Effects of cytosolic Mg^{2+} on halothane-induced Ca^{2+} release from the sarcoplasmic reticulum in isolated mechanically skinned rat skeletal muscle

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Previous studies have shown that the skeletal muscle ryanodine receptor (RyR) is strongly inhibited by Mg^{2+} at levels within the normal cytosolic range (0.8–1 mM). This may explain why high levels of caffeine (40 mM) do not induce a maximal release of Ca^{2+} from the SR unless $[Mg^{2+}]$ is simultaneously reduced (Lamb, 2000).

In the present study we have investigated the effects of cytosolic Mg^{2+} on halothane-induced Ca^{2+} release from the SR. Rats (200–250 g) were humanely killed (Schedule 1). Single extensor digitorium longus (EDL) muscle fibres were mechanically skinned and perfused with solutions approximating to the intracellular milieu containing (mM): KCl, 100; Hepes, 25; EGTA, 0.15; phosphocreatine, 10; ATP, 5. The free $[Ca^{2+}]$ was 100 nM and the free $[Mg^{2+}]$ was 1 or 0.1 mM (pH 7.0, 22 °C). Changes in $[Ca^{2+}]$ within the fibre were detected using fura-2. Preparations were initially perfused with a solution containing 1 mM Mg^{2+}. Following a 2 min Ca^{2+} loading period, application of 1 mM halothane failed to induce Ca^{2+} release from the SR. Indeed, in the presence of 1 mM Mg^{2+}, levels of halothane as high as 20 mM did not induce Ca^{2+} release ($n = 12$). However, when $[Mg^{2+}]$ was reduced to 0.1 mM, application of 1 mM halothane induced a substantial release of Ca^{2+} from the SR. The amplitude of the halothane-induced fluorescence transient was 60 ± 9 % ($n = 6$, mean ± S.E.M.) of that induced by a maximal response to 40 mM caffeine. A similar release was obtained when $[Mg^{2+}]$ was reduced to 0.1 mM in the halothane-containing solution, while the $[Mg^{2+}]$ of the Ca^{2+} loading solution was maintained at 1 mM. Therefore, the effect of reducing $[Mg^{2+}]$ appears to involve facilitation of RyR activation by halothane, rather than an indirect action on the SR Ca^{2+} uptake mechanism.

These results suggest that even during deep anaesthesia or induction, when the [halothane] may reach 1.2 mM (Franks & Lieb, 1996), SR Ca^{2+} release is unlikely to be significant due to the potent inhibitory effect of cytosolic Mg^{2+} on the RyR. However, in malignant hyperthermia (MH) clinical levels of halothane or other volatile anaesthetics can induce a SR Ca^{2+} release, resulting in a sustained contracture. Interestingly, recent work suggests that inhibition of the RyR by Mg^{2+} is substantially reduced in porcine (Owen *et al.* 1997) and human (Duke & Steele, 2002) MH. The present study suggests that a decrease in Mg^{2+} inhibition may substantially increase the sensitivity of the RyR to halothane.

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All procedures accord with current UK legislation.

Role of the Na^+–K^+ pump in maintaining T-tubular excitability in rat skeletal muscle

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Intensive contractile activity is associated with a significant loss of K^+ from the working muscle fibres, causing an increase in interstitial and T-tubular $[K^+]$. The ensuing depolarisation may interfere with fibre excitability and cause fatigue (Sejersted & Sjogaard, 2000). On the sarcolemma, a work-induced increase in Na^+–K^+ pump activity reduces the depressing effect of high K^+ on excitability (Overgaard & Nielsen, 2001). Because the ratio between membrane area and T-tubular volume is very large, the build-up of K^+ is likely to be augmented in the T-tubules. Little is known, however, about the significance of elevated extracellular K^+ and Na^+–K^+ pump activity for the excitability of the T-tubular membrane. To examine this, the sarcolemma of single fibres from rat extensor digitorum longus was mechanically removed in such a way that the T-tubular system was sealed off and polarised, making it possible to elicit contractions by setting up action potentials in the T-tubular system with electrical stimulation (Posterino *et al.* 2000). The fibres were placed on an isometric transducer at room temperature and stimulated with 2 ms square pulses (50 V cm^{-1}). The activity of the Na^+–K^+ pump was manipulated by use of solutions with 10 to 50 mM Na^+. Animals were killed humanely.

