease of muscles in which the protein dystrophin is absent. Dystrophic muscles are more susceptible to stretch-induced muscle damage and the stretch-activated channel seems to be one pathway for the increases in intracellular  $\text{Ca}^{2+}$  and  $\text{Na}^+$  which are a feature of this disease. We have recently shown that blockers of the stretch-activated channel can minimize some of the short-term damage in muscles from the *mdx* mouse, which also lacks dystrophin. Currently we are testing whether blockers of the stretch-activated channels given systemically to *mdx* mouse can protect against some features of this disease.

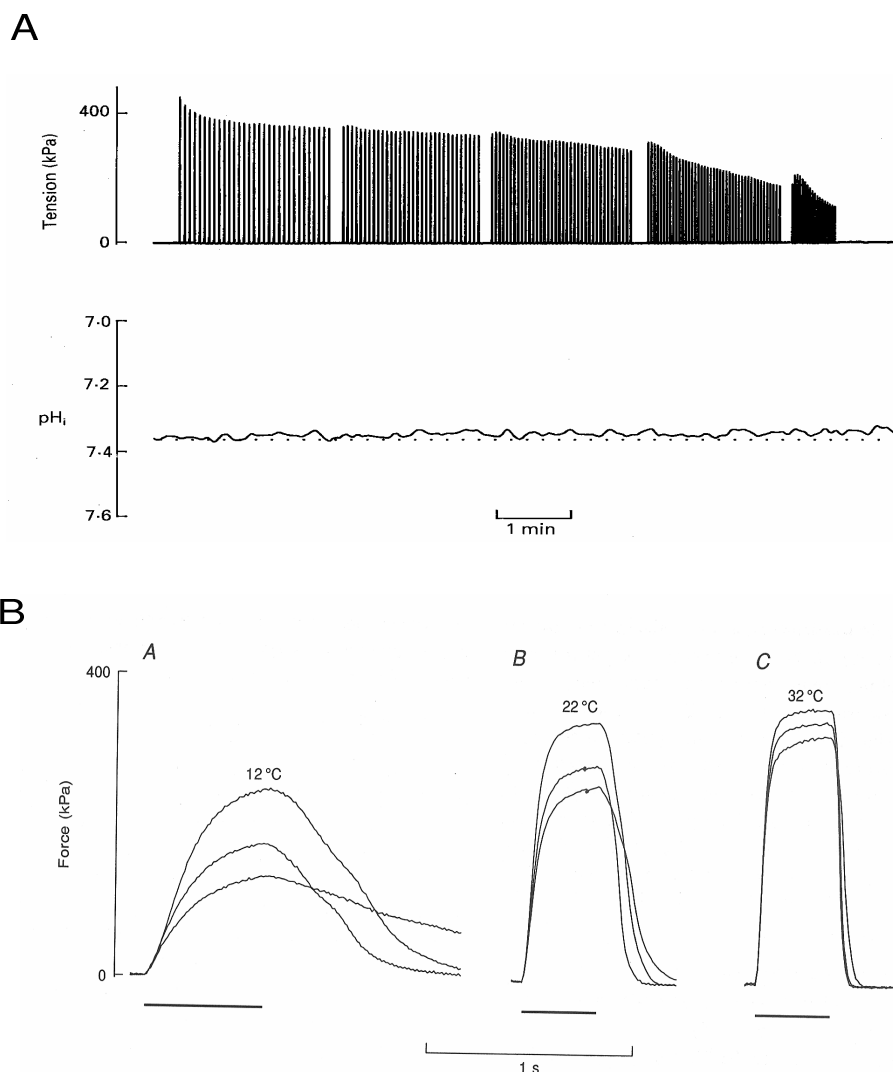
### Introduction

Ionic changes are central to the activity of muscle. The action potential is caused by rapid movements of  $\text{Na}^+$  into the cell and  $\text{K}^+$  out of the cell. The action potential in the T-tubules triggers rapid release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum into the myoplasm where it binds to troponin initiating cross bridge cycling. An early source of energy is the anaerobic breakdown of glycogen whose products are lactate and protons. Thus changes in the intracellular concentrations of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{H}^+$  all occur as part of normal muscle activity. In the studies described in this review we are concerned with the changes in muscle function which accompany repeated activity. We show that each of the above cations can change during repeated muscle activity and analyse how this changes contribute to muscle function.

Our approach to these issues has been to develop the single mammalian muscle fibre preparation first described by Lännergren and Westerblad<sup>1</sup>. Single fibres are dissected from the *flexor brevis* muscle of the mouse, clips are attached to the tendons at either end and the muscle fibre can then be attached to a tension transducer and a motor to impose length changes. Electrodes running parallel to the fibre allow stimulation. Normally fibres are continuously perfused by a physiological salt solution with pH buffered by  $\text{HCO}_3^-/\text{CO}_2$ . These fibres can be penetrated with microelectrodes and microinjected with fluorescent dyes or many other substances e.g. ions, drugs, peptides, proteins, DNA plasmids. At the end of the experiment fibres can be fixed for light or electron microscopy or subject to immunofluorescence. The attractions of this approach are that any sequence of stimulation (twitches, tetani, repeated in any pattern) or contraction type (isometric, shortening or lengthening) can be imposed on the fibre and the force and fluorescence can be monitored from a single cell during activity. In the experiments described in this review we have used fluorescent  $\text{Ca}^{2+}$ ,  $\text{Na}^+$  or pH indicators to allow continuous measurements of these ions. With use of an imaging microscope the distribution of these ions within a single cell can also be determined.