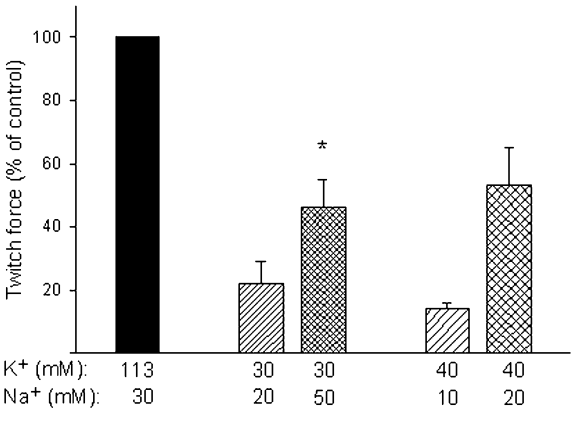

Figure 1. Effect of $[K^+]$ and $[Na^+]$ on twitch force in skinned muscle fibres. Na^+ and K^+ were replaced with NH_4^+. At each $[K^+]$ (30 mM, 5 fibres and 40 mM, 4 fibres), the same fibres were tested at both the indicated $[Na^+]$. Columns show average ± S.E.M.

In control solution that mimicked the intracellular environment (30 mM Na^+ and 113 mM K^+, for details, see Posterino *et al.* 2000), twitch force was 36 ± 3 % of the force induced by saturating levels of Ca^{2+} ($n = 9$). Figure 1 shows that lowering the chemical gradient for K^+ by reducing K^+ in the buffer to 30 or 40 mM produced a large force deficit, indicating a reduction in T-tubular excitability. The reduction in force depended, however, on the $[Na^+]$ of the solution. Thus at 30 K^+ the force produced at 50 mM Na^+ was 2-fold larger than at 20 mM Na^+ ($P < 0.03$, paired t test). At 40 mM K^+ a similar effect was obtained by increasing Na^+ from 10 to 20 mM. These results indicate that the sensitivity of T-tubular excitability to a lowering of the chemical gradient for K^+ can be reduced by increasing the activity of the Na^+–K^+ pump. This suggests that during exercise, the increase in the activity of muscle Na^+–K^+ pumps protects

T-tubular excitability against the depressing effect of elevated extracellular K^+.

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Posterino, G.S. *et al.* (2000). *J. Physiol.* **527**, 131–137.
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All procedures accord with current National and local guidelines.

Effects of caffeine on power output of isolated mouse extensor digitorum longus muscle during recovery from fatigue

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Caffeine has previously been shown to cause rapid recovery of isometric muscle force generation following fatigue (Westerblad & Allen, 1991), eliciting speculation that caffeine supplementation may directly increase *in vivo* force in athletes (Graham, 2001). However, these *in vitro* studies used higher concentrations (mM) of caffeine than the micromolar concentrations found in human athletes during dynamic muscular actions. In this study, we examined the effect of both 10 mM (high) and 70 μM (physiologically relevant) caffeine concentrations on power production of mouse extensor digitorum longus (EDL) muscle during recovery from fatigue. Female CD1 8- to 10-week-old mice were killed by cervical dislocation and the EDL muscle was isolated in oxygenated Krebs-Henseleit solution (Barclay, 1996) at 35°C. Muscle stimulation was optimised to produce maximum power output at a work loop cycle frequency of 5 Hz using a total strain of 10 % of the muscle's resting length (James *et al.* 1995). The muscle was then subjected to a fatigue run consisting of 50 work loop cycles. Immediately after the fatigue run, the bathing solution was replaced with either standard Krebs, Krebs containing 10 mM caffeine or Krebs containing 70 μM caffeine ($n = 8$ muscles for each treatment). Recovery from fatigue was monitored by regularly subjecting the muscle to a set of four control work loop cycles. After 10 min the bathing solution was replaced with standard Krebs solution to allow recovery to be further monitored during a wash-out period. The effect of caffeine treatment (5 and 10 min after fatigue) was analysed using ANOVA (with a Bonferroni *post-hoc* test). Power output was significantly higher in 10 mM treated EDL 5 min after the fatigue run (Fig. 1; $P = 0.035$). In contrast, 70 μM caffeine had no significant effect on EDL power output (Fig. 1).

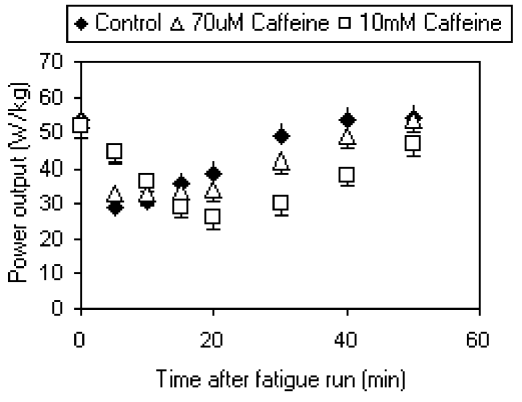

Figure 1. Effects of caffeine on muscle power output during recovery from fatigue. Values represent means ± S.E.M.

The depressant effect of fatigue on force in EDL was partially reversed by exposure to 10 mM caffeine. However, 70 μM caffeine did not influence EDL force, suggesting that the concentrations of caffeine found in human athletes are unlikely to directly affect skeletal muscle.

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All procedures accord with current UK legislation.

Effects of Mg^{2+} and SR luminal Ca^{2+} on caffeine-induced Ca^{2+} release in skeletal muscle from humans susceptible to malignant hyperthermia

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Regulation of the ryanodine receptor (RYR) by Mg^{2+} and SR luminal Ca^{2+} was studied in mechanically skinned malignant hyperthermia susceptible (MHS) and non-susceptible (MHN) fibres from human vastus medialis. Muscle was obtained by open biopsy from patients attending for investigation of MH susceptibility at St James's Hospital Leeds, UK. This was done with institutional Research Ethics Committee approval and informed patient consent. All procedures were carried out according to the Declaration of Helsinki. Approximately 1 g of the muscle used in the *in vitro* contracture test (IVCT), which was performed according to the criteria for malignant hyperthermia (MH) research of the European MH Group (The European Malignant Hyperpyrexia Group, 1984). The IVCT was used as the primary method of categorising MHN and MHS tissue. Single mechanically skinned fibres were prepared from the remaining 0.2 g of tissue. Preparations were perfused with solutions mimicking the intracellular milieu and changes in $[Ca^{2+}]$ were detected using fura-2 fluorescence. MHS fibres had a higher sensitivity to caffeine (2–40 mM) than MHN fibres. For example, in MHS fibres, the amplitude of the caffeine-induced Ca^{2+} transient resulting from rapid application of 10 mM caffeine was 86.7 ± 6.7 % ($n = 9$, mean ± S.E.M.) of the maximum response (to 40 mM/zero Mg^{2+}), compared with 37 ± 5.9 % ($n = 10$, mean ± S.E.M.) in MHN fibres. The inhibitory effect of Mg^{2+} on caffeine-induced Ca^{2+} release was studied by increasing the $[Mg^{2+}]$ of the solution containing 40 mM caffeine. Increasing $[Mg^{2+}]$ from 1 to 3 mM reduced the amplitude of the caffeine-induced Ca^{2+} transient by 77 ± 7.4 % ($n = 8$, mean ± S.E.M.) in MHN fibres. However, the caffeine-induced Ca^{2+} transient decreased by only 24 ± 8.1 % ($n = 9$, mean ± S.E.M.) in MHS fibres. In MHN fibres, reducing the Ca^{2+} loading period from 4 to 1 min (at 1 mM Mg^{2+}) decreased the fraction of the total SR Ca^{2+} content released in response to 40 mM caffeine by 90.4 ± 6.2 % ($n = 6$). However, in MHS fibres the response was reduced by only 31.2 ± 17.4 % ($n = 6$, mean ± S.E.M.) under similar conditions. These results suggest that human MH is associated with reduced inhibition of the RYR by (i) cytosolic Mg^{2+} and (ii) SR Ca^{2+} depletion. Both of these effects may contribute to increased sensitivity of the RYR to caffeine and volatile anaesthetics.