### Muscle fatigue

It is a common experience that the performance of muscle gradually declines when muscles are used repeatedly at near their maximum force. This decline of performance, or muscle fatigue, is reflected in reduced force production, reduced shortening velocity and a slower time course of contraction and relaxation. Of course muscles



**Figure 1. pH has minimal role in muscle fatigue.**

Panel A shows measurement of myoplasmic pH throughout a period of fatigue caused by repeated brief tetani. Data from single mouse muscle fibre at 22°C. Note that myoplasmic pH changed little despite the development of fatigue. Data from Westerblad & Allen<sup>6</sup>. Panel B shows tetanic force from mouse single fibres at three different temperatures (12°C, 22°C and 32°C). At each temperature three tetani are shown with intracellular pH modified by changes in external [CO<sub>2</sub>]. From above down, the intracellular pHs are respectively 0.5, 0 and -0.5 greater than the resting level. Note that the effect of a 1 pH unit change in intracellular pH is much greater at 12°C than at 32°C. Tension or force measurements are normalized as the force per cross-sectional area of the fibre and are quoted in units of kPa where a Pascal is one Newton metre<sup>-2</sup>. Data from Westerblad et al.<sup>8</sup> (reproduced with the permission of the copyright holder).

can be used near their maximal capacity in many different activities e.g. maximal continuous isometric contractions such as lifting a piano, repeated contractions such as running 100 m or a marathon, repeated stretched contractions such as walking down a mountain and it would be expected that these different activities would affect muscle function in different ways. Equally important many different diseases cause skeletal muscle weakness e.g. muscular dystrophies, cardiac failure, renal failure, starvation, chronic infections etc. and surveys show that complaints about muscle weakness and fatigue are among

the commonest presenting symptoms in medical consultations<sup>2</sup>. Of particular importance is the fact that all elderly humans suffer a gradual loss of muscle mass and the consequent weakness and rapid fatigue during every day activities contribute to the loss of mobility and independence. Single fibres can be useful in the investigation of many of these situations by appropriate choice of conditions.

The present review will consider the muscle fatigue caused by repeated short isometric tetani e.g. Figs 1 & 2. These figures show that when short (0.3 s) maximal tetani

are repeated every few seconds, the force produced declines to 50% within a few minutes. The time scale of this experiment is similar that involved when running 1-2 km or swimming 200-500 m and it seems reasonable to suppose that the intracellular mechanisms within the muscles are similar.

### Lactic acid as the cause of skeletal muscle fatigue

Since the pioneering research of A.V. Hill, the accumulation of intracellular lactic acid has been a dominant theory of muscle fatigue<sup>3</sup>. Lactic acid accumulates in many intense fatiguing regimes and can lead to an intracellular acidosis of about 0.5 pH units. There are two major lines of evidence that have been used to link this decline of intracellular pH to the contractile dysfunction in fatigue. First, studies on human muscle fatigue of rapid onset have often shown a good temporal correlation between the decline of intracellular muscle pH and the reduction of force or power production. Second, studies on skinned skeletal muscle fibres have shown that acidification reduces the isometric force by a direct effect on the isolated myofibrillar proteins<sup>4</sup>.

We therefore measured the intracellular pH in our mouse single fibre model of fatigue and found, to our initial surprise, that there was only a small acidosis of around 0.06 pH units (Fig. 1A). Later we showed in the same preparation that if the duty cycle (fraction of time the fibre is stimulated at 100 Hz) was increased the muscles fatigued more rapidly and the acidosis was greater<sup>5</sup>. We also found that blocking the lactate transporter with cinnamate, substantially increased the magnitude of the resulting acidosis<sup>6</sup>. Both these results suggest that lactic acid is produced during intense stimulation but can leave the cell at a substantial rate on the lactate transporter and consequently the intracellular acidosis is reduced. Because it is intracellular acidosis which affects the contractile proteins, it would be predicted that in longer or less intense stimulation protocols the acidosis would be smaller and that fatigue caused by this component would also be reduced. These experiments are compatible with the idea that when intracellular acidosis does occur it contributes to fatigue but, more important, they make it clear that there must be alternative mechanisms of fatigue which dominate when the timecourse is greater than a few minutes and are unrelated to acidosis.

Recent experiments have cast further doubt on the lactic acid theory. Early experiments showing that acidosis reduced the force produced by the myofibrillar proteins were generally performed at room or lower temperatures. When such experiments were repeated nearer body temperature, the magnitude of the inhibitory effect of acidosis was found to be much lower<sup>7</sup>. This is also true for intact fibres and Fig 1B illustrates the inhibitory effects of changes in intracellular acidosis produced by changes in extracellular CO<sub>2</sub><sup>8</sup>; in each panel the smallest tetanus is 1 pH unit more acid than the largest tetanus. Note that at 12°C an intracellular acidosis of 1 pH units reduces force by about 47% whereas at 32°C the same acidosis only

reduces force by about 11%.

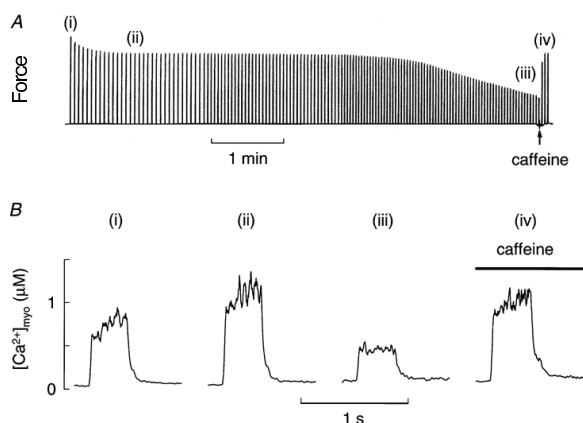
To sum up, acidosis has little direct effect on the force production in mammalian muscles studied at physiological temperatures (for review see <sup>9</sup>). However it remains true that production of lactic acid is of great importance in exercise physiology and the training of athletes. When glycogen is consumed anaerobically to produce lactic acid, the ATP production is 3 ATP per glycosyl unit whereas aerobic metabolism within the mitochondria supplies 39 ATP per glycosyl unit. Thus, the glycogen store is more rapidly depleted when large amounts of lactic acid are produced anaerobically and muscle performance is severely depressed at low glycogen levels. Also high levels of lactic acid in the blood contribute to the discomfort and breathlessness when performing at close to maximum levels of oxygen consumption<sup>10</sup>.