The European Malignant Hyperpyrexia Group (1984). *Anesthesiol.* **56**, 1267–1269.

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Effect of temperature on the sensitivity of muscle contractions to extracellular K^+

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Loss of K^+ from active muscles, leading to increased extracellular K^+ concentration ($[K^+]_o$), has been proposed to contribute to muscle fatigue by reducing excitability. Since strenuous exercise increases muscle temperature, it is of interest to know how temperature elevation modifies the sensitivity of skeletal muscles to increased $[K^+]_o$.

Intact soleus muscles from humanely killed 4-week-old Wistar rats were mounted isometrically on force transducers and stimulated 2 s every 10 min with pulses of 0.2 ms duration. Choice of stimulation frequency (30–90 Hz) was based on a force frequency analysis to make certain that full tetanic force was obtained at all temperatures. During the experiments muscles were exposed to controlled changes in $[K^+]_o$ and temperature while force production was recorded. Compound action potentials were measured and M-wave area was taken as an expression of excitability.

As shown in Fig. 1, increasing $[K^+]_o$ from 4 to 10 mM at 20 °C reduced tetanic force production to 14 ± 4 % and M-wave area to 10 ± 7 % of the control level. At 10 mM K^+ temperature elevation from 20 to 30 °C restored force production and M-wave area to 67 ± 14 and 65 ± 12 %, respectively. Force and M-wave area were closely correlated ($r^2 = 0.955$, $P < 0.001$). Other experiments showed that when temperature was elevated from 20 to 35 °C the level of $[K^+]_o$ required to reduce tetanic force by 50 % increased from 8.8 to10.7 mM ($n = 6$, Student's two-tailed t test).

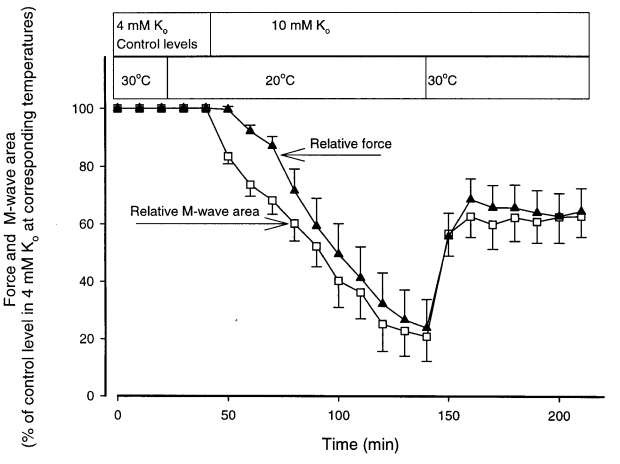

Figure 1. Temperature-specific $[K^+]_o$ sensitivity. Force production (▲) and M-wave area (□) at 10 mM K^+ were related to the 4 mM K^+ control level at the corresponding temperature (20 or 30 °C). Values are means with S.E.M. $n = 3$.

The protective effect of elevated temperature on force production disappeared when ouabain (10^{-3} M) was added to muscles, indicating that a temperature-dependent increase in the Na^+–K^+ pump activity contributed to the protective effect. Measurements of ouabain-suppressible $^{86}Rb^+$ uptake showed that increasing the temperature from 20 to 30 °C augmented Na^+–K^+ pump activity by 93 % ($n = 9$).

It is concluded that the sensitivity of skeletal muscles to high $[K^+]_o$ is reduced by elevated muscle temperature. We suggest that this effect is due to a protection of the excitability in part mediated by increased Na^+–K^+ pump activity. In addition, a reduction in slow inactivation of voltage-gated sodium channels associated with high muscle temperatures, may contribute to the observed reduction in sensitivity to extracellular K^+ (Ruff, 1999).

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All procedures accord with current National and local guidelines.