### Role of intracellular calcium in skeletal muscle fatigue

Given that changes in intracellular pH are not the main cause of fatigue, we examined the role of intracellular calcium. The classic work of Eberstein and Sandow (1963) first suggested that changes in activation played an important role in fatigue<sup>11</sup>. They fatigued intact muscles with repeated tetani until force was greatly reduced and then increased the level of activation by increasing extracellular K<sup>+</sup> or application of caffeine; both agents cause increased Ca<sup>2+</sup> release from the sarcoplasmic reticulum (SR). Both these manoeuvres increased force substantially in the fatigued muscle suggesting that a reversible failure of activation was an important contributor to fatigue. A recent example of this approach is shown in Fig. 2A which illustrates how a moderate concentration of caffeine can reverse much of the decline of force in a fatigued muscle. The rise in intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) which activates the contractile proteins (Fig. 2B(i)), initially increases tetanic [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 2B(ii)), but then tetanic [Ca<sup>2+</sup>]<sub>i</sub> declines during fatigue (Fig. 2B(iii)). Agents such as caffeine, which increase the opening of the SR Ca<sup>2+</sup> release channels (ryanodine receptors), can increase the amplitude of tetanic [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 2B(iv)) and thus overcome much of fatigue. Thus the partial failure of SR Ca<sup>2+</sup> release is accepted to be one of the causes of muscle fatigue<sup>12-14</sup>.

What causes the reduced Ca<sup>2+</sup> release which can be reversed by caffeine? In recent years it has become increasingly clear that increased inorganic phosphate (P<sub>i</sub>) can affect fatigue development by acting on SR Ca<sup>2+</sup> handling (for review see<sup>15</sup>). Studies in intact muscles show that the resting [P<sub>i</sub>]<sub>i</sub> is 1-5 mM<sup>16</sup> while during intense contraction it can rise to 30-40 mM<sup>17</sup>. It is already established that increasing [P<sub>i</sub>]<sub>i</sub> reduces crossbridge force and Ca<sup>2+</sup>-sensitivity of the myofilaments<sup>18</sup> and probably contributes to the early fall in force (within 1 min) shown in Fig. 2A. There are several mechanisms whereby P<sub>i</sub> might influence SR Ca<sup>2+</sup><sup>15</sup>; here we consider only the Ca<sup>2+</sup> precipitation theory.

The solubility product of Ca<sup>2+</sup> and P<sub>i</sub> is 6 mM<sup>2</sup><sup>19</sup> and this product can be exceeded in the extracellular space



**Figure 2. Muscle fatigue is partly caused by failure of SR  $\text{Ca}^{2+}$  release.**

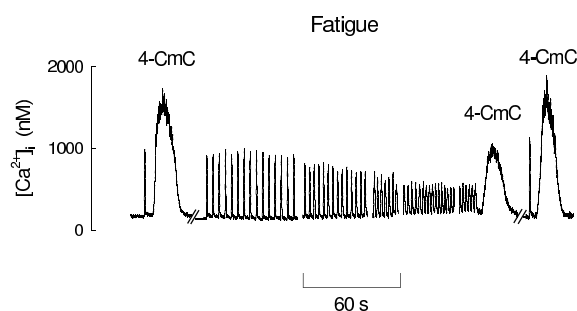
Panel A shows force production from a mouse single fibre stimulated to give repeated brief tetani at gradually reducing intervals until force had declined to ~40% of control. At that time caffeine (10 mM) was applied which reversed much of the decline of force. Panel B shows  $[\text{Ca}^{2+}]_i$  records of selected tetani from experiments similar to Panel A. (i) is the first tetanus, (ii) is at the end of the early decline of force, (iii) is a fatigued tetanus just before the addition of caffeine, and (iv) is in the presence of caffeine. These data show that a caffeine-reversible decline in tetanic  $[\text{Ca}^{2+}]_i$  is responsible for much of the late phase of decline of force. Figure reproduced from Allen & Westerblad<sup>15</sup>.

resulting in the production of bone. In the intracellular environment the very low  $[\text{Ca}^{2+}]_i$  generally prevents precipitation but in the SR the  $[\text{Ca}^{2+}]_{\text{SR}} = 1 \text{ mM}$ , so if  $[\text{P}_i]_{\text{SR}}$  exceeds 6 mM then  $\text{CaP}_i$  will start to precipitate. Thus if  $\text{P}_i$  enters the SR during fatigue, this could result in  $\text{CaP}_i$  precipitation and hence decrease the  $\text{Ca}^{2+}$  available for release.

This mechanism has recently gained support from studies using many different experimental approaches. In initial experiments on skinned fibres with intact T-tubular-SR system it was shown that increased  $\text{P}_i$  could depress SR  $\text{Ca}^{2+}$  release<sup>19</sup>. These authors also provided indirect evidence that  $\text{P}_i$  may reach a concentration in the SR high enough to exceed the threshold for  $\text{CaP}_i$  precipitation. A second indication that  $\text{P}_i$  has effects other than directly on the myofilaments came from a study in which  $\text{P}_i$  was directly injected into muscle cells<sup>20</sup>. We were expecting to see reduced force and  $\text{Ca}^{2+}$ -sensitivity due to the direct effects of  $\text{P}_i$  on the myofilaments but, to our surprise, these effects were hardly apparent and instead there was a drastic reduction in SR  $\text{Ca}^{2+}$  release which caused a fall in force. Since the expected effects of  $\text{P}_i$  on myofilaments were largely absent, we reasoned that most of the injected  $\text{P}_i$  had entered the SR, precipitated as  $\text{CaP}_i$ , and consequently reduced SR  $\text{Ca}^{2+}$  release.

Another approach to this issue has been to measure

SR  $\text{Ca}^{2+}$  stores in the expectation that the  $\text{Ca}^{2+}$  available for release might decline if free  $\text{Ca}^{2+}$  became sequestered as precipitated  $\text{CaP}_i$  within the SR. 4-Chloro-*m*-cresol (4-CmC) is a drug which, like caffeine, rapidly opens the SR  $\text{Ca}^{2+}$  channels allowing most of the rapidly-releasable SR  $\text{Ca}^{2+}$  to enter the myoplasm. Thus in Fig. 3 the initial vertical line represents the rise in  $[\text{Ca}^{2+}]_i$  caused by tetanic stimulation while the application of 4-CmC produces a larger and slower rise in  $[\text{Ca}^{2+}]_i$  whose magnitude represents the  $\text{Ca}^{2+}$  available for release in the SR. Note that during fatigue the peak tetanic  $[\text{Ca}^{2+}]_i$  signal rises and then falls and that in the fatigued muscle, a second 4-CmC application shows the SR  $\text{Ca}^{2+}$  content to be reduced. Both the tetanic  $[\text{Ca}^{2+}]_i$  and the SR  $\text{Ca}^{2+}$  content recover over the next 20 min. Measurements of the SR  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{SR}}$ ) using a  $\text{Ca}^{2+}$  indicator located in the SR have also shown a decrease in fatigued cane toad fibres<sup>18</sup>.



**Figure 3. SR  $\text{Ca}^{2+}$  stores decline during fatigue.**

$[\text{Ca}^{2+}]_i$  recorded from a single cane toad fibre from the lumbrical muscle at 22°C. The first record shows a single short tetanus followed by ~10 s application of 4-chloro-*m*-cresol (4-CmC). This drug opens SR  $\text{Ca}^{2+}$  release channels and the large rise in  $[\text{Ca}^{2+}]_i$  represents the amount of rapidly releasable SR  $\text{Ca}^{2+}$ . Similar results can be obtained with caffeine. The fibre was then rested for 20 min and then fatigued with repeated brief tetani until the tetanic force (not shown) was reduced to 40%. 4-CmC was then reapplied and the amount of rapidly releasable SR  $\text{Ca}^{2+}$  was reduced compared to control. The fibre was then rested for 20 min and showed a recovery of tetanic  $[\text{Ca}^{2+}]_i$  and the rapidly releasable  $\text{Ca}^{2+}$ . These data show that the rapidly-releasable  $\text{Ca}^{2+}$  in the SR store declines during fatigue and recovers after a period of rest. Adapted from Kabbara & Allen<sup>68</sup>.

Dahlstedt and colleagues have made use of the creatine kinase knockout mouse as another way to investigate this possibility<sup>21</sup>. In this animal, because of the absence of creatine kinase, the usual rise of  $\text{P}_i$  observed during fatigue is absent. They found that in muscles which lack the rise of  $\text{P}_i$ , the late decline of tetanic  $[\text{Ca}^{2+}]_i$  during fatigue was delayed. Thus results obtained with a variety of experimental approaches suggest that  $\text{CaP}_i$  precipitation in the SR is a possible cause of reduced tetanic  $[\text{Ca}^{2+}]_i$  in fatigue.

The  $\text{CaP}_i$  precipitation theory is obviously dependent on the ability of  $\text{P}_i$  to move from the myoplasm to the SR. The SR membrane contains small conductance chloride channels, which conduct  $\text{P}_i$ <sup>22</sup> and may be the pathway involved<sup>23</sup>. Interestingly the open probability of these channels increases at low ATP. This dependence on ATP can explain one apparent weakness of the hypothesis that raised  $[\text{P}_i]_i$  causes  $\text{CaP}_i$  precipitation in the SR:  $[\text{P}_i]_i$  increases relatively early during fatiguing stimulation while the decline of tetanic  $[\text{Ca}^{2+}]_i$  generally occurs quite late. Moreover, in mouse fibres the decline of tetanic  $[\text{Ca}^{2+}]_i$  temporally correlates with an increase in  $\text{Mg}^{2+}$ , which presumably stems from a net breakdown of ATP<sup>24</sup>, and it is not obvious why  $\text{CaP}_i$  precipitation in the SR should show a temporal correlation with ATP breakdown. The ATP-dependence of the presumed SR  $\text{P}_i$  channels can explain both why  $\text{P}_i$  enters the SR with a delay and why there is a temporal correlation between declining ATP and declining tetanic  $[\text{Ca}^{2+}]_i$ .

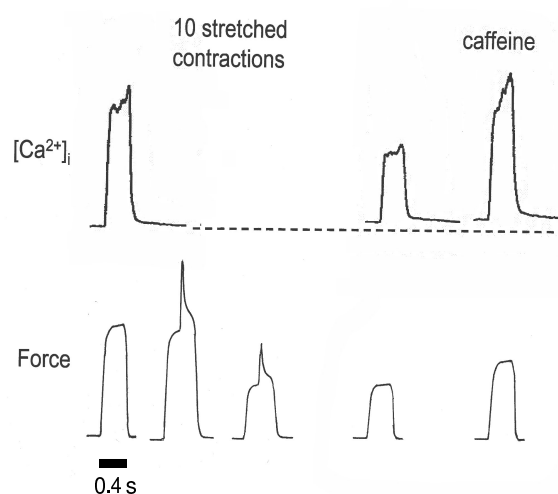
### Stretch-induced muscle damage

Muscle damage is a common consequence of intense muscular activity<sup>25</sup> and is more severe when the activity involves stretch of contracting muscles (eccentric contraction). In this review the term 'stretched contractions' is used to mean contractions in which the muscle is stretched by an external force<sup>26</sup>. Following repeated stretched contractions, particularly by untrained subjects, the muscles exhibit an immediate weakness and over the subsequent days they remain weak but also become tender, painful and stiff<sup>27</sup>. These changes can take a week to fully recover.