Protective effects of acidosis and Na^+–K^+ pump activation on force in K^+-depressed skeletal muscle

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Intensive exercise leads to large increases in $[K^+]_o$, which may reduce excitability and contractility of skeletal muscles. Intensive exercise also leads to an increase in the activity of muscle Na^+–K^+ pumps and to accumulation of lactic acid. Individually, Na^+–K^+ pump stimulation (Clausen & Flatman, 1977) and lactic acidosis (Nielsen *et al.* 2001) have been shown to counteract the depressing effects of high $[K^+]_o$ on muscle excitability and force via an increase in the chemical Na^+ gradient (Overgaard *et al.* 1997) and a reduction in pH_i, respectively. To estimate the sensitivity of active muscles to the depressing effects of high $[K^+]_o$ we examine here whether the protective effects of acidosis and Na^+–K^+ pump stimulation are additive.

Isolated rat soleus muscles were mounted on isometric force transducers and incubated in HCO_3^--buffered Krebs-Ringer at 30 °C. Contractions were evoked using 30 Hz pulse trains of 2 s duration. Na^+–K^+ pump stimulation was elicited by the addition of supramaximal doses of adrenaline (10^{-5} M). Muscle excitability was assessed by extracellular compound potentials (M-wave). Animals were humanely killed. The statistical significance of difference between two groups was ascertained using Student's two-tailed t test.

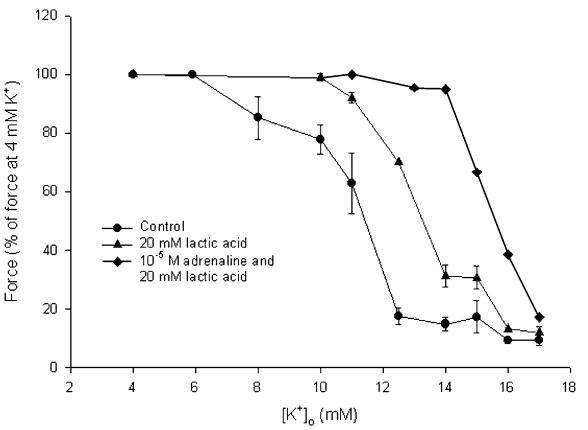

Figure 1. Effects of lactic acid and adrenaline on the relation between tetanic force and $[K^+]_o$. Muscles were incubated at different $[K^+]_o$ values until a steady force was obtained.

Control muscles, buffer pH 7.4 (n = 4–6); 20 mM lactic acid added, buffer pH 6.8 (n = 8–10); 20 mM lactic acid and 10^{-5} M adrenaline added, buffer pH 6.8 (n = 8). Symbols show means ± S.E.M.

As shown in Fig. 1, an increase in $[K^+]_o$ from 4 to 11.1 mM led to a 50 % reduction in tetanic force. In the presence of 20 mM lactic acid, the level of $[K^+]_o$ necessary to reduce tetanic force by 50 % (IC_{50}) increased by almost 2 mM to 13.0 mM. An even further increase in IC_{50} of 2.8 mM was seen when, in addition, the Na^+–K^+ pump was stimulated by using adrenaline. Measurement of extracellular compund potential showed that the protection of force against increased $[K^+]_o$ by low pH_i and Na^+–K^+ pump stimulation was caused by an improvement of muscle excitability. An increase in $[K^+]_o$ to 11.5 mM reduced M-wave area to 18.2 ± 1.1 % in controls but only to 89.7 ± 5.6 and 99.5 ± 2.1 %, respectively, in muscles treated with lactic acid and with adrenaline in addition to lactic acid (n = 2).

These results show that the protective effects of lactic acid and Na^+–K^+ pump stimulation on the depressing effects of $[K^+]_o$ on muscle force are additive. Since intensive muscle activity is associated with both accumulation of lactic acid and activation on the muscle Na^+–K^+ pump, this indicates that during activity muscles become much less sensitive to the inhibitory effects of exercise-induced hyperkalaemia.

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Overgaard, K. *et al.* (1997). *Eur. J. Physiol.* **434**, 457–465.

All procedures accord with current National and local guidelines.