The cellular mechanisms which underlie the immediate weakness and the subsequent muscle damage following stretched contractions have two separate components. Fridén (1981) performed electron microscopy on humans muscle biopsies following stretched contractions and showed that the sarcomere structure was disturbed with overstretched sarcomeres and wavy Z-lines distributed randomly throughout the affected fibres<sup>28</sup>. Morgan (1990) pointed out that sarcomeres are unstable on the descending limb of the length-tension curve, particularly when undergoing stretch<sup>29</sup>, and that this can lead individual weak sarcomeres to suddenly stretch (popping sarcomeres). Such overstretched sarcomeres normally reinterdigitate during relaxation but after repeated stretched contractions increasing numbers of sarcomeres will fail to reinterdigitate and areas of overstretched sarcomeres may gradually extend.

The earliest changes of sarcomere structure can be observed within a single stretched contraction<sup>30,31</sup> and are probably the initiating factor in damage. Nevertheless there is good evidence that changes in excitation-contraction coupling also contribute to the muscle weakness caused by stretched contractions<sup>32,33</sup>. For instance when intracellular calcium was measured during tetani before and after stretched contractions it was found that the resting  $[\text{Ca}^{2+}]_i$  was increased while the tetanic  $[\text{Ca}^{2+}]_i$  was reduced (Fig.

4). To test whether the reduction of tetanic  $[\text{Ca}^{2+}]_i$  contributed to the reduced force production, caffeine was applied and shown to increase both the tetanic  $[\text{Ca}^{2+}]_i$  and force after stretched contractions. This result establishes that part of the weakness following stretched contractions is caused by reduced  $\text{Ca}^{2+}$  release which can be overcome by caffeine. The results also suggests that the SR  $\text{Ca}^{2+}$  store is not greatly affected since caffeine was capable of increasing  $\text{Ca}^{2+}$  release and implies that the defect in release lies in the action potential or its coupling to the release channel or the release channel itself. However the mechanism of the disturbance to excitation-contraction coupling remains uncertain.



**Figure 4. Stretched contractions cause a decline of tetanic  $[\text{Ca}^{2+}]_i$  which can be reversed by caffeine.**

Figure shows representative tetanic  $[\text{Ca}^{2+}]_i$  and force records from a mouse single fibre. Initial  $[\text{Ca}^{2+}]_i$  and force are an isometric control obtained at  $L_o$  (the length at which maximal tetanic tension is observed). The next two force records are the first and last (10th) stretched contractions in which the fibre was stretched by a motor from  $L_o$  to  $L_o + 40\%$  over 100 ms. The next pair of records are an isometric tetanus at  $L_o$  after 20 min recovery. The last pair of records show that caffeine can overcome the reduced tetanic  $[\text{Ca}^{2+}]_i$  and partially restore the force. These data show that stretch-induced muscle damage causes a reduction in tetanic  $[\text{Ca}^{2+}]_i$  which is partly responsible for the decline of force. Time scale applies to each tetanus; the time between tetani was variable. Adapted from Balnave & Allen<sup>33</sup>.

Another component of stretch-induced damage is an increase in membrane permeability. For example, both serum albumin and the fluorescent dye orange procion have been shown to enter some damaged fibres after a series of stretched contractions<sup>34</sup>. Resting  $[\text{Ca}^{2+}]_i$  increases within 10 minutes of eccentric contractions and, although the mechanism has not been established, this might also be a consequence of increased membrane permeability. The rise in resting  $[\text{Ca}^{2+}]_i$  might initiate the impairment of excitation-contraction coupling by activating proteases

which damage the sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  release channel<sup>35-37</sup>. It has also been proposed that  $\text{Ca}^{2+}$ -activated proteases might damage membranes and contribute to the increased membrane permeability.

We recently showed that following a series of stretched contractions, muscles developed vacuoles which filled with an extracellular marker (sulphorhodamine B) suggesting that they were attached to the T-system<sup>38</sup>. Such vacuoles had previously been observed under a range of situations in which a muscle was eliminating an osmotic load<sup>39,40</sup>. We proposed that stretch-induced damage produced T-tubular tears allowing the intracellular  $\text{Na}^+$  ( $[\text{Na}^+]_i$ ) to rise and that vacuoles were a consequence of the osmotic load caused by the  $\text{Na}^+$  pump extruding the excess  $\text{Na}^+$  along with osmotically-equivalent water.

To test these ideas we measured  $[\text{Na}^+]_i$  in muscle subjected to stretched contractions<sup>41</sup>. Ten isometric tetani had no significant effect on resting  $[\text{Na}^+]_i$  but 10 stretched contractions caused a significant increase in  $[\text{Na}^+]_i$  (Fig. 5A) from about 7 to 15 mM. The rise was surprisingly slow taking several minutes to reach a maximum and only starting to decline after about 20 min. By removing the extracellular sodium, we showed that this rise in  $[\text{Na}^+]_i$  was caused by increased  $\text{Na}^+$  influx from the extracellular space. To test whether the  $\text{Na}^+$  entry arose through T-tubular or membrane tears we imaged  $[\text{Na}^+]_i$  expecting to observe localized elevations of  $[\text{Na}^+]_i$  close to the putative tears. However we never observed any obvious areas of elevated  $[\text{Na}^+]_i$  suggesting that the  $\text{Na}^+$  influx was via multiple small sources below the spatial resolution of the confocal microscope. For this reason we decided to test blockers of various channels through which  $\text{Na}^+$  might enter. Skeletal muscle is known to contain stretch-activated non-specific cation channels<sup>42</sup> so we tested whether known blockers of this channel could prevent the rise of  $[\text{Na}^+]_i$  following stretched contractions. Fig. 5B shows that 20  $\mu\text{M}$   $\text{Gd}^{3+}$ , applied after the stretched contractions, was capable of preventing the rise of  $[\text{Na}^+]_i$ . While  $\text{Gd}^{3+}$  is an established blocker of stretch-activated channels it is also capable of blocking many other channels (for review see<sup>43</sup>), it is therefore important to note that  $\text{Gd}^{3+}$  had no effect on resting  $[\text{Na}^+]_i$  (Fig. 5B) or on tetanic force<sup>41</sup> in wild-type fibres. In addition, we showed that a chemically-unrelated blocker of stretch-activated channels, streptomycin, was also able to prevent the rise of  $[\text{Na}^+]_i$ <sup>41</sup>.

The above results suggest that entry of  $\text{Na}^+$  may occur through a class of channels activated by the preceding stretch. Most stretch-activated channels open rapidly ( $<1$  s) after distortion of the membrane and typically close briskly when the distortion is removed<sup>44</sup>. A notable feature of the present experiments is that the rise of  $[\text{Na}^+]_i$  occurred mainly after the stretches and that blockers applied after the stretches were capable of preventing the rise of  $[\text{Na}^+]_i$ . This suggests that the channels were opened by connection to a membrane component or cytoskeletal element which remains distorted long after the initial stretched contractions. For instance long lasting distortion of the T-tubular system following stretched contractions has been demonstrated by electron-microscopy<sup>45</sup> and by diffusion of

fluorescent markers<sup>38</sup>. Alternatively changes in cytoskeletal elements such as desmin and titin have been observed after stretched contractions<sup>46</sup>.

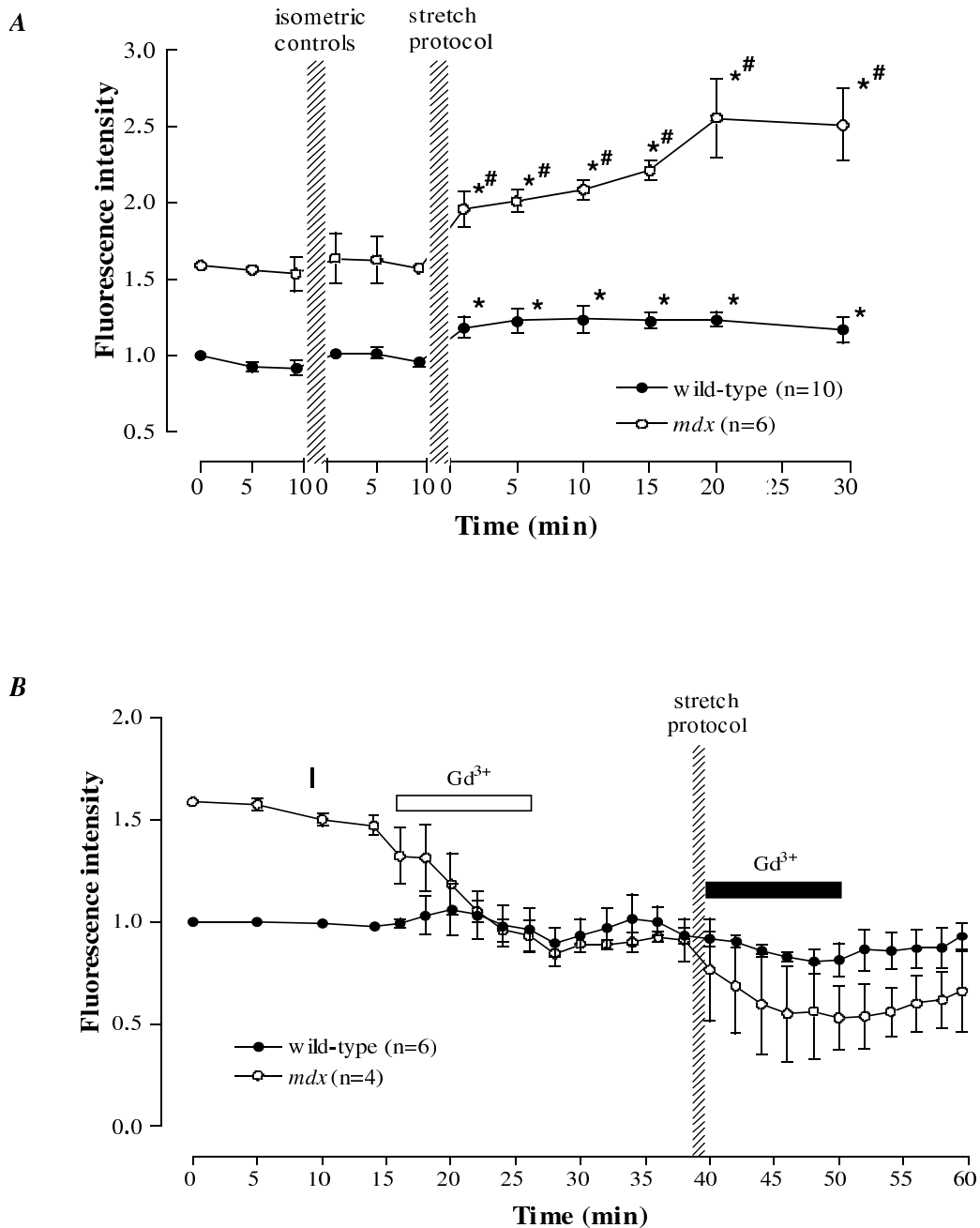
The above results also raised the possibility that the ionic changes associated with the stretch-contractions might be implicated in the reduction of force. For instance, if  $\text{Ca}^{2+}$  ions also entered through the same channel, this would provide an explanation for the raised resting  $[\text{Ca}^{2+}]_i$  and we have previously speculated that this might cause the reduced  $\text{Ca}^{2+}$  release which is one of the causes of the reduced force. This possibility was tested by comparing the recovery of force after stretch-contractions with and without blockers of stretch-activated channels. Either  $\text{Gd}^{3+}$  or streptomycin increased the recovery of force from 36 to 49% strongly suggesting that ionic entry has some part in the processes which reduce the force.

Stretched contractions are a normal part of the repertoire of muscle activities and have an important role in the training of muscles<sup>47</sup>. It has long been recognized that the damage caused by a series of stretched contractions is reduced on a second repeat<sup>48</sup>. The mechanism of this training effect has been the subject of much investigation and one component is that stretched contractions seem to elicit a recovery in which synthesis of additional sarcomeres in series occurs<sup>49,50</sup>. The net result of this is a shorter sarcomere length at a given muscle length thereby reducing the propensity to damage<sup>51</sup>. These findings suggest that stretched contractions stimulate a specialized sub-set of gene activation. Since  $[\text{Ca}^{2+}]_i$  appears to be intimately involved in gene regulation<sup>52</sup> this raises the possibility that  $\text{Ca}^{2+}$  entry by stretch-activated channels, perhaps because of some unidentified spatial or temporal feature, activates a group of genes which result in synthesis of additional sarcomeres in series.

## **Muscular dystrophy**

Duchenne muscular dystrophy is an X-linked condition that affects approximately 1 in 3500 male births<sup>53</sup>. It is a degenerative muscle disease causing death through respiratory and cardiac failure by the end of the second decade. The discovery that the disease was caused by absence of the protein dystrophin has revolutionized understanding of the disease and given new impetus to therapy<sup>54</sup>. The effectiveness of gene replacement therapy has been demonstrated in mouse models of dystrophy but in humans gene therapy has so far proved of limited value because of the difficulties of obtaining adequate expression of the very large dystrophin gene in human muscle (for review see<sup>55</sup>).

The mechanism by which the absence of dystrophin exacerbates stretch-induced damage is unclear. It is widely accepted that excessive  $\text{Ca}^{2+}$  entry is a feature of dystrophic muscle (for review see<sup>56</sup>). One theory to explain the excessive  $\text{Ca}^{2+}$  entry is that stretch-induced contractions lead to membrane tears which then allow ionic entry<sup>34</sup>. On this theory, the absence of dystrophin is assumed to increase the membrane fragility so that membrane tears are more frequent and lead to greater ionic entry. Another



**Figure 5. Intracellular sodium in wild-type and mdx mouse after stretched contractions.**

$[Na^+]_i$  indicated by fluorescence intensity; the higher initial value in mdx fibres represents their higher  $[Na^+]_i$ . Panel A shows that a series of isometric contraction had no significant effect on  $[Na^+]_i$  whereas a series of stretched contractions produced a rise of  $[Na^+]_i$ . Note that the rise in  $[Na^+]_i$  caused by the stretched contractions was larger in mdx fibres compared to wild-type fibres. \* significantly larger than initial level. # significantly larger increase compared to wild-type ( $P < 0.05$ ). Panel B shows first the effect of  $Gd^{3+}$  on resting  $[Na^+]_i$  in both mdx and wild-type fibres.  $Gd^{3+}$  had no effect on wild-type fibres but lowered  $[Na^+]_i$  in the mdx fibres to about the level of the wild-type fibres. The fibres then underwent a series of stretched contractions and  $Gd^{3+}$  was applied immediately for 10 min (indicated by the bar).  $Gd^{3+}$  eliminated the rise of  $[Na^+]_i$  caused by stretched contraction in both wild-type and mdx fibres. Values are mean  $\pm$  S.E.M. Data adapted from Yeung et al.<sup>41,65</sup>.

possibility is that the stretch-activated channel in wild-type fibres<sup>42</sup> has altered properties in the mdx fibres so that it becomes more readily activated by stretch<sup>57</sup>. The

observation that mdx fibres have increased permeability to divalent cations using the  $Mn^{2+}$  quench approach would be consistent with either of these hypotheses<sup>58</sup>.

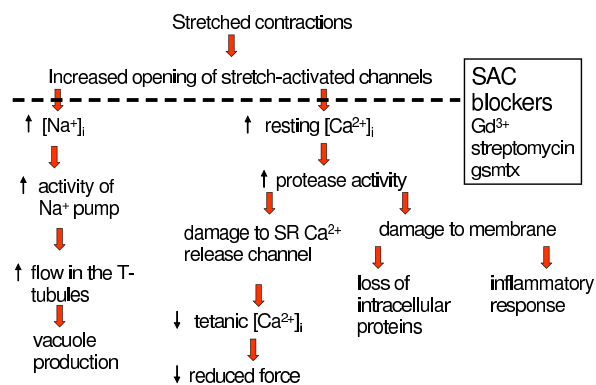
Studies of muscular dystrophy are very dependent on animals models of which the most widely used is the *mdx* mouse. This spontaneous mutant lacks dystrophin due to the presence of stop codon early in the sequence (for review see <sup>59</sup>). Although the genotype is similar to the human disease the phenotype is much milder. *Mdx* mice are fertile, live a near normal lifespan and show few overt signs of the disease. However, the creatine kinase levels are elevated and histology shows that the mild signs of muscle damage including centralized nuclei. Stretch-induced damage is generally more severe in *mdx* mice compared to wild-type mice<sup>60-62</sup> and the delivery of a dystrophin mini gene to *mdx* fibres reduces stretch-induced damage<sup>63</sup>. The reasons that the *mdx* phenotype is so much milder than Duchenne muscular dystrophy are not entirely clear but one possibility is that utrophin can substitute to a limited extent for dystrophin and seems to be overexpressed in the *mdx* mouse<sup>59</sup>. Another interesting observation is that muscle damage does not become apparent until about 3 weeks after birth which is close to the time when the animals are weaned and become more mobile, suggesting the possibility that mechanical factors contribute to the damage.

To investigate the mechanism of damage in *mdx* muscle we have measured  $[Na^+]_i$  in single fibres dissected from *mdx* mice. An initial finding was that resting  $[Na^+]_i$  was higher in *mdx* compared to wild-type fibres, confirming an earlier observation<sup>64</sup>. An interesting feature was that the elevated resting  $[Na^+]_i$  of the *mdx* muscle was reduced by  $Gd^{3+}$  or streptomycin (Fig. 5B) strongly suggesting that it is caused by increased opening of the same class of stretch-activated channels considered earlier. When the *mdx* fibres were exposed to our standard protocol of stretched contractions the rise in  $[Na^+]_i$  started from a higher level and showed a significantly greater rise (Fig. 5A). Either  $Gd^{3+}$  or streptomycin were capable of preventing this rise and seemed to lower the  $[Na^+]_i$  back towards the level observed in wild-type fibres (Fig 5B). Just as in wild-type fibres we found that either  $Gd^{3+}$  or streptomycin could prevent one component of the reduced muscle force after stretch-induced damage<sup>65</sup>. We interpret these findings to mean that muscle contains a stretch-activated channel whose open probability is enhanced in the *mdx* mouse. Patch-clamp studies on *mdx* fibres have produced similar findings<sup>57</sup>. Furthermore the channels seem to be more sensitive to the effects of stretch contractions in *mdx* fibres compared to wild-type fibres. Thus these channels may explain the elevated resting  $[Na^+]_i$  in *mdx* mice and also the elevated  $[Ca^{2+}]_i$  reported by others<sup>57</sup>. These channels appear to be further opened by stretch and the ionic entry associated with this pathway appears to have a role in the reduction of force observed after muscle stretch.

Some of the pathways we hypothesize to be active in muscle as a consequence of stretch-activated channels are illustrated in Fig. 6. As discussed above, we suggest that stretched contractions lead to a persistent opening of stretch-activated channels. The mechanisms involved are unclear at present but could involve membrane stretch as a result of popped sarcomeres or T-tubular vacuoles or perhaps changes in the cytoskeleton which modify channel

activity. Since these channels are permeable to both  $Na^+$  and  $Ca^{2+}$ <sup>42</sup> we would expect to observe increases in the resting  $[Na^+]_i$  and  $[Ca^{2+}]_i$  and, as discussed above, both have been observed. On this basis we would expect that blockers of the stretch-activated channels would be capable of preventing the rise of  $[Na^+]_i$  and  $[Ca^{2+}]_i$  and we have demonstrated that both  $Gd^{3+}$  and streptomycin can block the rise of  $[Na^+]_i$  in *mdx* muscle<sup>65</sup>. The functional changes in muscle as a consequence of raised  $[Na^+]_i$  are not clear but we have previously suggested above that the vacuoles present after stretched contractions are because the  $Na^+$  pump extrudes the excess  $Na^+$  with osmotically equivalent water and this hydraulic load in the T-tubules causes dilation and vacuolation<sup>38</sup>. The elevated  $[Na^+]_i$  would be expected to reduce the amplitude of the action potential and might therefore reduce SR  $Ca^{2+}$  release. In addition a raised  $[Na^+]_i$  will affect all  $Na^+$  linked transporters with a range of possible consequences for the cell. Whether the changed properties of the T-tubules affect muscle function is unclear<sup>38,45</sup>.

Current hypothesis



**Figure 6. Role of stretch-activated channels in stretch-induced muscle damage.**

Schematic to illustrate some of the pathways thought to be involved in the muscle damage caused by stretched contractions. For description see main text.

In Fig. 6 we propose that the elevated  $[Ca^{2+}]_i$  activates proteases with consequent effects on intracellular proteins and membranes. This possibility has been extensively considered but a difficulty is that the proteases which have been described do not have sufficient  $Ca^{2+}$  sensitivity to be activated by the observed rises in  $[Ca^{2+}]_i$ <sup>66-67</sup>. One protein which might be damaged is the ryanodine receptor (SR  $Ca^{2+}$  release channel) and, as discussed earlier, there is good evidence that increases in  $[Ca^{2+}]_i$  can lead to reduced SR  $Ca^{2+}$  release<sup>35-37</sup>. We also speculated in Fig. 6 that the loss of intracellular proteins and the inflammatory response are secondary consequences of membrane damage.



### Therapeutic possibilities

Our experimental data so far show that in isolated single fibres stretch-induced damage involves changes in  $[Na^+]_i$  and  $[Ca^{2+}]_i$  which are probably attributable to stretch-activated channels. When these channels are blocked some of the reduced force associated with stretch-induced damage can be prevented. These results raise the possibility that if the damage in muscular dystrophy is partly or predominately through the same pathway then blockers of the stretch-activated channels may reduce part of the muscle damage. We are currently testing this idea by using the stretch-activated channel blocker to *mdx* mice and testing whether the muscle damage is reduced.

### Conclusions

Single fibre preparations have proved a powerful experimental approach for studies of muscle function. The ability to make ionic measurements with good temporal and spatial resolution in single functioning fibres has greatly facilitated understanding of the functional changes during repeated muscle activity. Increasingly it will be possible to measure ionic concentrations in defined regions such as mitochondria, nuclei, SR and the near-membrane region. The possibilities of fluorescent tagging of signaling molecules, transcription factors, mRNA and proteins and following the distribution of these substances during different kinds of muscle activity offer exciting directions for the future.

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